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ARTICLE *in* ANALYTICAL CHEMISTRY · OCTOBER 1997

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Simultaneous Topographic and Fluorescence Imagings of Recombinant Bacterial Cells Containing a Green Fluorescent Protein Gene Detected by a Scanning Near-Field Optical/Atomic Force Microscope

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A scanning near-field optical/atomic force microscope (SNOAM) system was applied for simultaneous topographic and fluorescence imaging of biological samples in air and liquid. The SNOAM uses a bent optical fiber simultaneously as a dynamic mode atomic force microscopy cantilever and as a scanning near-field optical microscopy probe. Optical resolution of this system was about 50−100 nm in fluorescence mode for fluorescent latex beads on a quartz glass plate. Green fluorescent protein (GFP) is a convenient indicator of transformation and should allow cells to be separated by fluorescence-activated cell sorting. The gene coding to GFP was cloned in recombinant *Escherichia coli*. The SNOAM system used 458- or 488-nm irradiation from a multiline Ar ion laser for excitation of GFP, since a native GFP has been known to give a maximum at 395 nm and a broad absorption spectrum until 500 nm. Topographic and fluorescence images of recombinant *E. coli* were obtained simultaneously with a high spatial resolution which was apparently better than that of a conventional confocal microscope. A nanoscopic GFP fluorescence spectrum was obtained by positioning the optical fiber probe above the bright area of the *E. coli* cells. Comparing topographic and fluorescence images, it can be seen that individual *E. coli* cells expressed different fluorescence intensities. Fluorescence obtained by SNOAM indicated that GFP oxidation possibly occurred near the cell surface. A SNOAM system also indicated the possibility of precise imaging of native cells in liquid.

In the bioanalytical field, there is a growing need for high sensitivity, high spatial resolution, and in vivo monitoring. Electron microscopy has been a powerful tool for giving high resolution and sensitivity. In vivo monitoring, however, is not possible because of the fatal pretreatment of drying and metal coating. Optical microscopy has also clearly supplied powerful tools for analyses of biological targets. Conventional optics had a limit of resolution (about 250 nm) which was based on half the wavelength of visible light, while a near-field optical microscope (NSOM) has demonstrated spatial resolution of only a few tens of nanometers and sensitivity down to the single molecule level.[1−3] This technique is analogous to scanning tunnel microscopy (STM) and atomic force microscopy (AFM). Various kinds of NSOMs have developed by varying the method to control tip−sample separation, such as utilizing STM,[4] lateral shear force,[1,2] and contact-mode AFM.[5] We have already developed a scanning near-field optical microscope (scanning near-field optical/atomic force microscope, SNOAM) in which the dynamic mode AFM method

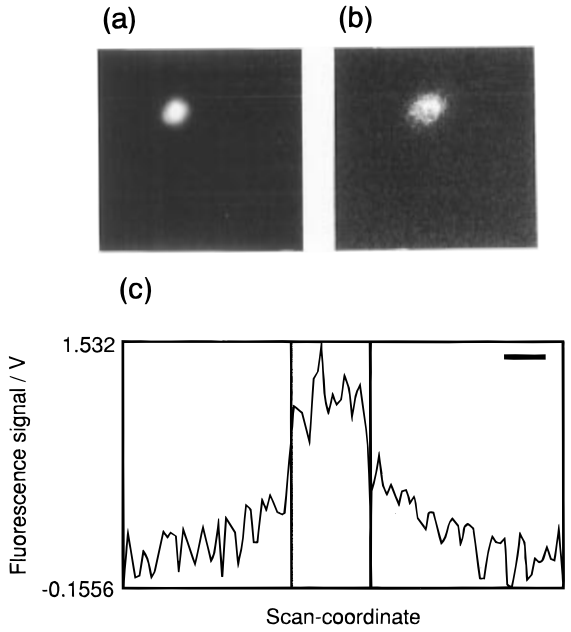

Figure 1. Images of a fluorescent latex bead on a glass plate spread with PVA film: (a) topography, (b) near-field fluorescence, (c) scan line through the center of the bead in (b). Scale bar, 100 nm.

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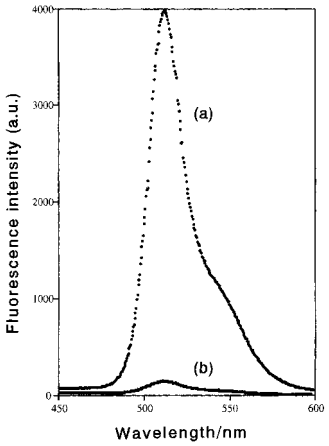

Figure 2. Fluorescence emission spectra of *E. coli*-generated GFP with (a) and without (b) IPTG induction. Emission spectra were recorded with an excitation wavelength of 395 nm.

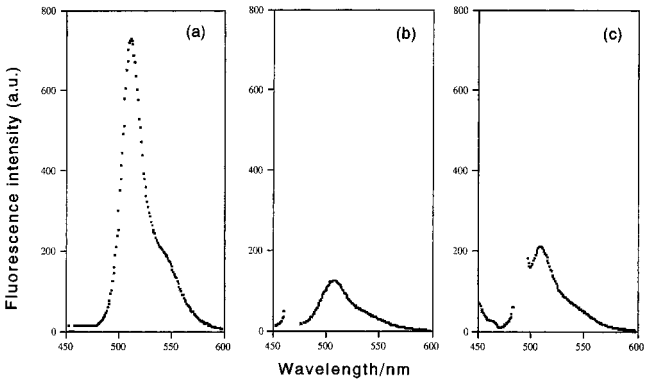

Figure 3. Fluorescence emission spectra of *E. coli*-generated GFP, obtained by collecting excitation at wavelengths of 395 (a), 458 (b), and 488 nm (c). The optical density (660 nm) of the *E. coli* suspension was fixed at 1.2.

was used to control the tip−sample separation.[6,7] An optical fiber with a sharpened tip was bent to allow the probe to be used as a cantilever for AFM, and the vibration amplitude of the cantilever was held constant during scanning. Our group has already demonstrated a SNOAM system which can make simultaneous AFM topographic and fluorescence imaging. Our topographic imaging is more realistic than that based on shear force control. This point has been also supported by our previous report on human chromosome imaging.[8] The SNOAM may be superior in biological observation to other NSOM systems because this system operates excellently in liquids.[9] It is safely applicable for observation of soft samples with great variations in height, such as cultured cells. The SNOAM may be also superior in liquid to other cyclic contact AFM (e.g., tapping mode); the latter uses flat type cantilevers,[7,10,11] while the optical fiber cantilever of SNOAM is round, which helps to reduce viscositic resistance of the liquid. Green fluorescent protein (GFP) was originally isolated from the jellyfish *Aequorea victoria* and has become a useful reporter molecule for monitoring gene expression and protein localization in vivo and in real time.[12,13]

In this paper, spatial analyses of recombinant *Escherichia coli* containing the GFP gene are done using our SNOAM system. Our interest point is how GFP molecules give fluorescence activity inside *E. coli* cells. The *E. coli* cell is the most fundamental cell in molecular biology; however, far-field photomicroscopic imaging is not sufficient for spatial analyses due to its small size. The near-field technique overcomes the limit of resolution of far-field photomicroscopy. The addition of topographic AFM imaging makes our method more quantitative, because cell volume, surface structure, and overall shape of individual cells can be estimated and effective excitation and fluorescence can be discussed.

EXPERIMENTAL SECTION

SNOAM System. The SNOAM system is essentially the same as that reported previously.[10,14] A polychromator and an intensified CCD (ICCD) camera were connected with the previous system in order to obtain nanoscopic spectra. The optical fiber cantilever is mounted on a bimorph and vibrated vertically against the specimen stage at the resonant frequency (typically 15−40 kHz). The vibration voltage applied on the bimorph was between 0.1 and 5 ACV for a 0.11 nm/V bimorph. The vibration amplitude is monitored by detecting the deflection of the laser beam, which is reflected on the ground surface of the optical fiber cantilever. The probe−sample distance is controlled by decreasing the vibration amplitude to an appropriate level when the distance between the probe and the sample decreases. This operation was controlled by a commercialized AFM controller (Model SPI 3700, Seiko Instruments Inc.). Laser beams (458, 488, and 514.5 nm) from a multiline Ar ion laser (maximum 150 mW) were selected by a polychromatic AO modulator and coupled to the optical fiber on the other side of the optical fiber probe. Signal light from the sample is corrected by the objective lens (typically 100× oil immersion type) and separated by the dichroic mirror to the CCD camera and detectors. A photomultiplier and an ICCD camera with spectrometer were connected as the detectors. A liquid chamber was designed for the present experiment. Water and cell culture media were held between the glass plate and an upper window. Both the probe and the sample were immersed in solution. The probe was prepared as described previously.[8] Briefly, an optical fiber was sharpened by chemical etching to make a tip and then bent with irradiation from a CO_2 laser. The probe was coated with a 100−200-nm-thick metal layer (aluminum or gold), and an aperture was made at the point of deposition of metal. Gold was used particularly for the probe operated in liquid. The spring constant of the probe was 2−20 N/m, as calculated on the basis of its shape (rod) and Young's modules for quartz glass. The *Q* factor is typically 200−600 in the air and 40−200 in

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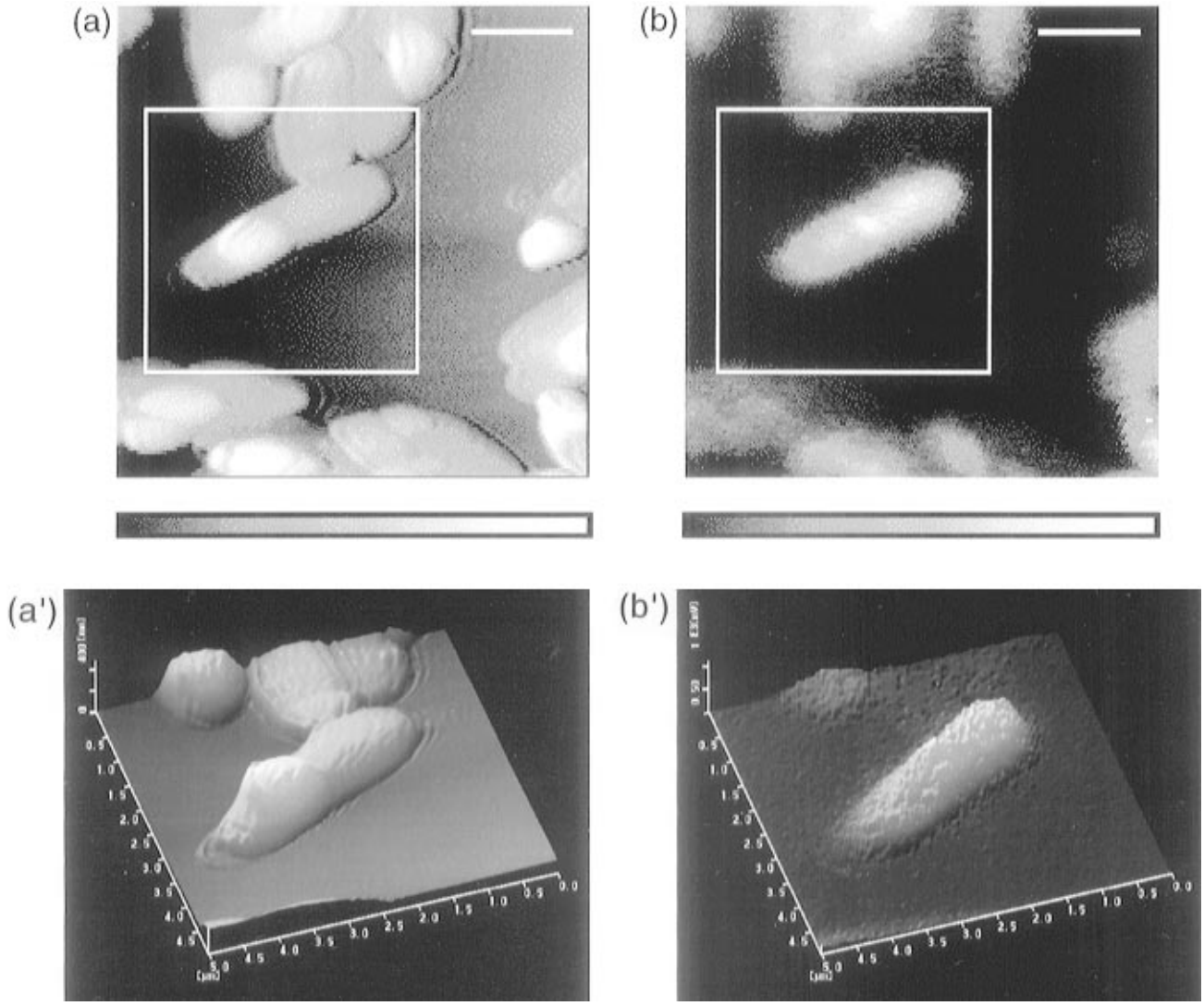

Figure 4. Topographic (a,a′) and near-field fluorescence (b,b′) images of *E. coli*-generated GFP. (a,b) and (a′,b′) were obtained in 7.5 μm × 7.5 μm and white box regions, respectively. The maximum height is 478.6 nm. A fluorescence image was observed at an excitation wavelength of 488 nm. Scale bar, 2 μm.

water.[14] The oscillation amplitude employed was between 10 and 100 nm (0.1−1 in the air or 0.5−5 ACV in water for driving the bimorph). Under typical imaging conditions, the amplitude of the vibration was controlled in a range 80−96% of the maximum vibration amplitude for controlling the sample−probe separation. The interaction force between the probe and the sample is as small as that for normal cyclic contact mode AFM.

Recombinant *E. coli* Containing the GFP Gene. Plasmid pGFP contains the *Eco R*I fragment encoding the GFP complementary DNA. The GFP gene is controlled under *lac* z promoter. This plasmid was purchased from CLONTECH. The *E. coli* strain JM109 was transformed with pGFP and grown at 37 °C. Isopropyl β-D-thiogalactoside (IPTG) was used as an inducer for effective expression of GFP in *E. coli*. For observation with SNOAM, *E. coli* cells were immobilized onto a glass coverslip in the following two ways. A drop of *E. coli* suspension was put onto the coverslip and allowed to semidry when we observed *E. coli* under air. *E. coli* cells were chemically bound onto the glass coverslip when we observed them in liquid. In this case, glass plates were pretreated with 5% γ-aminopropyltriethoxysilane/acetone solution and subsequently 5% glutaraldehyde solution. The *E. coli* suspension was left on the treated glass plate for 30 min and then replaced by water.

Fluorescent Latex Beads and Spectrofluorometer. FluoSpheres fluorescent latex beads were purchased from Molecular Probes Inc. Their particle size was 0.093 μm ±7.4%. Approximate dye excitation and emission wavelengths were 490 and 515 nm, respectively. Fluorescence spectra were obtained with a spectrofluorometer (JASCO FMM, Japan). The emission spectra of the *E. coli* suspension measured from 450 to 500 nm with various fixed excitation wavelengths of 395, 458, and 488 nm.

Confocal Microscopy. The *E. coli* cells were examined using a Zeiss LSM410 confocal laser scanning microscope equipped with an argon ion laser (488 nm/10 mW) and a high-resolution objective (Plan-Apochromat, 63×, NA =1.40). A sequence of eight confocal images was obtained by optical sectioning at different levels in the specimen at intervals of 0.325 μm.

RESULTS AND DISCUSSION

Topographic and fluorescence images of 100-nm-diameter fluorescent beads are shown in Figure 1a,b, where beads were scattered in poly(vinyl alcohol) (PVA) film on a glass plate. The topographic image shows a round bead shape. The fluorescence image was observed with a 488-nm laser beam for excitation and clearly showed the round shape of the fluorescent bead. The profile of fluorescence intensity of the bead shows that the half-

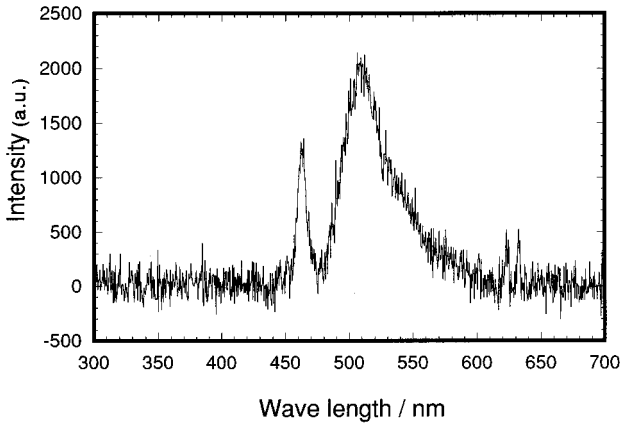

Figure 5. Fluorescence spectrum recorded by positioning the optical fiber probe above the bright area of the *E. coli* cell shown in Figure 7b. The excitation wavelength was 458 nm.

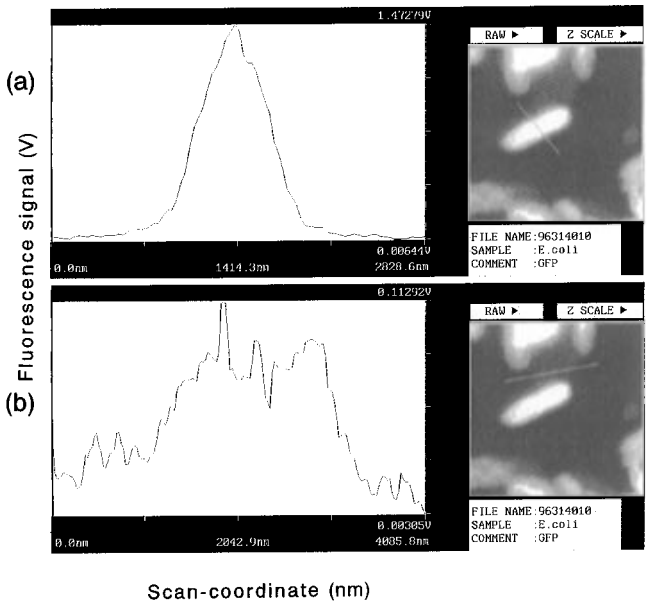

Figure 6. Comparison of profiles of fluorescence intensity between bright (a) and dark (b) *E. coli* cells. Each profile was measured for the bar in the fluorescence image.

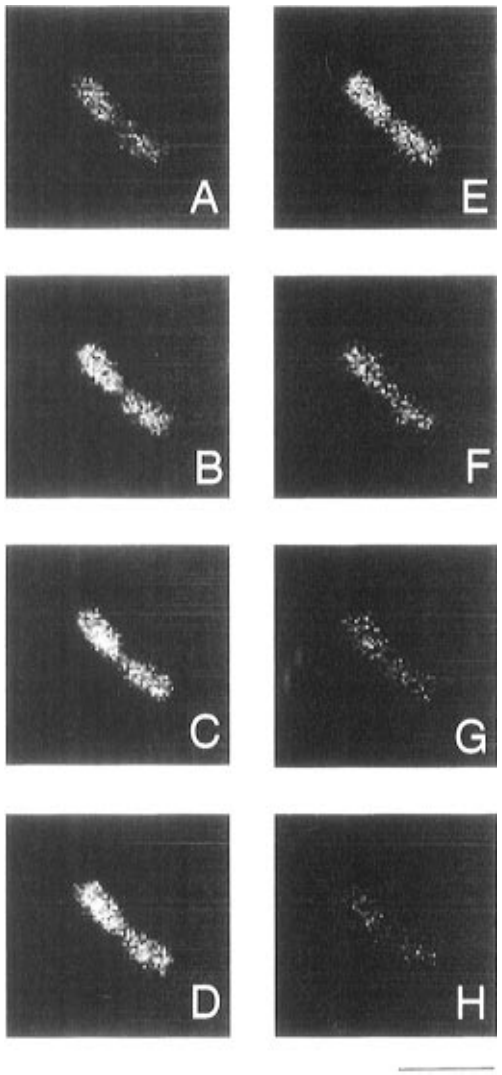

Figure 7. Sequence of eight confocal laser scanning microscopic images obtained by optical sectioning at different levels in the specimen at intervals of 0.325 μm. Scale bar, 2 μm.

width of the fluorescence peak for the bead is about 170 nm (Figure 1c). In this case, the diameter of the bead is approximately 100 nm, and the aperture of the probe we used was 50−100 nm; therefore, a width of about 170 nm is reasonable.

The GFP gene has already been cloned in several kinds of cells, and fluorescence has been measured using purified GFP or the native form expressed in recombinant intact cells.[12] Expression of GFP in *E. coli* results in a readily detected green fluorescence.[12] Figures 2 and 3 show far-field fluorescence spectra which were obtained to determine what wavelength of multiline argon laser is effective for excitation of GFP. Figure 2 shows emission fluorescence spectra of the suspension of recombinant *E. coli* containing plasmid pGFP. Maximum and shoulder peaks were observed around 505 and 540 nm, respectively. The production of GFP gives a clear enhancement in fluorescence over the low-level background in wild-type *E. coli*. IPTG has been known to be an inducer of gene expression. Actually, fluorescence intensity increases by more than 20 times after addition of IPTG. Native GFP gives a maximum at 395 nm and a broad absorption spectrum until 500 nm.[12] Figure 3 shows both wavelengths of 458 and 488 nm were useful for excitation of GFP, and 458 nm gave a better separation between excitation and fluorescence spectra. The SNOAM system uses 458 or 488 nm from a multiline Ar laser as excitation wavelengths. Topographic and fluorescence images of recombinant *E. coli* were obtained simultaneously with high spatial resolution which was apparently better than that of a conventional optical microscope (Figure 4a,b). Figure 5 shows a fluorescence spectrum recorded by positioning the optical fiber probe above the bright area of the *E. coli* cell shown in Figure 4b. This spectrum was estimated to be from a microarea whose diameter was approximately 50−100 nm. This result indicates that the optical near-field technique can surely realize microscopic analyses. Comparing topographic and fluorescence images, it is clear that individual *E. coli* expressed different fluorescence intensities. Figure 6 shows intensity profiles of bright (A) and dark (B) cells. Cell A expressed 10 times higher fluorescence than cell B. A cellular fluorescence expression is dependent on regular transcription, translation, and postmodificaion of protein. The time required for posttranslational maturation of the GFP chromophore can be lengthy and appears to vary in different cell.[15] On the other hand, high-level expression of GFP in *E. coli* may result in incorporation of GFP into inclusion bodies which are nonfluores-

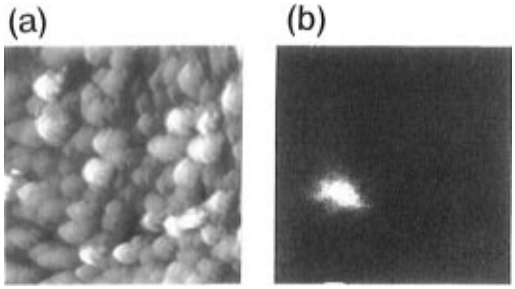

Figure 8. Topographic (a) and near-field fluorescence (b) images of *E. coli* cells without IPTG induction. Fluorescence was recorded at an excitation wavelength of 488 nm.

cent.[15] In Figures 4 and 6, the results of spatial analyses of recombinant *E. coli* containing the GFP gene, done using our SNOAM system, are shown. Spatial resolution is apparently excellent compared to that of confocal fluorescence images shown in Figure 7. SNOAM mainly detects the fluorephore within a tip diameter of roughly 50−100 nm. Oxidative modification of GFP apoprotein is required for expression of green fluorescence.[15] Our result suggests that GFP oxidation is easier near the outer membrane due to rapid oxygen diffusion. To be exact, our SNOAM fluorescence intensity was obtained by excitation light consisting of a near-field component with an exponential evanescent field and a constant far-field background. Obermuller et al. reported on photosignal profile obtained with a 90-nm tip aperture.[16] An exponential decrease of the signal intensity in the first 100 nm is attributed to the near-field component of the light transmitted through a tip aperture. A decay length is estimated to be 20 nm for $\lambda = 633$ nm. The far-field component of the signal is ~10% of the total integrated photosignal at $z = 0$.

On the other hand, confocal microscopy has limits of the depth of field of ~1 μm and diameter at the focal plane of ~0.5 μm.[17,18] *E. coli* cells are approximately 2 μm long, 1 μm wide, and 0.5 μm thick, estimated from topographic images (Figure 4). The spatial resolution of confocal microscopy is not sufficient for imaging of *E. coli* cells due to their small size (Figure 7). Fluorescence obtained by SNOAM indicated that GFP oxidation may have occurred near the cell surface.

Figure 8 shows images of collected *E. coli* cells which were incubated without addition of IPTG. Depression of transcription activity reduced the total fluorescent intensity, but a highly fluorescent cell was rarely observed in Figure 8, indicating that expression of GFP fluorescence was different in the individual cells. A conventional fluorescent microscope was not available for detection of fluorescence from the same sample. As reported previously, our system can be operated in liquid.[9] A scanning probe microscope generally requires biological samples to be immobilized onto a sample plate. In this work, *E. coli* cells were chemically attached to the glass plates and followed by observation in liquid. Figure 9 indicates the possibility of in vivo imaging of biological cells. A topographic profile in liquid was compared with that in the air.

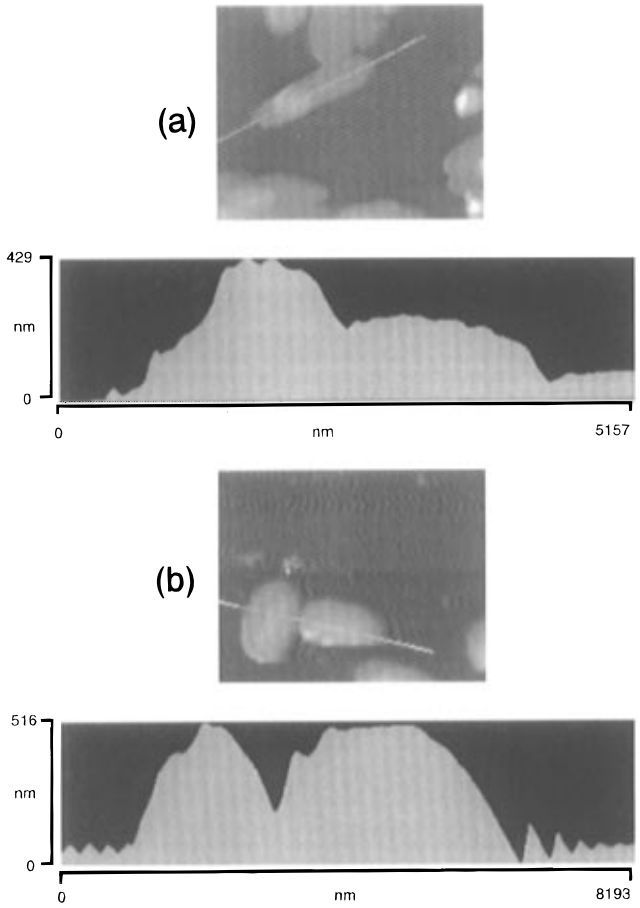

Figure 9. Topographic images and profiles of semidried and aqueous *E. coli* cells. Each profile was recorded for the bar in the topographic image. These cells were fixed onto glass plates as described in the Experimental Section.

As a fusion tag, GFP can be used to localize target proteins, to follow their movement, or to study the dynamics of the subcellular compartments to which these proteins are targeted.[13] SNOAM will be a powerful tool for in vivo monitoring of topographic and fluorescence profiles which give novel information on molecular dynamics of receptor−ligand interaction in living cells. We are incorporating a pico- to millisecond time-correlated single-photon counting capability into microscopy to investigate the quenching of the fluorescence as well as the energy transfer dynamics of the GFP molecules inside biological cells.[19,20]

ACKNOWLEDGMENT

This study was supported in part by the Original Industrial Technology R&D Promotion from the New Energy and Industrial Technology Development (NEDO) of Japan.

Received for review January 17, 1997. Accepted June 20, 1997.[⊗]

AC970060W

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[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1997.