Localization of membrane-bond OPN using Force Spectroscopy Analysis

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Abstract. At this study we present molecular recognition method which is based on force spectroscopy analysis for biological markers on the whole cell level. The presented method allows recognition of specific cell surface proteins and receptor sites by nanometer accuracy level. Here we demonstrate specific recognition of membrane-bond Osteopontin (OPN) sites over a whole Preosteogenic cell membrane. By merging specific force detection map of the proteins and topography image of the cell, we create a new image (recognition image), which demonstrate the exact locations of the proteins relative to the cell membrane. The recognition results indicate on the strong affinity between the modified tip and the target molecules, therefore, it enables the use of an AFM as a remarkable nanoscale tracking tool at the whole cell level.

1. Introduction

AFM (atomic force microscopy) is a well recognized tool for its high resolution images and also a powerful tool for the detection of Pico scale molecular interactions in variety of applications. The ability to detect minute forces provides the possibility to measure and quantify inter molecular forces at the single molecule level \cite{1-2}. Interactions at molecular level can be investigated by various force spectroscopy methods \cite{3-4}. One of the common techniques is based on modified AFM tip, which contains specific ligand such as antibody against the target molecule. Using force distance measurement it is possible to monitor the changes in interaction level between antibody and target.

In this work we present an advanced recognition method for biological analysis. The method is based on specific force measurements between modified AFM tip and a mammalian cell. Using this technique we are able to identify the exact location and distribution (by nano meter resolution) of OPN (Osteopontin proteins) over Preosteogenic cell membrane. The structural information, which has been demonstrated in this work, could provide information about the distribution of OPN in variety of biological mechanisms like bone formation and destruction, immune system function and behavior, and some cancer types \cite{5}.

2. Recognition method

The goal of our method was to develop a multi-image technique which based on merging of high resolution topographic image and the force detection map of the scanned object. Anti-OPN antibody is attached to the AFM tip prior to the scan. In the case when recognition event between the antibody and
the OPN occurs; the AFM detects the "pull-off" (retraction) force which is required to break the interaction between them.

During the recognition process (Figure 2) each force distance curve is transformed to a row of numerical data; the data point which indicates the "pull-off" force between the tip and the sample is isolated and used to the creation of the Force matrix. Every component in the Force matrix represents the "pull-off" value of single Force distance curve. The Force image represents an 8-bit transformation of the force matrix. It means that every component in the Force matrix has a numerical value between 0 to 255, which corresponds to a high level of interaction event (high force) and a low value respectively, which is related to low level of interaction (low force). The data which is collected during the scan is used to the creation of both the topography of the scanned cell and the force matrix which indicates the measured "pull-off" force levels over the cell membrane. The force matrix is transformed to 8-bit representation. Numerical filtering process, which is based on statistical analysis of the force measurements, is applied in order to isolate the specific recognition sites from the force image. After the isolation process a new binary matrix is created, each point in the binary matrix (recognition matrix) indicates about the exact location of single target site. The recognition image is created by merging the recognition matrix and the topography image. The location of all the target sites (relative to the cell membrane) is demonstrated by the red dots over the image.

3. Results

The level of adhesion interaction was measured through a series of probe modifications in order to evaluate the effect of background interferes on the specific interaction of OPN. Using Table 1 we summarized the evolution of adhesion measurements during all the modification steps. In addition, the level of unspecific binding was checked through the use of unspecific IgG antibody, and BSA (Bovine Serum Albumin) coating treatment of the cells surface.

The distribution of adhesion forces reveals clear separation between high and low events, which can be attributed/translated to OPN localization over the membrane. Low adhesion events (10-50pN) may correspond to interaction between the coated probe and non/low reactive domains on the membrane. The high events (85-135 pN, ~80% of the high adhesion measurements) rise from both specific and unspecific interactions of anti OPN with the cell surface. Based on the high affinity of OPN, which is one of the highly conserved proteins among vertebrates and the unspecific IgG control, the probability of unspecific binding (false sites) seems low. On the other hand, the BSA control suggests possible contribution of charge interactions which can yield (in addition to anti-OPN specific binding) high adhesion values. The mead rang values (70-80 pN, ~10% of the high adhesion measurements), which
were not affected from both the controls, were probably raised from partial/unsuitable binding interactions and therefore exhibit relative decreasing in adhesion.

Table 1: characterization of adhesion measurements

| Probe modification       | Surface blocking | Adhesion range (pN) | Mean adhesion value (\( \bar{x} \)) | STD (\( \sigma \)) |
|--------------------------|------------------|---------------------|--------------------------------------|------------------|
| Without (bare tip)       | None             | 5-51                | 21                                   | 7                |
| Silane                   | None             | 7-62                | 29                                   | 14               |
| Silane+ Glutaraldehyde   | None             | 9-58                | 27                                   | 12               |
| Silane+ Glutaraldehyde   | BSA              | 7-53                | 23                                   | 8                |
| Anti OPN                 | None             | 5-56, 67-145        | 26, 107                              | 11, 16           |
| Anti OPN                 | BSA              | 7-55, 73-125        | 24, 94                               | 9, 11            |
| Unspecific IgG           | None             | 9-73                | 32                                   | 12               |

Isolation of the specific binding events enables localization of the exact OPN sites on the cell membrane. The localization process is based on isolation of specific segment (“recognition frame”) from the whole adhesion range, which mostly contains data regarding specific interaction of OPN. The boundaries of the frame are important factors, which influence about both recognition yield and error, and therefore, should be set carefully. In order to develop a criterion regarding the setting of the correct recognition frame, we preformed a series of force experiments between randomly distributed proteins and their matching antibody (data not shown). Based on those results, we found that suitable recognition frame for protein molecules is found mostly at the range of ±15 pN around the average adhesion force (here the mean was calculated based on adhesion events greater than 75 pN). Using this frame, we achieved recognition of ~85% of the molecules, and recognition error of ~8% (only one of 12 recognition events was considered false).

Here the recognition frame was chosen to be 100±15 pN. Using this frame we still keep on the mean binding force of OPN, and also minimize the affects of unspecific events on the recognition accuracy. After the setting, each pixel in the force image is compared to the limitation values of the frame. If the value is located within the frame the pixel is considered as a specific interaction and will get the binary value ‘1’, otherwise, the value will be ‘0’. The new binary image which is known as “recognition matrix” demonstrates the exact location of all OPN sites relative to the measurements order. The recognition image (Figure 2) has been created by direct merging of the recognition matrix and the topography image, this image demonstrates the exact location of OPN sites relative to the cell membrane.

4. Discussion

AFM is a powerful tool for exploring the forces and the dynamics of the interaction between individual ligands and receptors, either on isolated molecules or on cellular surfaces. Previous AFM studies demonstrated spectroscopy analysis of membrane proteins on microbial and yeast cells [6-7]. The study of mammalian cells is therefore challenging for such analysis and thus the results presented here are new. The resolution at the nanometer scale provides information about function-related proteins existent on the surface of live cells. The mapping of membrane proteins relies on quantification of force distribution and probing after specific recognition forces at the surface of living cells. This unique mapping provides a new insight into the molecular mechanisms of cell membrane structure. In addition, such analysis provides an enhanced knowledge of cell adhesion, interaction with other cells and ECM that regulates cell growth and differentiation. Knowledge about the surface properties of cells is a key to understand their functions in cell-cell interaction and the environmental effects that exploit them in differentiation processes.

OPN contains a conserved RGD (Arg-Gly-Asp) sequence, and can bind to preosteogenic cells via Integrin-mediated mechanisms [8]. Therefore, the distribution of OPN can be referred to specific sites,
which demonstrate binding with Integrin receptors. In fact, the OPN molecules did not disconnect from the Integrin receptor sites during the measurements, indicates, that the binding force between these two molecules is much higher then the force between the OPN and its antibody. The recognition image (Figure 2) reveals the distribution and location of OPN-Integrin complexes over preosteogenic cell membrane. These complexes exhibit strong tendency to arrange in several groups, which are located mainly between the cytoskeleton filaments. It is also interesting to reveal that Integrin receptors (which have been considered as one of the important receptor groups) are expressed, mostly over flat sections of the cell membrane; this unique expression may enhance their ability to bind their substrate. The relatively high number of Integrin receptor is not so surprising due to the high number of cell activities, which are controlled by the Integrin family. Most of these activities are related to signal transduction, which is very important to cell function and surviving [9].

Figure 2: (a) AFM image demonstrates the topography of the whole preosteogenic cell (30 µm x 30 µm). The black frame on the right corner (1 µm x 1 µm) shows the chosen recognition zone. (b) High resolution topography of recognition zone. (c) Recognition matrix demonstrates the location of all specific binding events between anti-OPN tip and the OPN proteins on the cell surface. (d) The recognition image was created by merging the binary matrix and high resolution topography of the recognition zone. (image b + image c) The image reveals the location of all OPN sites over the recognition zone, each black point indicates about the location of single OPN site.

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