High sensitivity detection of protein molecules picked up on a probe of atomic force microscope based on the fluorescence detection by a total internal reflection fluorescence microscope

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Abstract We developed a method to detect and identify proteins on a probe of the atomic force microscope (AFM) with a high sensitivity. Due to a low background noise of the total internal reflection fluorescence microscope employed as a detecting system, we were able to achieve a high enough sensitivity to detect zeptomole orders of protein molecules immobilized on the tip. Several different methods to immobilize protein molecules to AFM-probes were tested, meant for a wide range of applications of this method. Furthermore, we demonstrated that different proteins were clearly distinguished by immunofluorescence microscopy on the probe using their specific antibodies.

Keywords: Bcl-2; Evanescent illumination; Fibronectin; GFP; Immunofluorescence; Sensitive protein detection

1. Introduction

The atomic force microscope (AFM) [1] is a versatile tool capable of taking topological images of unstained biological samples under physiological conditions [2–5] as well as measuring their mechanical properties at nanometer and pico- to nano-newton ranges [6–8]. It has been applied to measure such properties of proteins and other molecules [9–15] and to probe inter-molecular interactions [16–19] both at the single-molecular level and to manipulate biological samples [20] such as chromosomes [21,22] and DNA [23]. Affinity mapping with the AFM under physiological conditions has been carried out on targeted molecules on a plasma membrane of a single cell and the results were compared between two types of cells [24]. Use of AFM enables us to observe the same sample in real time under physiological conditions and to probe a local area of each cell with high precision and resolution.

The total internal reflection fluorescence microscope (TIRFM) [25] working in an evanescent illumination field allows us to detect the single-molecule fluorescence [26,27] with due mainly to its very low background noise. Although it is regarded as an ultimately sensitive detection system of molecules, it has not been used as a biochemical analytical tool for the detection and identification of biomolecules in combination with other methods, while other detection systems such as the immunoassay or the mass spectrometry are widely used as downstream detection systems. One of the reasons for the current lack of such application may be in the absence of a universal way to bring samples to the TIRFM for detection and identification. Very recently, using immuno-nanoparticle and DNA tag, target proteins in solution were detected at attomolar concentrations [28]. We develop a system for direct identification of biomolecules picked up from biosamples such as intact cell surface and extracts. Our system is also ultra sensitive to a very low population of molecules.

We recently constructed a combined tool of an AFM and a TIRFM [29] to further improve our investigations on receptor mapping [30,31], manipulation of chromosomes [22], and extraction and identification of membrane proteins [32,33]. Here, we report that the presence of GFP protein at the zeptomole level on an AFM-probe was fluorometrically detected with an evanescent illumination. Detection and identification of non-fluorescent proteins were achieved by an immunofluorescence method using specific antibodies to the test proteins which were labeled with fluorescent dyes. We discuss the sensitivity and possible applications of our system that captures protein with a functionalized probe and identifies it subsequently.

2. Materials and methods

2.1. Preparation of GFP

GFP was engineered to introduce a histidine tag at its C-terminus so that the proteins should form complexes with Ni^{2+}–NTA and in the engineered GFP, Cys48 was also replaced with alanine to block reaction between thiol moiety and a gold surface. The modified gene for GFP was prepared by the PCR-based site-directed mutagenesis using "Megaprimer" method [34] with an original GFP gene containing 35SΩ-TP-sGFP (S65T)-nos3′ (pUC18) [35] as a template. The constructed gene was subcloned into an expression vector, pET-24b (encoding histidine tag in original vector) (Novagen Inc., WI, USA) and the nucleotide sequence was verified. The engineered protein was expressed in *Escherichia coli* after the transformation of the plasmid vector [36] and was purified by metal chelate affinity chromatography on Ni–NTA agarose (Qiagen Inc., CA, USA) according to the manufacturer's instruction manual. The purity of the protein was checked on SDS–PAGE [37]. Biotinylated GFP was prepared by a reaction between the engineered GFP (1.4 nmol) and biotinamidohexanoic acid 3-sulfo-*N*-hydroxysuccinimide ester sodium salt (Sulfo-NHS-LC-

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Abbreviations: AFM, atomic force microscope; TIRFM, total internal reflection fluorescence microscope; PDPH, 3-(2-Pyridyldithio) propionyl hydrazine; PBS, phosphate buffer saline

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Biotin) (8 nmol) (Sigma–Aldrich, MO, USA) in 0.1 M borate buffer (pH 8.3, 220 μl) for 2 h at room temperature. The Ni–NTA column (Qiagen) was used to remove unreacted reagents and purify the GFP with imidazole for elution from the column, and the obtained GFP fraction was equilibrated to 0.1 M borate buffer (pH 8.3).

2.2. Modification of AFM-probe with proteins

(both side) Gold-coated AFM-probes (TR400-PB, Olympus, Tokyo, Japan) were used for the following modifications after gentle washing with chloroform and ethanol for 5 min each. Five μmol of 3-(2-Pyridyldithio) propionyl hydrazine (PDPH) (Pierce, IL) in 0.1 M borate buffer (pH 8.3) was reduced on the Reduce-Imm^{TM} column (Pierce, IL) activated with DTT according to the manufacturer's instruction manual, and the reduced PDPH fractions which showed absorbance at 343 nm were collected. For preparing PDPH-probe (Fig. 1), probes were incubated in reduced PDPH solutions for 4 days at room temperature and were gently washed three times for 5 min with fresh milli-Q water in each step. NHS-probe (Fig. 1) was prepared by incubation of PDPH-probe in 10 mM 3,3′-dithiobis (sulfosuccinimidylpropionate) (Pierce, IL) solution in phosphate buffer saline (PBS, pH 7.5) for 1 h at room temperature and was washed with fresh PBS in a similar manner as describe above. For the preparation of Tris/NHS-, GFP/NHS- and bovine serum albumin (BSA)/NHS-probes, NHS-probes were incubated with 1 M tris (hydroxymethyl) aminomethane (Tris), 10 μM GFP or 150 μM BSA overnight at room temperature and subsequently with 150 μM BSA for 3 h at room temperature. In the last step of each reaction, probes were washed three times with PBS. For the preparation of AB-probes, PDPH-probes were incubated with 10 mM biotinamidohexanoic acid 3-sulfo-*N*-hydroxysuccinimide ester (Sulfo-NHS-LC-Biotin sodium salt) (Sigma–Aldrich, MO) for 1.5 h at room temperature, washed with Milli-Q water and subsequently reacted with Streptavidin (3.3 mg/ml) in 0.1 M borate buffer (pH 8.3) and washed three times with the same buffer. For the preparation of btGFP/AB-probes, AB-probes were incubated with biotinylated GFP (0.1 mg/ml) for 1 h at room temperature and washed with 0.1 M borate buffer (pH 8.3). For the preparation of NTA-probes, gold-coated probes were incubated with *N*-[5- (2′-thiolethylamido)-1-carboxypentyl] iminodiacetic acid which was produced by reducing 10 μmol of Dithio (C_2-NTA) (Dojindo, Kumamoto, Japan) in 0.1 M borate buffer (pH 8.3) overnight at room temperature, then successively washed with the borate buffer, Milli-Q water and 1 mM NaOH. The probes were then incubated in 50 mM $NiSO_4$ for 1 h and washed with Milli-Q water.

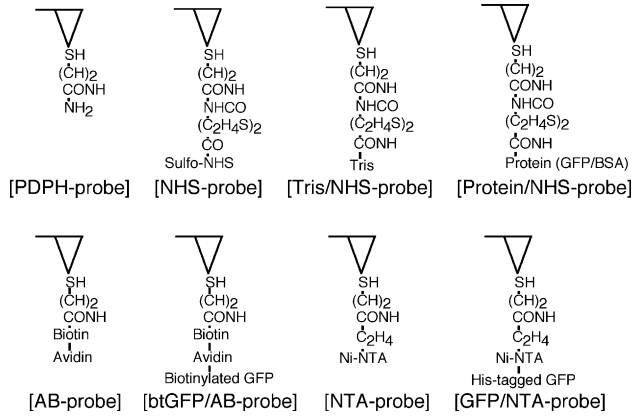

Fig. 1. Modified AFM-probes. The modified probes were constructed on both side gold-coated AFM-probes (TR-400PB, Olympus, Tokyo, Japan). PDPH-probe is functionalized with an amino group and NHS-probe has an amino-reactive NHS (N-hydroxysuccinimide ester) group. Tris/NHS-probe and protein/NHS-probe were obtained from the reaction between NHS-probe and tris (hydroxymethyl) aminomethane (Tris) or a protein such as GFP and BSA, respectively. btGFP/AB-probe was made from the specific interaction between the biotinylated GFP and the streptavidin of AB-probe which was obtained from the reaction between a streptavidin and a biotinylated PDPH-probe. GFP/NTA-probe was obtained from complex between Ni–NTA on a NTA-probe and histidine tag of the engineered GFP. The methods in detail for the modification were described in Section 2.

GFP/NTA-probes were prepared by incubation of NTA-probes with 10 μM GFP for 1 h at room temperature.

2.3. Labeling of monoclonal antibodies with fluorescent dye

Monoclonal antibodies against GFP (JL-8, Clontec Lab., Inc., CA) and human fibronectin (FNH3-8, TAKARA SHUZO, Shiga, Japan) were labeled with Alexa Fluor 532 (Molecular Probes Inc., OR) according to the manufacturer's instruction manual, and FITC-labeled antibodies (Santa Cruz Biotechnology, Inc., CA) against Bcl-2 were directly used in our detection system. Before the labeling, the IgGs of the monoclonal antibodies were isolated from amine contaminants

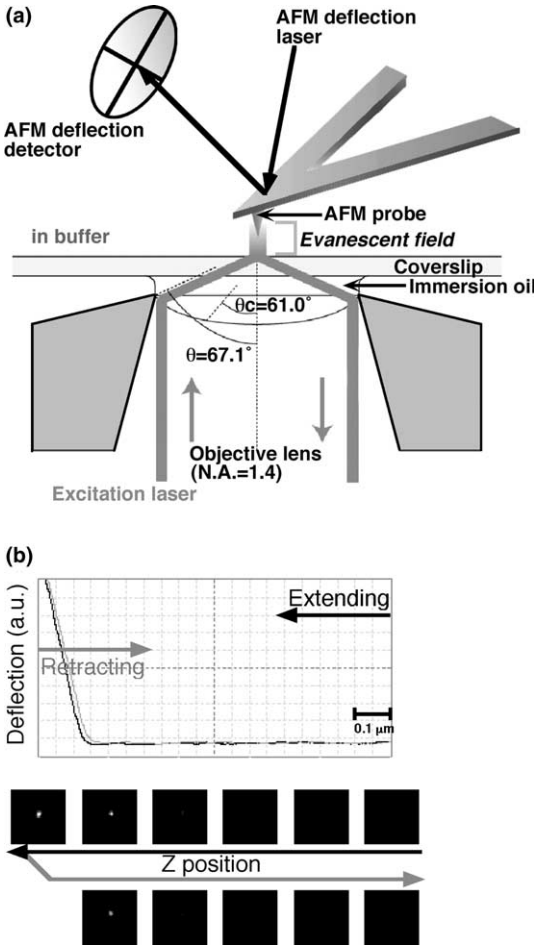

Fig. 2. Detection system of a protein molecule on AFM-probe in evanescent field. (a) Schematic diagram of the detection system that combines AFM with TIRFM. The incident laser was totally reflected using the objective lens with 1.4 in N.A. with over critical angle (θ_c), producing an evanescent field very near to the interfere between buffer and glass surface. The limited incident angle (θ) is calculated as 67.1° from the N.A. and the minimal depth of the evanescent field was calculated as 89 nm from the incident angle and the wavelength (488 nm) of the incident laser. Fluorophore on the probe is excited in the evanescent field very near to the surface of the coverslip. (b) Fluorescence on AFM-probe was observed during measuring a force curve (a curve in the upper column) by AFM (NVB 100, Olympus, Tokyo, Japan) with a probe movement within 1 μm from the glass surface at 0.5 Hz in rate. *Z* position is of the fixed end of the probe. The right end of *X*-axis is the far from the surface. Black and gray lines represent force curves for approaching and retracting from the surface, respectively. Fluorescence images in evanescent field on the glass surface with an excitation at 488 nm were sequentially taken with 0.2-s intervals. Upper and lower sequential panels represent the sequence images in approaching and retracting from the surface, respectively.

such as Tris and BSA using protein G column (Amersham Bioscience, Uppsala, Sweden).

2.4. GFP-containing agarose gel

GFP solutions with 10 times dilutions from 0.05 to 50 μM were separately added to aliquots of 3% SeaPlaque GTG Agarose gel (final concentration = 2.4 w/v%) (TAKARA SHUZO, Shiga, Japan) melted in 0.1 M borate buffer (pH 8.3) at 35 °C, then mixed homogeneously and was dropped on a coverslip (30 × 50 mm, thickness/No.1, MATSUNAMI GLASS, Osaka, Japan). GFP containing agarose gels formed on coverslip (s) were kept from drying in dark until use.

2.5. Microscope

The objective-type TIRFM equipped with an AFM [29] was used in this research as illustrated in Fig. 2. An AFM (NVB 100, Olympus, Tokyo, Japan) was mounted on the stage of an inverted optical microscope (Olympus, IX70, Tokyo, Japan) equipped with a high numerical aperture objective lens (Plan Apo (100×, N.A. = 1.40), Olympus, Tokyo, Japan) for producing an evanescent field. An image intensifier (VS4-1845, VIDEOSCOPE, Sterling, VA), an SIT camera (C2741-08, HAMAMATSU, Shizuoka, Japan) and a digital video cassette recorder (DVCAM, SONY, Tokyo, Japan) were also used for the detection of an emission light and recording the signal. A blue laser (Sapphire 488-20, COHERENT, CA) with 488 nm and 20 mW in wavelength and output power, respectively, and a green laser (Model 4301, Uniphase, CA) with 532 nm and 50 mW in wavelength and output power, respectively, were introduced into the microscope for the excitation of fluorophores. The intensity of blue and green lasers was reduced to 30% of their original power using ND filters for prevention of a rapid bleaching of fluorophores. Scanning electron microscopic images of AFM probes (TR400-PB, Olympus, Tokyo, Japan) were taken with an ELIONIX ERA-800FE (Tokyo, Japan).

3. Results and discussion

3.1. Detection of GFP picked up from agarose gel with AFM-probe

GFP is a good model protein for our fluorescence detection system excited by an evanescent illumination. A modified GFP having a histidine tag at its C-terminus for the formation of complexes with Ni^{2+}–NTA and an Ala replacing Cys48 for preventing –SH from reacting with gold surface was prepared in *E. coli* using a site-directed mutagenesis and expression. The expressed GFP was purified by metal chelate affinity chromatography on a Ni–NTA column. Gold-coated probes (TR400-PB, Olympus, Tokyo, Japan) were functionalized with bifunctional covalent crosslinkers reactive on one end toward amino groups and having a thiol group on the other that reacts with a gold surface. The engineered GFP was immobilized on a probe made amino-reactive with NHS (NHS-probe in Fig. 1, see Section 2). GFP fluorescence on the probe was detected on the TIRFM combined with an AFM (Fig. 2(a)). In this system, fluorescence becomes observable when the AFM-probe is driven into the evanescent field present very near to the glass surface AFM (Fig. 2(b)).

To see if functionalized probes with NHS (Fig. 1) can be used for picking up GFP molecules from agarose gel, one of the probes was driven into agarose gel that contained GFP and kept in the same position for 15 min at 25 °C keeping the depth of indentation to 1 μm as controlled by AFM (Fig. 3(a) and (b)). The probe was then pulled out of the gel and placed in a buffer solution deposited on a coverslip for the examination by the TIRFM (Fig. 2), and the result is given in (Fig. 3(c)). The GFP fluorescence was clearly observed when the concentration of GFP in the gel was 1 μM but it was not detectable when the protein concentration was 0.1 μM or when the functionalized probe was treated with Tris to deactivate amino-reactive crosslinkers prior to incubation in GFP containing gel as shown in Fig. 3(c). The result shows that the functionalized probe was able to pick up GFP from GFP-containing gel.

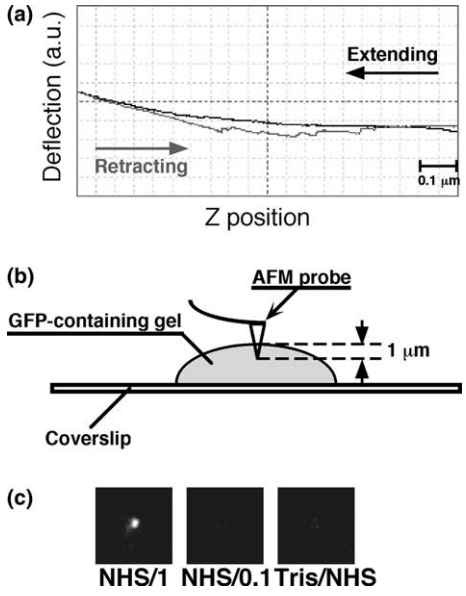

Fig. 3. Picking up GFP from an agarose gel by AFM. (a) A force curve obtained during picking up GFP containing in an agarose gel which was taken with 1 μm scan range and 0.1 Hz scan rate. (b) Schematic image illustrated in the pickup process of GFP by AFM from the agarose gel. The concentration of agarose was 1.5 w/v%. Picking up was carried out by keeping the probes in the agarose gel of 1 μm in depth from the gel surface for 15 min. (c) GFP fluorescence on AFM-probe picked up from the agarose gel. The fluorescence on the probe was detected and took images as described in Fig. 2. The fluorescence was detected on the NHS-probe with the 1 μM GFP containing gel (NHS/1) not with 0.1 μM (NHS/0.1) one and not on the probe having amino-reactive groups blocked with Tris (Tris/NHS).

3.2. Specific detection of proteins on AFM-probe with antibodies

To distinguish different proteins immobilized on a probe, GFP, fibronectin and Bcl-2 were separately immobilized on individual probes using the above-described method and examined using their monoclonal antibodies labeled with fluorescent dyes (Fig. 4). Fibronectin and Bcl-2 are important factors in cell adhesion and apoptosis fields. We prepared the fluorescence-labeled antibodies with an estimated ratio of 3 to 5 dyes to a protein molecule on the basis of absorbance of protein at 280 nm and that of the dye at 530 nm. The labeled proteins were then reacted with NHS-modified probes (Fig. 1) and after the reaction was over the remaining amino-reactive groups on the probe were blocked with BSA. Subsequently the probes were treated with fluorescently labeled antibodies at an appropriate concentration. To examine positive binding of antibodies to the proteins, each probe was examined on the TIRFM for the fluorescence from the antibodies by repeatedly bringing its apex into the evanescent field (see Fig. 2(b)).

In the experiment using anti-GFP antibodies (Fig. 4(a)–(d)), fluorescence was observed from the GFP/NHS-probe but not from probes treated with Tris, fibronectin and Bcl-2, thus strongly indicating that GFP on the probe was specifically recognized by anti-GFP antibodies. Similar specific fluorescence emission was also observed from fibronectin/NHS-probes (Fig. 4(g)) and from Bcl-2/NHS-probes (Fig. 4(k))

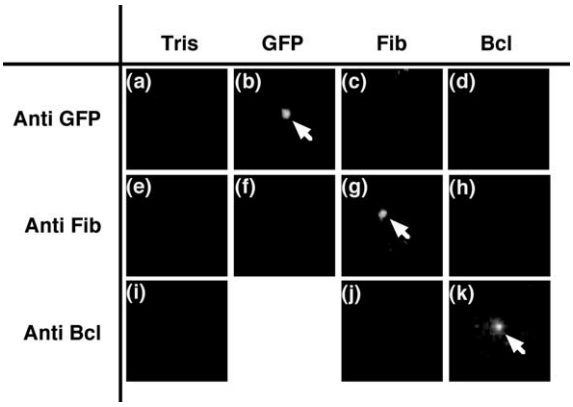

Fig. 4. Specific detection of proteins immobilized on AFM-probe by their specific antibody labeled with fluorescence dye under TIRFM. Tris (hydroxymethyl) aminoethane (Tris) (a,e,i), GFP (b,f), recombinant fibronectin (Fib) (c,g,j) and Bcl-2 (Bcl) (d,h,k) immobilized on the AFM-probe were fluorometrically recognized with anti-GFP antibody (anti-GFP) (a–d), anti-Fibronectin (anti-Fib) (e–h) and anti-Bcl-2 (anti-Bcl) (i–k) in evanescent field. GFP, Fib and Bcl-2 were recognized only by their specific antibody, though fluorescence was not observed on Tris-probe. AFM detection laser leaked from the backside of the cantilever was observed in some panels. The fluorescence on the probe was clearly distinguishable from the AFM laser because of the position and shape of the laser light. The fluorescence-observation in the sequential experiment with each antibody was carried out in the same sensitivity. IgGs for anti-GFP and for anti-fibronectin were labeled with Alexa Fluor 532 (Molecular Probes, OR), and for anti-Bcl-2 was used FITC-labeled one commercially available (Santa Cruz Biotechnology, CA).

when their specific antibodies were used. Fluorescence was not detected on the probes treated with Tris. (Fig. 4(a), (e) and (i)), verifying that the level of non-specific binding was low. These results have shown that different proteins immobilized on the apex of an AFM-probe could be clearly distinguished by their specific antibodies.

3.3. Immobilization of GFP with different binding fashions on AFM-probe

We further examined suitability of different immobilization methods of proteins onto AFM-probes using GFP as a model protein to develop the sensitive molecular detection system on an AFM-probe. As descried above, functionalization of the probes was carried out through binding between thiol group and the surface of gold-coated probes. When amino groups were required on the probe surface, PDPH having an amino and a dithio group was reacted with gold-coated probes after reduction. Although it was reported that the reaction between thiols and a gold surfaces produces a strong bond rather quickly [38], we reacted the gold-coated probe with a long incubation time as long as 4 days with reduced PDPH, which stabilized GFP on the probe.

To confirm the presence of immobilized GFP on the probe, its specific fluorescence was examined on the TIRFM–AFM system (Fig. 2(a)). Probes having immobilized GFP by different methods as described above showed fluorescence (Fig. 5), whereas those treated with tris-(hydroxymethyl) amino methane (Tris) (Tris/NHS-probe in Fig. 1 and Fig. 5) or BSA (BSA/NHS-probe in Fig. 1) had no fluorescence. The fluorescence of GFP immobilized with Ni^{2+}–NTA/histidine-tag was released from the probe by the addition of EDTA, resulting in no fluorescence on the probe (Fig. 5). These results showed GFP

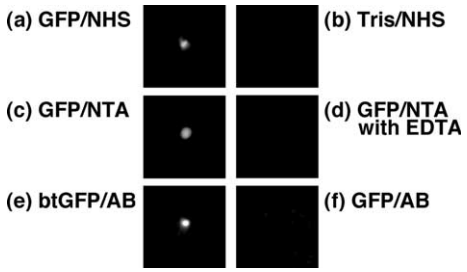

Fig. 5. Fluorescence of GFP on AFM-probe immobilized by different fashions. Fluorescence of GFP were observed on the probe after reaction between GFP and NHS-probe in (a) but nor Tris/NHS-probe in (b), whose active group was blocked with tris (hydroxymethyl) aminoethane as a negative control. The fluorescence was observed on GFP/NTA-probe in (c) and the fluorescence disappeared by addition of 0.1 M EDTA after 30 min (d), and was observed on the AB-probes (Fig. 1) treated with biotinylated GFP producing btGFP/AB-probe (Fig. 1, e) but not with GFP (Fig. 1, f) as a negative control.

was successfully immobilized to AFM-probes in different manners and protein molecules on the tip of the probe can be fluorometrically detectable in our detection system.

3.4. Sensitive detection of GFP on AFM-probe

We have demonstrated that the combined detection system of TIRFM and AFM used in the present study has a very high sensitivity compared with other detection systems such as mass spectrometry or immunoblot methods available to date. We estimated the sensitivity of our system in terms of the detectable number of protein molecules on a probe. For this purpose, we repeated binding experiment of GFP to AFM-probes from agarose gels containing GFP at different concentrations from 0.1 to 1 μM, and concluded GFP fluorescence was detectable with confidence at the lowest concentration 0.25 μM GFP in gel with higher sensitive detection (Fig. 6). The immuno-detection using an anti-GFP antibody labeled with a

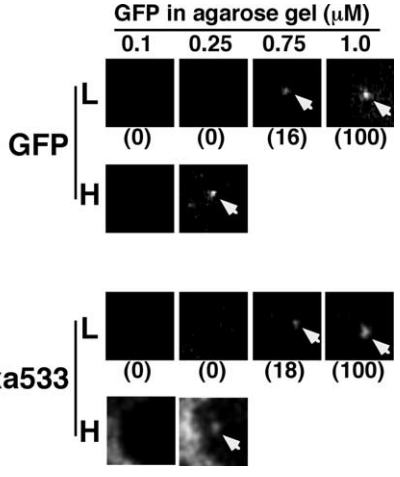

Fig. 6. Sensitive detection of GFP on AFM-probe from agarose gels with different concentrations of GFP. GFP on AFM-probes was detected using GFP fluorescence (GFP) and anti-GFP antibody (Alexa533) after picking up GFP from agarose gels with different concentrations (0.1–1.0 μM) of GFP with NHS-probes (see Figs. 3 and 4). L and H represent higher and lower sensitive detection, respectively. Intensity of the fluorescence measured by NIH image is shown as relative values (%) in parentheses. GFP on AFM-probe was observed in over 0.25 μM GFP gel by GFP fluorescence and the antibody.

fluorescence dye (Alexa533) also gave similar results (see Section 3.2). We need only a small area and volume to pickup protein molecules on an AFM-probe. If we approximate the indented part of the AFM-probe by a cone with both the hight and bottom diameter of 1 μm, as referred from an SEM image of the probe, the required volume was calculated to be 0.26 μm^3. This volume contains about 40 GFP molecules as calculated for the concentration of 0.25 μM.

The upper limit of GFP molecules captured on a probe is calculated on the surface area of the probe illuminated in the evanescent field and the bottom surface area of a barrel structure of GFP. The bottom area of GFP was calculated as 9.6×10^{-18} m^2 from the values of 5 nm in height and 3.5 nm in diameter of the barrel measured by RasMol software and protein data (PDB ID: iemb) [39]. The depth of the evanescent field was calculated as approximately 100 nm when blue laser at 488 nm was totally reflected with an objective lens of N.A. = 1.4. The probe surface area in the evanescent field (depth = 100 nm) was calculated as 1.8×10^{-14} m^2 comparable with the bottom area for 1800 molecules of GFP, indicating that at least the sensitivity was at zeptomole level though rough estimation.

The 1800 molecules of GFP for 0.25 μM would occupy 12 μm^3 in the gel that was around 50 times larger than the conical volume described above. Considering diffusion of the protein molecule with a diffusion coefficient of 4 $\mu m^2/s$ [40], however, about a half thousand of molecules were possible to come to the tip of the probe illuminated with evanescent field within a second. As we had no method to quantify molecules on AFM so far, we could only estimate the upper limit, which was already quite low (highly sensitive). Mass spectrometry has sensitivity at a high attomole level. The newest chemiluminescent detection may be the only comparable method. Our detection system is, however, distinguished for a direct detection without transfer to a membrane and exposure to any film.

Our system owes its high sensitivity to TIRFM, which has low background noise and is known to be able to detect single-molecular fluorescence. In single-molecular detection, one molecule out of many on glass slip was observed, while in our system we have to not only detect but also identify the specific molecules picked up on the probe. Fluorescence background on commercial AFM-probes can also prevent from higher sensitive detection. We believe, however, that our AFM–TIRFM combined microscope will be improved 10–100 times more sensitive by brushing it up. This is the first example showing the universal way a target molecule is captured by AFM-probe and is brought to a ultrasensitive detection system, TIRFM for its identification.

4. Conclusion

We successfully detected protein molecules on an AFM-probe picked up from an agarose gel with a quite high sensitivity, which was estimated to be a zeptomole level (10^2–10^4 molecules) and established their identities using specific antibodies under TIRFM. Proteins were immobilized on a probe in different manners such as through amide bonds, Ni^{2+}–NTA/histidine-tag complex and avidin–biotin interaction.

We have developed a method where the manipulation of protein molecule with AFM is combined with the simultaneous molecular detection fluorometrically in the evanescent illumination. The method shows that AFM is a useful tool to bring a trace amount of sample from quite small area to a sensitive detection system such as TIRFM. Mass spectrometry is used as a sensitive detection device at high attomole (10^7 molecules) level. Our system is, however, 10–1000 times sensitive than the mass spectrometry and is almost the same or somewhat better in sensitivity to a new chemiluminescent detection with immunoblot, which is, so far the most sensitive detection system as a downstream detection system.

We believe that an antibody library can be constructed from the proteins derived from the information of genome sequences, and any protein molecule on the probe is distinguishable by the specific antibody of the antibody library. Our technique can be also applied to detect a protein such as a receptor or biomolecules picked up from a local area of a single cell with AFM in situ for studying dynamics of molecules in post-genome sequencing.

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