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Research paper

Rapid recognition and functional analysis of membrane proteins on human cancer cells using atomic force microscopy

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ABSTRACT

Understanding the physicochemical properties of cell surface signalling molecules is important for us to uncover the underlying mechanisms that guide the cellular behaviors. Atomic force microscopy (AFM) has become a powerful tool for detecting the molecular interactions on individual cells with nanometer resolution. In this paper, AFM peak force tapping (PFT) imaging mode was applied to rapidly locate and visually map the CD20 molecules on human lymphoma cells using biochemically sensitive tips. First, avidin–biotin system was used to test the effectiveness of using PFT imaging mode to probe the specific molecular interactions. The adhesion images obtained on avidin-coated mica using biotin-tethered tips obviously showed the recognition spots which corresponded to the avidins in the simultaneously obtained topography images. The experiments confirmed the specificity and reproducibility of the recognition results. Then, the established procedure was applied to visualize the nanoscale organization of CD20s on the surface of human lymphoma Raji cells using rituximab (a monoclonal anti-CD20 antibody)-tethered tips. The experiments showed that the recognition spots in the adhesion images corresponded to the specific CD20–rituximab interactions. The cluster sizes of CD20s on lymphoma Raji cells were quantitatively analyzed from the recognition images. Finally, under the guidance of fluorescence recognition, the established procedure was applied to cancer cells from a clinical lymphoma patient. The results showed that there were significant differences between the adhesion images obtained on cancer cells and on normal cells (red blood cell). The CD20 distributions on ten cancer cells from the patient were quantified according to the adhesion images. The experimental results demonstrate the capability of applying PFT imaging to rapidly investigate the nanoscale biophysical properties of native membrane proteins on the cell surface, which is of potential significance in developing novel biomarkers for cancer diagnosis and drug development.

1. Introduction

Cells sense and respond to the stimuli from the external environments via the various cell surface signalling molecules (such as receptor and ion channel). To fulfill their versatile functions, these molecules have heterogeneous properties (e.g., structural, biophysical and biochemical properties) that can change dynamically as required by the cell (Pfreundschuh et al., 2014). Hence understanding the dynamics of molecular activities on the cell surface is important for us to uncover the underlying mechanisms that guide the cellular behaviors. However, due to the lack of adequate techniques, knowledge of the nanoscale molecular interactions on single cells is scarce. The advent of atomic force microscopy (AFM) provides an exciting instrument for investigating the individual receptor–ligand interactions on single living cells in aqueous solution (Heinisch et al., 2012) and this technique is called single-molecule force spectroscopy (SMFS) (Muller et al., 2009). In SMFS, by obtaining force curves on the cell surface via tips carrying ligands (or antibodies), the binding force between single receptor and single ligand (or antibody) can be directly measured. By controlling the tip to obtain arrays of force curves at different points on the cell surface, the distribution maps of receptors can be constructed and this technique is called force volume method (El-Kirat-Chatel et al., 2014). However, the force volume method requires a lot of time to produce a distribution map (often more than 10 min), thus considerably reducing the efficiency of experiments.

In recent years, a new AFM imaging mode called peak force tapping (PFT) (Heu et al., 2012; Dufrene et al., 2013) emerges as a powerful method to simultaneously obtain multiple parameters (e.g., topography, elasticity, adhesion, deformation and energy dissipation) of biological

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samples in a relative short time. In the PFT mode, the vibrating tip indents the samples to record an array of force curves for each sampling points on the samples. By real-time analyzing the different parts of the force curve (Fig. 1A), multiple parameters that reflect the physical and chemical properties of the samples can be obtained simultaneously. Since the advent, researchers have applied PFT imaging to investigate the physicochemical properties of different biological samples, e.g., membrane proteins (bacteriorhodopsin on the purple membrane of bacterial (Rico et al., 2011; Medalsy et al., 2011), erythrocyte membrane (Picas et al., 2013) and living cells (keratinocyte (Heu et al., 2012), yeast (Alsteens et al., 2012), and *Escherichia coli* bacteria (Alsteens et al., 2013). However, these researches were commonly performed using ordinary tips (Heu et al., 2012; Rico et al., 2011; Medalsy et al., 2011; Picas et al., 2013) that cannot identify the specific membrane proteins on the cell surface. In recent studies, researchers (Alsteens et al., 2012, 2013) have applied PFT imaging to investigate the molecular interactions on bacterial cells using functionalized tips. Although, studies that demonstrate the accuracy and reliability of the specific molecular interactions detected using PFT imaging mode with functionalized tips are still needed. Besides, to our knowledge, studies that applying PFT imaging to investigate the specific membrane proteins on human cancer cells have not been reported. In this work, we applied PFT imaging to rapidly visualize the organization of CD20 antigen molecules on human lymphoma cells using biochemically sensitive tips. First, avidin–biotin system was used to test the reliability of using PFT imaging to detect the specific molecular interactions. Second, the established procedure was applied to visualize the organization of CD20 molecules on the surface of lymphoma Raji cells with rituximab-tethered tips (rituximab is a monoclonal antibody against CD20). Finally, the established procedure was applied to locate and quantify the CD20 molecules directly on cancer cells prepared from the bone marrow of a clinical lymphoma patient.

2. Materials and methods

2.1. Cell and reagents

Lymphoma Raji cell line and rituximab (10 mg/mL) were obtained from Affiliated Hospital of Military Medical Academy of Sciences (Beijing, China). Avidins were purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). The spacer molecules (Biotin-PEG-NHS and MAL-PEG-NHS) were purchased from JenKem Technology Co., Ltd. (Beijing, China). Reagents (aminopropyltriethoxysilan (APTES), *N*,*N*-diisopropylethylamin, triethylamine, *N*-succinimidyl 3-(acetylthio) propionate (SATP)) of tip functionalization were purchased from Sigma-Aldrich Corporation (Saint Louis, MO, USA) and Thermo Fisher Scientific Inc. (Rockford, IL, USA).

2.2. Avidin-coated mica

Avidins were adsorbed onto mica according to the protocol in the reference (Riener et al., 2003). Avidin powder was dissolved in 0.1 M phosphate buffered saline (PBS) to a stock concentration of 1 mg/mL. Then the stock solution was diluted with 100 mM NaCl to a final concentration of 1 μg/mL. Freshly cleaved mica was immersed in this solution for 20 min and subsequently rinsed with PBS for three times. The avidin-coated mica was glued to a glass slide which was then placed in a petri dish containing PBS.

2.3. Cell sample preparation

Raji cells were cultured in RPMI-1640 containing 10% fetal bovine serum at 37 °C (5% CO_2). After 24 h of culture, Raji cells were harvested at 1000 rpm for 5 min. Harvested Raji cells were dropped to poly-L-lysine-coated glass slides and then fixed by 4% paraformaldehyde for 30 min. After being rinsed by PBS, the glass slide was placed in a petri dish containing PBS. Normal human cells were prepared from the peripheral blood of healthy volunteers. The preparation process of normal cell sample was the same as the Raji cell sample.

2.4. Patient biopsy cell sample preparation

Bone marrow cells from a B-cell lymphoma patient with bone marrow invasion were used. This study was approved by the ethical committee of the Affiliated Hospital of Military Medical Academy of Sciences (Beijing, China). Bone marrow cells were dropped onto glass slides which were coated by poly-L-lysine. After fixing the cells with 4% paraformaldehyde for 30 min, ROR1 fluorescence labeling experiments were performed to recognize the cancer cells, as described previously (Li et al., 2013a). Briefly, the cells were firstly blocked by donkey serum. Then goat-anti-human ROR1 antibody solution (R&D systems, Minneapolis, USA) was added to the slides and incubated for 3 h at room temperature. After washing the slides with PBS for three times, the donkey-anti-goat IgG solution (KangChen company, Shanghai, China) was added to the slides and incubated for 30 min at room temperature. After washing the slides with PBS for three times, the slides were put on the stage of an inverted fluorescence microscope (Ti, Nikon, Japan) for experiments. ROR1 is a cellular surface marker which is expressed only on some types of cancer cells (such as chronic lymphocytic leukemia cell, mantle cell lymphoma cell, follicular lymphoma cell, and marginal zone lymphoma cell) but not expressed on normal cells (Uhrmacher et al., 2011; Baskar et al., 2012). Hence, for lymphoma patients whose bone marrow had been invaded by cancer

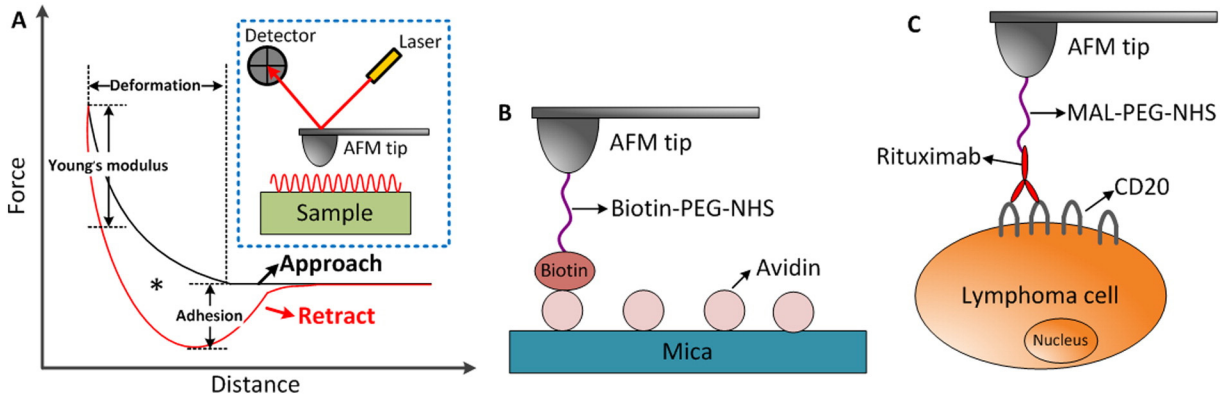

Fig. 1. Principle of recognizing proteins using PFT imaging with functionalized tips. (A) Schematic diagram of a force curve which includes an approach curve and a retract curve. PFT imaging extracts different properties of samples by analyzing the different parts of the force curve. The Young's modulus and adhesion force are obtained from the retract curve. The deformation is obtained from the approach curve. The energy dissipation is equal to the area between the approach curve and the retract curve (denoted by the asterisk). The inset is the schematic diagram of PFT imaging. The tip intermittently contacts the sample to obtain an array of force curves for each sampling point. (B) Probing the avidins adsorbed on mica by linking biotins to the AFM tip. (C) Probing the CD20s on the surface of lymphoma cell by linking rituximabs to the AFM tip.

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