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Single Molecule Imaging of Fluorescently Labeled Proteins on Metal by Surface Plasmons in Aqueous Solution

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(Received 18 August 1997)

We report the first real time imaging of single fluorophores attached to protein molecules on metal surfaces in aqueous solution using surface plasmon resonance fluorescence microscopy. The fluorescence was enhanced by the surface plasmons as theoretically predicted for gold and silver. Active movement of single molecules of the fluorescently labeled motor protein, coupled to the ATPase reaction, was observed on the surfaces of gold and aluminum. This microscopy should prove a powerful tool to directly detect single molecule processes in biomolecule systems organized on a metal surface. [S0031-9007(98)06069-4]

PACS numbers: 87.64.–t, 33.50.–j, 87.22.Jb

Metal surfaces play a unique role in biomolecular activities such as the self-assembly and self-organization of proteins and lipids, a topic which has recently attracted a great deal of attention. Some types of self-assembly and self-organization are achieved by covalent bonding of sulfur-bearing compounds on zero-valent metals such as gold, silver, platinum, and copper [1–5]. The controlled construction of ordered supermolecular organic structures on metal surfaces will help our understanding of two-dimensional systems, such as biomembranes that play important roles in molecular recognition, signal transduction, and signal processing which are essential for cellular functions. It also has potential applications in the fields of biosensors and molecular electronics.

In this work, we developed a new technique for directly imaging single molecules of fluorescently labeled proteins on a metal surface by optical microscopy. An aqueous environment is essential in order to observe the events of active biomolecules and hence an optical microscope has been generally used for observing biological materials. However, the size of biomolecules such as protein, lipid, and nucleic acid (less than tens of nm) is below the limit of optical resolution of the microscopes, so single biomolecules cannot be directly observed. In this study, single protein molecules have been visualized as fluorescence images by labeling them with fluorophores. The number of photons emitted from a single fluorophore is large enough so that it can be visualized by a conventional high sensitivity camera. One obstacle is the large background light due to Raman scattering from water molecules, luminescence from dust in solution, and scattered incident light [6]. The background light has been reduced sufficiently enabling single fluorophores to be observed. This has been achieved by minimizing the illumination volume using total internal reflection microscopy [6–8], fine optical probe [9–11], confocal microscopy [12], and by epifluorescence microscopy equipped with carefully selected optical filters [13–17]. We used an evanescent field generated by a surface plasmon resonance at the interface between a thin metal film and solution. The evanescent field is localized near the metal surface with a $1/e$ penetration depth of 100–200 nm in solution. Furthermore, the electromagnetic field generated by the surface plasmons is stronger than that generated by total internal reflection.

Figure 1(a) shows a schematic drawing of surface plasmon resonance fluorescence microscopy. The fluorescently labeled protein molecule was bound to the metal surface on a quartz slide and excited by surface plasmons using the Kretschmann-Raether configuration [18] using an Ar laser (Stabilite 2017, Spectra-Physics, wavelength = 514.5 nm) or a frequency-doubled Nd:YAG laser (YAG laser, model 140-0532-100, Light Wave Electronics, wavelength = 532 nm) for excitation of tetramethylrhodamine (TMR) and a He-Ne laser (GLG5410, NEC, Japan, wavelength = 632.8 nm) for excitation of Cy5. The experimental setup was built on an inverted microscope (TMD300, Nikon, Japan). A laser beam was collimated by a lens. The polarization plane of light was changed by rotating a half wave plate. The laser beam was directed towards a quartz slide using a hemicylindrical quartz prism. The gap between the slide and the prism was filled with an index matching oil. The incident angle was changed by a mirror without changing the focus point using a computer. The beam spot was 100×300 μm at the specimen plane. The reflected light intensity was measured by a power meter head. TMR and Cy5 were simultaneously excited and these fluorescence images were simultaneously observed using dual-color microscopy [19]. The fluorescence was collected by an objective lens [NCF PlanApo $\times$ 100; numerical aperture (NA) = 1.4; Nikon, Japan], passed through dichroic mirrors [DM in Fig. 1(a), custom designed by Sigma Koki, Japan; green light is reflected and red

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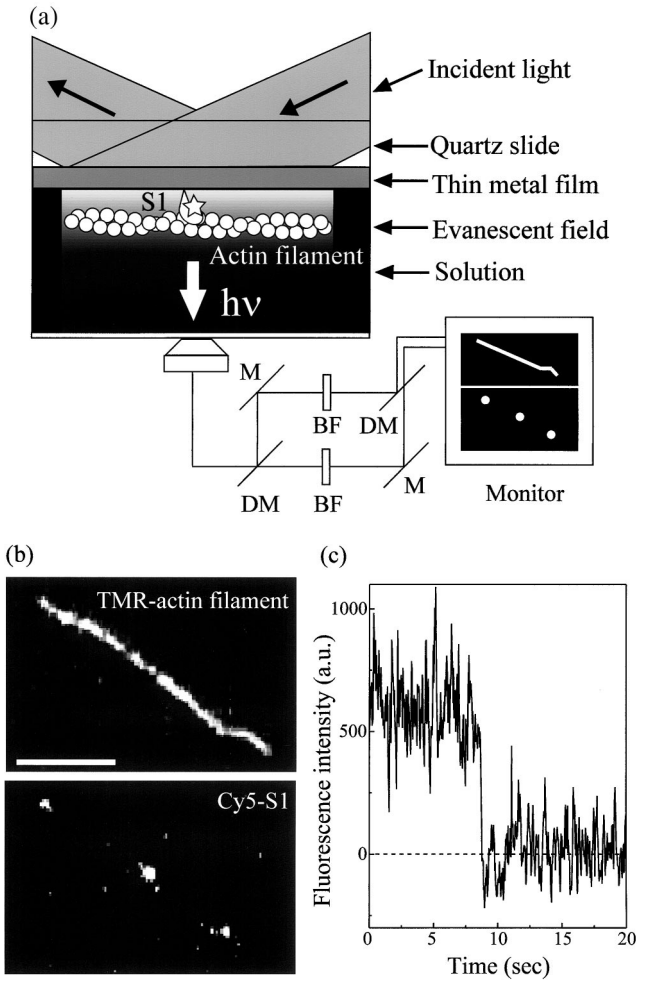

FIG. 1. Imaging of single fluorophores bound to protein molecules on metal by surface plasmons. (a) Schematic drawing of surface plasmon resonance fluorescence microscopy (not drawn to scale). The simultaneous fluorescence images were observed using dual-color microscopy [19]. M, DM, and BF represent mirror, dichoic mirror, and barrier filter, respectively. (b) Simultaneously observed fluorescence images of single fluorophores (Cy5) attached to myosin subfragments (S1) (lower panel) bound to a tetramethylrhodamine (TMR)-labeled actin filament (upper panel) on an aluminum surface with a thickness of 30 nm. The incident He-Ne laser power was 6 mW. 16 video frames were averaged. Scale bar, 5 μm. Medium: 25 mM CH_3CH_2COOK, 5 mM $MgSO_4$, 20 mM HEPES (pH 7.8), and an oxygen scavenger system [23]. (c) Time course of fluorescence intensity in arbitrary units (a.u.).

light is transmitted] to separate fluorescences from TMR and Cy5. The separated fluorescences were passed through barrier filters [BF in Fig. 1(a), 580DF30 for TMR, and 670DF40 for Cy5, Omega Optical, USA) to eliminate the background light [6], and finally imaged by a SIT camera (C2400-08, Hamamatsu Photonics, Japan), coupled to an image intensifier (VS4-1845, Videoscope, USA). For quantitative measurements of fluorescence intensity, an image-intensified CCD camera (C2400-87, Hamamatsu Photonics) was used. The metal was evaporated onto the quartz slides by resistive heating in a vacuum (JEE-400, JEOL, Japan) and the thickness was monitored during evaporation by a film thickness monitor (JTM-200R, JEOL, Japan).

Figure 1(b) shows simultaneously observed fluorescence images of single Cy5-labeled S1 molecules (lower panel) bound to TMR-labeled actin filament (upper panel) on the surface of a 30 nm thin aluminum film. Myosin, a motor protein which produces sliding movement with actin filaments coupled to the ATPase reaction in muscle, was extracted from chicken skeletal muscle and digested to subfragments (S1) by a protease (papain) [20]. S1 is soluble in aqueous solution and causes sliding movement of actin filaments (polymers of actin), as does myosin in the presence of ATP [21]. Actin was extracted from rabbit skeletal muscle [22]. Actin was polymerized to form double-helical filaments and labeled with TMR at a molar ratio of 1:1 [23], i.e., ~28 dye molecules per helical pitch of an actin filament (72 nm in length). S1 was labeled with Cy5 at the molar ratio of 1:1 [6,24]. Actin filaments, labeled with TMR, were decorated with a mixture of Cy5-labeled and unlabeled S1, appropriately adjusted to observe individual fluorescently labeled S1 molecules several μm apart. The fluorescence intensities from single Cy5-labeled S1 molecules were all similar, supporting the notion that the fluorescent spots were due to single fluorophores bound to S1 molecules. Figure 1(c) shows the time course of the fluorescence intensity of a single fluorophore (Cy5) attached to S1. The photobleaching occurred in a single step, providing strong evidence that single molecules had been observed [6]. Single Cy5-labeled S1 molecules could also be observed on silver and aluminum surfaces in the similar manner. Single S1 molecules could also be observed on the three kinds of metals for TMR-labeled S1.

The evanescent field generated by total internal reflection was enhanced by the surface plasmons. Table I shows the fluorescence enhancements of single actin filaments labeled with TMR on various metal surfaces at an incident angle of 81°. This experiment was carried out to confirm that the fluorescence intensity was actually enhanced by electromagnetic field by surface

TABLE I. Comparison of fluorescence intensities of actin filaments labeled with tetramethylrhodamine on various metal surfaces. Each fluorescence intensity has been normalized to the fluorescence intensity on quartz (no metal). Values are written as follows: mean ± standard deviation (n).

Surface	Normalized fluorescence intensity
Quartz (no metal)	1.00 ± 0.26 (86)
Silver 30 nm	12.6 ± 2.6 (64)
Gold 20 nm	1.57 ± 0.29 (37)
Aluminum 30 nm	2.33 ± 0.70 (52)

plasmons as theoretically predicted. The fluorescence intensity was enhanced by the surface plasmons approximately 13-fold on a silver film with a thickness of 30 nm, 1.6-fold for gold film 20 nm thick, and 2.3-fold for aluminum film 30 nm thick. The enhancement is explained by the fact that surface plasmons generally stay longer along the surface than the evanescent field produced by total internal reflection and the electromagnetic field generated by the surface plasmons is intensified near metal surface [18]. The enhancement of electromagnetic field intensity using the surface plasmons has been calculated based on the multiple-reflection theory of the electromagnetic field intensity [18]. The optical constants of metals used are described in a handbook of optical constants [25]. The enhancement T is given by the ratio of intensity transmittance in the presence (T_{metal}) and absence ($T_{\text{no metal}}$) of metal as

$$T = \frac{T_{\text{metal}}}{T_{\text{no metal}}} = \frac{\left|\frac{t_{01}t_{12}\exp(ik_{z1}d_1)}{1+r_{01}r_{12}\exp(2ik_{z1}d_1)}\right|^2}{|t_{02}|^2},$$

where t_{ij} and r_{ij} are Fresnel's transmission and reflectance coefficients, respectively, for one-boundary between medium i and medium j for light incident from i to j (0: quartz; 1: metal; 2: solution [see Fig. 1(a)]), d_1 is the thickness of the metal, and k_{zi} the z component (normal to the surface) of the wave number of the light. The fluorescence enhancement shown in this study agreed well with theoretically predicted enhancement values of electromagnetic field intensity, which were 14.3 for silver and 5.9 for gold. In the case of aluminum, the predicted enhancement for fluorescence was only 0.082. The discrepancy between the predicted and the measured value may be explained by either the uncertainty of the refractive index for the thin aluminum film or the interaction of the aluminum with fluorophores.

Fluorescence quenching of fluorophores near (within tens of nm) or on a metal surface has been reported by many investigators [26,27]. The mechanism of quenching can be explained by fluorescence energy transfer to the metal. However, fluorescence images of single fluorophores attached to protein molecules on the metal surface could be clearly observed. The fluorescence intensity was stronger than that on a glass surface excited by the evanescent field without the metal (Table I). The protein molecules used were ~20 nm in length (S1), ~7 nm in diameter (actin filament), ~10 nm in length (kinesin), and ~25 nm in diameter (microtubule). Therefore, the fluorophores attached to the protein molecules would be adequately positioned from the metal surface not to be significantly quenched.

In order to examine if proteins remain active on the metal surfaces, the sliding movement of single fluorescently labeled actin filaments was observed over myosin molecules bound to three different metal surfaces (silver, gold, and aluminum) in the presence of ATP. The velocities on gold and aluminum surfaces were similar to that on a glass surface (Table II) and in muscle [28], indicating that myosin and actin filaments remained active on the metal surfaces. No sliding movement of actin filaments was observed on the silver surface.

We also observed movement of other motor protein, namely kinesin, on a metal surface. Kinesin transports organelles along microtubules in cells. Kinesin fragments, containing a reactive cysteine were expressed in *Escherichia coli* [8], and the reactive cysteine was labeled with TMR. Tubulin was extracted from bovine brain and polymerized to tubular filaments, microtubules. Microtubules were labeled with Cy5 at the molar ratio of 10:1. Figure 2(a) shows a schematic drawing illustrating how single kinesin molecules were observed. Figure 2(b) shows a fluorescence image of a microtubule bound to an aluminum surface, and Fig. 2(c) shows sequential images of a single kinesin molecule moving along the microtubule shown in Fig. 2(b) in the presence of ATP. The images of the Cy5-labeled microtubule and the TMR-labeled kinesin were observed simultaneously [see Fig. 1(a)]. Table II summarizes the velocities of kinesins moving along microtubules bound to various metal surfaces. The velocities on the gold and aluminum surfaces were as large as those on glass and in a cell [8], indicating that kinesin and microtubules remained active on gold and aluminum surfaces. No movement was observed on a silver surface, suggesting silver is a potential poison for motor proteins.

The technique for imaging single molecules using local illumination can be applied not only to observing the location and movement of fluorophores but also for detecting individual association and dissociation events of the ligands, and chemical reactions of molecules attached to a surface as previously reported [6,7]. Furthermore, structural changes in individual molecules can be monitored directly by fluorescence spectroscopy [29,30]. Thus, the present method should be a very powerful tool for investigating surface chemistry and physics, especially for studies on biomolecules and their self-assemblies on metal surface despite many limitations stated above.

We thank Dr. Hiroshi Kano and members of ERATO project for helpful discussions and Dr. Jan West for critical reading of the manuscript and valuable comments.

TABLE II. Comparison of velocities of single actin filaments over myosin (25 °C) and single kinesin molecules along microtubules (23 °C). Values are written as follows: mean ± standard deviation (n).

Surface	Velocity (μm/s) Actin	Kinesin
Quartz (no metal)	3.7 ± 0.7 (30)	0.67 ± 0.10 (30)
Silver	...[a]	...[a]
Gold	2.2 ± 0.8 (19)	1.2 ± 0.4 (20)
Aluminum	4.7 ± 0.5 (35)	0.77 ± 0.16 (28)

[a]Movements were not observed.

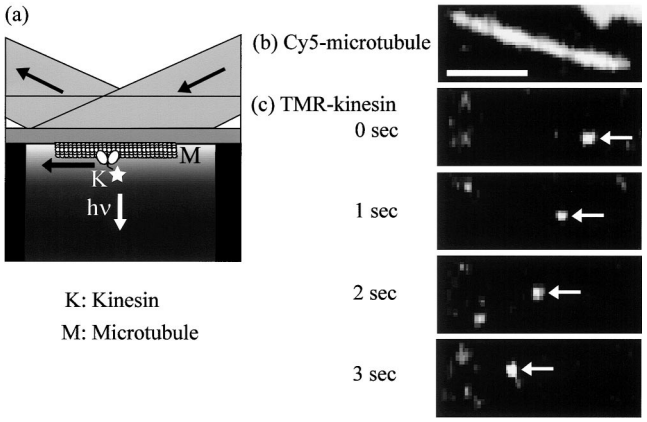

FIG. 2. Movement of a single kinesin molecule labeled with tetramethylrhodamine (TMR) along a Cy5-labeled microtubule bound to an aluminum surface. The incident Nd:YAG laser power was 10 mW. 16 video frames were averaged. (a) Schematic drawing showing how single kinesin molecules are observed. (b) Fluorescence image of Cy5-labeled microtubule. (c) Fluorescence images of a single kinesin molecule labeled with TMR during movement along the microtubule. The images of (b) and (c) were observed simultaneously. The arrows show the movements of a fluorescent spot at 1 sec intervals. Scale bar, 2.5 μm.

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