Protein patterning

Nanoscale Patterning of Protein Using Electron Beam Lithography of Organosilane Self-Assembled Monolayers**

Guo-Jun Zhang, Takashi Tanii, Tamotsu Zako,* Takumi Hosaka, Takeo Miyake, Yuzo Kanari, Takashi Funatsu, and Iwao Ohdomari*

The development of nanofabrication approaches to the immobilization of proteins on solid substrates on the nanometer scale is of great importance in the fundamental study of proteomics, diagnosis, and drug discovery. Such methods can enable the fabrication of protein nanoarrays of well-ordered feature size, shape, and pitch, hence offering the potential to develop protein biochips and biosensors more economically and efficiently. Recently, there have been several strategies developed for patterning surfaces with proteins on the nanometer scale using nanolithographic techniques.[1–10] Direct-write patterning methods (dip-pen nanolithography), which use a functionalized AFM tip for delivery of molecules from the tip to the surface, are a promising tool in the fabrication of protein-based nanostructures.[1–4] However, the main disadvantage of scanning-probe-based lithography is the long writing time. Moreover, with this methodology it is difficult to achieve an ultrahigh resolution down to several nanometers for location of a small number of molecules, down to a single molecule, inside one pattern as the feature size is controlled by wetting or diffusion. Such studies on the spatial and selective immobilization of a single molecule will benefit the understanding of single molecule events while retaining bioactivity.

Nanopatterning of self-assembled monolayers (SAMs) by electron beam (EB) lithography has been developed to produce organic templates for various nanofabrication processes.[11–13] Such organic templates can be selected to resist protein adsorption with the knowledge of the variation in protein affinity toward different SAMs.[8] However, little is known of protein adsorption on nanopatterned fluorinated SAMs. In addition, because of the use of ultrathin SAMs with monomolecular thickness as the resists, EB lithography of organosilane SAMs is capable of attaining sub-10-nm patterning to create high-density arrays while still retaining high throughput and reproducibility, which will bring the potential for reaching single molecule precision.[12] Therefore, EB lithography of SAMs provides a method of patterning substrates with proteins with ultrahigh resolution down to sub-10 nm on the scale of a single molecule. However, production of arrays of single molecules requires the maintenance of the biomolecules' native functionality once patterned.

Driven by these goals, we selected green fluorescent protein (GFP) as a model protein.[14] GFP is a spontaneous-fluorescence protein isolated from the Pacific jellyfish *Aequorea victoria*, and is unique among fluorescence proteins in the sense that its fluorophore is not a separately synthesized prosthetic group but composed of modified amino acid residues within the polypeptide chain. GFP emits light following the excitation of its internal fluorophore, which shows that GFP loses its activity if it has no fluorescence. In general, patterning technology requires that patterned proteins maintain their biological activity. Thus, GFP is an ideal protein for the elucidation of protein activity during patterning. In this study, we used a method of chemical vapor deposition (CVD) to deposit 1*H*,1*H*,2*H*,2*H*-perfluorodecyltriethoxysilane (FDTES) SAMs on a silicon surface for nanofabrication using EB lithography. Moreover, we introduced a cysteine residue at the c-terminal end of the protein by site-directed mutagenesis for the site-specific modification of protein with biotin. Then we developed a novel method of generating nanoscale protein patterns in a stepwise fashion with high resolution, by using the EB lithography of SAMs in combination with a high-affinity biotin–avidin system. As schematically illustrated in Figure 1, our technique produces nanoscale patterns of FDTES SAMs on a silicon substrate by EB lithography, followed by the covalent immobilization of biotin on the nanopatterned regions, the subsequent molecular recognition of streptavidin, and finally the binding of a biotinylated GFP through strong noncovalent biotin–avidin interactions. The nanopatterned proteins yielded by our method retain their biological activity, as shown by observing the resulting fluorescence of the immobilized GFP.

Organosilane SAMs have been used as ultrathin resists in EB lithography to produce nanoscale patterns.[11,12] Fluo-

[*] Dr. G.-J. Zhang, Prof. I. Ohdomari
Nanotechnology Research Center and
Institute of Biomedical Engineering
Waseda University
Waseda Tsurumaki-cho 513, Shinjuku-ku, Tokyo 162-0041 (Japan)
Fax: (+81) 352-869-076
E-mail: zhang@ohdomari.comm.waseda.ac.jp

Dr. T. Zako,[+] Prof. T. Funatsu
Laboratory of Bio-Analytical Chemistry
Graduate School of Pharmaceutical Sciences
The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033 (Japan)
Fax: (+81) 48-462-4658
E-mail: zako@riken.jp

Dr. T. Tanii, T. Hosaka, T. Miyake, Y. Kanari, Prof. I. Ohdomari
Department of Electronic Engineering and Bioscience
School of Science and Engineering, Waseda University
3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555 (Japan)

Prof. I. Ohdomari
Kagami Memorial Laboratory for Materials Science and Technology
Waseda University, 2-8-26 Nishi-waseda, Shinjuku-ku, Tokyo 169-0051 (Japan)

[+] Current address:
Bioengineering Laboratory, RIKEN (The Institute of Physical and Chemical Research)
2-1 Hirosawa, Wako, Saitama 351-0198 (Japan)

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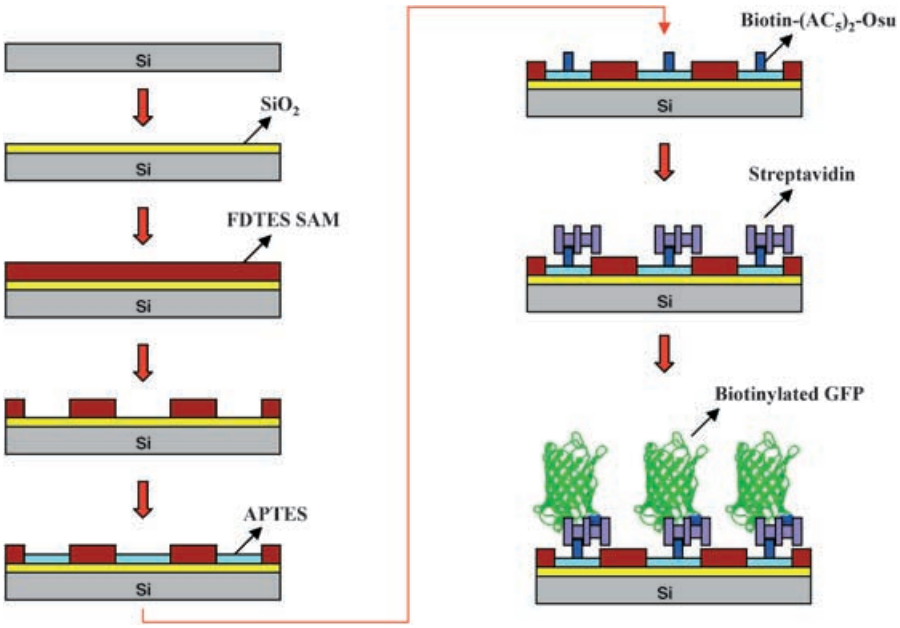

Figure 1. Process diagram of biotinylated GFP nanopatterning by EB lithography of organosilane self-assembled monolayers at a silicon surface (for details, see text).

rinated trichlorosilanes were studied previously and they were found to yield well-ordered monolayers on oxidized surfaces and serve as self-developing electron-beam resists by a liquid-phase deposition method on a modified silicon surface.[13] However, such a method always results in microdefects on the surface. To form a homogeneous monolayer, a CVD method was employed to deposit FDTES SAMs on a silicon substrate. To obtain a good silane monolayer, careful attention should be paid to the procedure, particularly with regard to temperature and humidity. In this experiment, a silicon oxide film was grown on n-type Si(100) wafers, which were activated using APM ($NH_4O_4/H_2O_2/H_2O$ (1:1:4)) solution, as described previously.[15] Then the wafers were placed inside a Teflon chamber filled with a solution of FDTES (300 µL). The chamber was sealed and incubated in an oven at 45 °C for 3 h in a N_2 atmosphere. The samples were then sonicated twice for 10 min in ethanol. Ellipsometer measurements were taken to check the thickness of the FDTES SAM formed. The resulting SAM observed by AFM was homogeneous (data not shown) and the mean thickness of the FDTES SAMs was approximately 1 nm.

EB lithography of the FDTES SAMs was performed using a Hitachi S-4200 instrument to produce nanopatterns by exposing the monolayer to an electron beam at a dose of 480 $\mu C\,cm^{-2}$ at 20 keV. The patterned monolayer was developed in buffered hydrofluoric acid (BHF) for 10 s, thus leaving exposed SiO_2 nanodots in the monolayer. The exposed oxide nanopatterns were selectively reacted with a solution of 2% 3-aminopropyltriethoxysilane (APTES) in 95% ethanol/water for 1 h after the substrates were activated. As a result, the nanopatterned regions were modified with amine groups, while the unpatterned regions were covered with FDTES. Field-emission scanning electron microscopy (FE-SEM) was employed to observe nanostructures composed of two different types of organosilane layers. Such chemical information in nanoscale regions plays an important role in the further fabrication of nanopatterned SAMs. Saito et al. observed the micropatterns composed of two different types of organosilane SAMs by FE-SAM.[16] However, they have not studied micro- or nanostructures composed of APTES/FDTES. Due to the FE-SEM contrast between APTES and FDTES caused by the number of emitted secondary electrons in the different regions, clear contrasts are visible between the two different layers by FE-SEM (S-4800, Hitachi) at low acceleration voltages (0.5 kV). Figure 2 shows FE-SEM images of binary-layer microstructures and nanostructures consisting of APTES/FDTES layers. Bright regions correspond to the areas modified with amine groups in the microstructures (Figure 2A) and nanostructures (Figure 2B), while dark regions correspond to the FDTES SAMs. However, the signal-to-noise ratio is slightly low in Figure 2B, probably

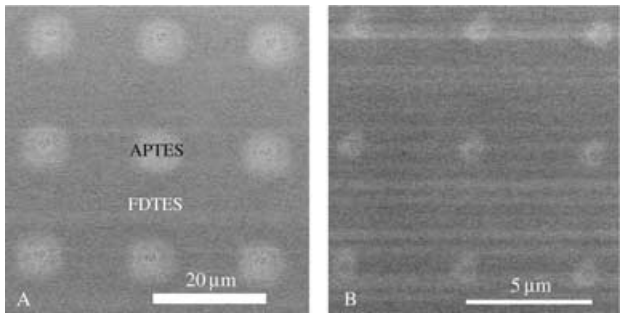

Figure 2. FE-SEM images of APTES/FDTES binary-layer micropatterns (A: 5 µm size, 20 µm pitch) and nanopatterns (B: 500 nm size, 5 µm pitch).

because the FE-SEM contrast between APTES and FDTES is not high. These results indicate that FE-SEM could provide information on the amine-modified regions of the nanopatterned SAMs with a monomolecular thickness.

Biotin was covalently bound to the amine-modified exposed regions of the patterned monolayer by reacting it with a solution of 5-[5-(*N*-succinimidyloxycarbonyl)-pentylamido]hexyl D-biotinamide (5 $mg\,mL^{-1}$, biotin-$(AC_5)_2$-Osu; Dojindo Laboratory, Japan) in DMF for 30 min as previously described.[17] To demonstrate immobilized biotin in the patterned regions, fluorescently labeled streptavidin-Cy5 conjugate was deposited and bound to the biotin layer by incubating the substrates with 1 µM streptavidin-Cy5 conjugate in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) for 30 min at room temperature. After molecular recognition, the samples were washed in PBS and imaged by epifluorescence microscopy, which confirmed that the streptavidin

was selectively bound to the biotin pattern on the surface. As shown in Figure 3A and B, protein nanostructures with dot arrays could be visualized easily, which indicates our ability to generate nanoscale protein patterns by EB lithography of the FDTES monolayers. To verify that the formation of streptavidin patterns was caused by the binding of the patterned biotin with streptavidin and not by nonspecific adsorption of the protein, a control experiment was performed in which streptavidin-Cy5 conjugate (1 μM) was applied to the amine-modified exposed regions of a patterned monolayer prior to biotin immobilization. Another control experiment, wherein a biotin pattern was incubated with streptavidin-Cy5 conjugate (1 μM) that had previously been treated with D-biotin aqueous solution (50 mM, Molecular Probes) to block four binding sites for biotin was conducted. In both control experiments, the fluorescence of streptavidin-Cy5 conjugate was absent in nanopatterned regions (Figure 3C and D), which confirmed the specificity and selectivity of streptavidin binding to biotin in the patterning process.

Biotin and streptavidin binding has been confirmed and the attachment of streptavidin to surface-immobilized biotin leaves two biotin binding sites that can be used to subsequently pattern other biotinylated proteins. Prior to biotin–GFP patterning, a streptavidin pattern was deposited and bound to the biotin pattern by molecular recognition during a 1 min incubation period with streptavidin (100 $\mu g\,mL^{-1}$; Pierce) in PBS. To demonstrate the biological functionality of patterned streptavidin, the resulting streptavidin pattern was incubated with biotin–GFP (500 nM) for 1 min. Green fluorescence (from GFP) was observed exclusively from the patterns. GFP nanostructures with dot arrays can clearly be observed in Figure 4A–C. In particular, GFP was successfully patterned onto 100-nm features, which showed that GFP does not lose its bioactivity while exhibiting strong green fluorescence even in such small features (Figure 4C). Notably, during a short incubation time, the strong affinity of biotinylated GFP for streptavidin allows GFP to bond strongly to a streptavidin pattern, while GFP in the unexposed, hydrophobic regions is rinsed off. However, fewer proteins are patterned inside a 100-nm nanostructure, which leads to the rather lower fluorescence intensity visible in Figure 3C compared with that in Fig-

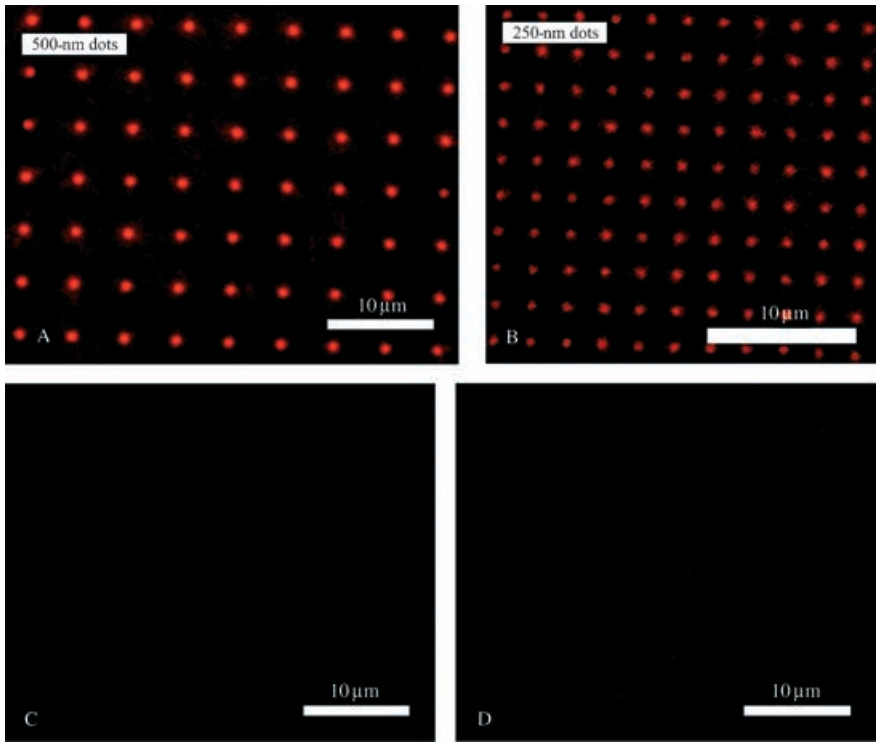

Figure 3. Epifluorescence images of Cy5-labeled streptavidin bound to patterns of immobilized biotin on silicon. A) Dot arrays: 500-nm dots, 5-μm pitch; B) dot arrays: 250-nm dots, 2.5-μm pitch. C) No adsorption of streptavidin-Cy5 conjugate on the amine-modified nanopatterns without biotin immobilization. D) No binding between a biotin pattern and streptavidin-Cy5 conjugate on which the four active binding sites are blocked.

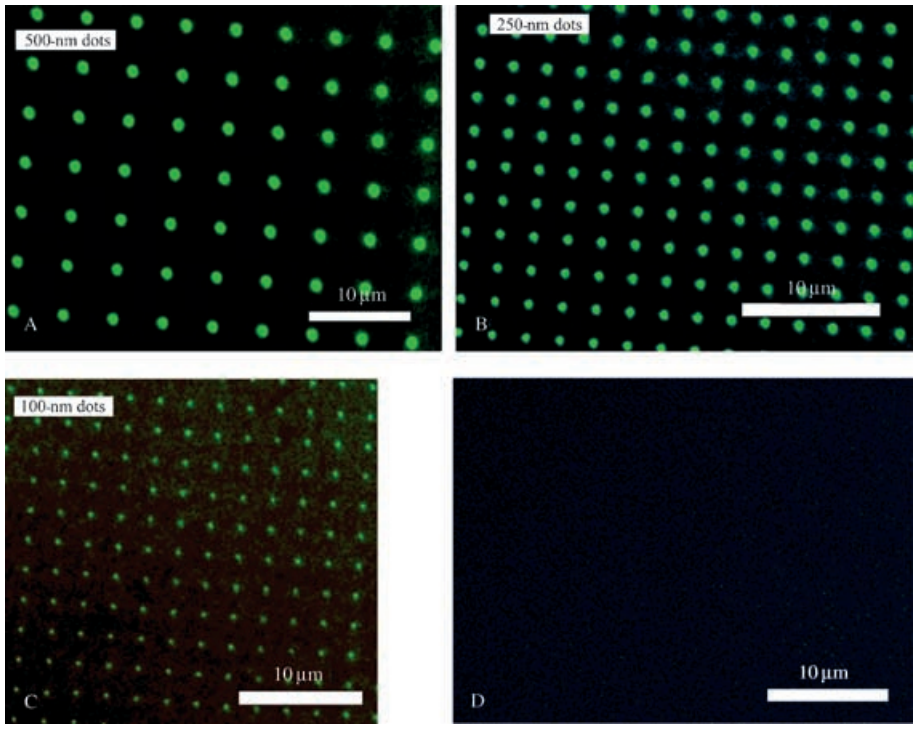

Figure 4. Epifluorescence images demonstrating the maintained bioactivity of patterned GFP. Biotinylated GFP was patterned using the high-affinity biotin–avidin system in 500-nm dots with a 5-μm pitch (A), 250-nm dots (B), and 100-nm dots (C) with a 2.5-μm pitch; no green fluorescence is visible without streptavidin as a bridge (D).

ure 3A and B. To confirm the specific binding of biotin–GFP to the streptavidin pattern, biotin–GFP was incubated directly with the biotin pattern without treatment with streptavidin. Green fluorescence was hardly observed in the nanopatterned regions (Figure 4D). These experiments with biotin–GFP demonstrate that a protein can be patterned on the nanoscale using the streptavidin–biotin system while maintaining the protein's activity.

One of the important criteria for protein patterning is the ability to avoid nonspecific binding of proteins. Namely, the technique has to produce a high density of proteins in patterned regions while preventing adhesion of those proteins in unpatterned regions. Basically, protein adhesion depends on the nature of the protein and the properties of the surface. Usually proteins strongly adsorb on hydrophobic surfaces if given enough time, which likely leads to many difficulties in achieving nanoscale protein patterning with hydrophobic SAMs. However, it is found that only a higher concentration and longer reaction time might result in protein attachment to methyl-terminated surfaces.[8] Consequently, nonspecific protein binding is controllable on hydrophobic surfaces by controlling the reaction conditions. As demonstrated by another group,[18] the correction of adsorption of protein with the surface hydrophobicity was compared for different materials, and the protein adsorbed on an octadecyltrimethoxysilane (ODS) surface was approximately twice that adsorbed on a fluorinated surface.

The EB lithography of ODS SAMs has been developed for the patterning of DNA nanostructures on a silicon surface.[11] Alternatively, a parallel experiment was performed using the EB lithography of an ODS monolayer instead of the FDTES monolayer for sequentially patterning biotin, streptavidin, and finally biotinylated GFP. After patterning with GFP, the surface was imaged by epifluorescence microscopy (see Figure 5). As shown in Figure 5B, 250-nm dots are difficult to distinguish from the background, so we were unable to observe sub-250-nm dots. The fluorescence images of the GFP patterns showed a very low signal-to-noise ratio on the nanoscale due to stronger adhesion of GFP to ODS SAMs than to FDTES SAMs. The results further demonstrate the different adsorptions of proteins onto ODS and the fluorinated monolayers, as described.[18] However, GFP adsorbed on the ODS SAM outside nanostructures is still somewhat fluorescent and the fluorescence state of GFP does not seem to be sensitive to the environment, probably because the fluorophores of GFP are protected by the cage itself.

In conclusion, we have developed a technique of immobilizing proteins with nanoscale resolution by streptavidin–biotin binding. The biological activity of the immobilized proteins is maintained in each patterning process. The use of the EB lithography of a fluorinated SAM as an ideal template enables the high-resolution patterning of proteins with a high signal-to-noise ratio. Feature sizes down to 100 nm in diameter have been observed. Since our method uses a high-affinity biotin–streptavidin binding system, it can be extended to pattern any proteins of interest by introducing a tag sequence at the c-terminal end. This versatile and highly specific technique of producing nanoscale protein patterns enables the integration of bioactive proteins into biosensors. More importantly, we believe that this technique is capable of producing arrays of single molecules by further miniaturizing the feature sizes.

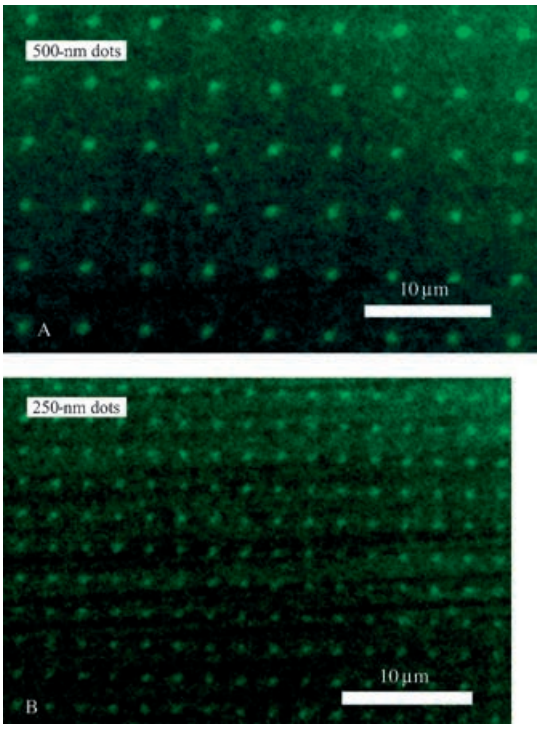

Figure 5. Epifluorescence images of patterned GFP on an ODS SAM. A) Dot arrays: 500-nm dots, 5-μm pitch; B) dot arrays: 250-nm dots, 2.5-μm pitch.

Experimental Section

GFP is composed of a single chain of 238 residues or amino acids. To obtain biotinylated GFP, Lys 238, which is the c-terminal residue of GFP, was replaced by cysteine (termed GFPcys) and labeled with biotin maleimide. GFPcys was obtained using a QuikChange site-directed mutagenesis kit (Stratagene). Thirty-six nucleotide primers, 5′-ATGGATGAACTATACTGTTAGGAATTCGAGCTCCGT-3′ and 5′-ACGGAGCTCGAATTCCTAACAGTATAGTTCATCCAT-3′ (mutated nucleotides are underlined), were used for codon substitution, to TGT (Lys to Cys) in pET-GFP.[19] The mutation was confirmed by DNA sequencing, and the mutant DNA was designated as pET-GFPK238C. GFPcys was expressed using *Escherichia coli* BL21 (DE3), transformed with pET-GFPK238C. Expression of GFP was induced by the addition of isopropyl-β-D-thiogalactopyranoside (1 mM, Wako, Osaka, Japan) at the middle log-growth phase (≈0.5 absorbance at 600 nm) and culturing at 37 °C for 2 h. GFP was purified from the disrupted *E. coli* cells using a Butyl-Toyopearl column (Tosoh, Tokyo, Japan) as described.[20] GFP was stored as a 65 % saturated ammonium sulfate suspension until use. Before labeling, GFPcys was pretreated with dithiothreitol (5 mM). After removal of the dithiothreitol by gel filtration through a Sephadex G-25 column equilibrated with buffer (25 mM HEPES of pH 7.4, 100 mM KCl), GFPcys was incu-

bated with biotin-$PEAC_5$-maleimide (Dojindo Laboratory, Japan) for 1 h at room temperature. The labeled GFPcys (bio-GFP) was separated from unreacted reagents by gel filtration. GFPcys and bio-GFP showed similar absorption spectra to that of the wild-type GFP, which suggested that GFP was not functionally affected by the mutation and modification (data not shown).

Keywords:
biorecognition · nanolithography · patterning · protein design · self-assembled monolayers

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