Engineered Green Fluorescence Proteins for Proteomics and Biomolecular Electronic Applications

Ranieri Bizzarri,*[1] *Vittorio Pellegrini*,[1] *Caterina Arcangeli*,[1] *Aldo Ferrari*,[1] *Riccardo Nifosì*,[1] *Pasqualantonio Pingue*,[1] *Valentina Tozzini*,[1] *Mauro Giacca*,[2] *Fabio Beltram*[1]

[1] NEST-INFM, Scuola Normale Superiore, Piazza dei Cavalieri 7, I-56126 Pisa, Italy
E-mail: r.bizzarri@sns.it
[2] Molecular Medicine Laboratory, International Center for Genetic Engineering and Biotechnology, Padriciano 99, I-34012 Trieste, Italy

Summary: The discovery of the photochromic characteristics of engineered green fluorescent proteins (GFPs) allows new proteomics and biomolecular electronic applications. In particular, photoreversibility among two distinct optical states can lead to the realization of a bio-optical high density storage memory. Here we review our recent work on an optically bistable GFP and we report the recent developments of self-assembly methods for spatial immobilization of proteins into well-definite 2D patterns.

Keywords: fluorescence; lithography; molecular dynamics; nanotechnology; protein photophysics; self-assembly

Introduction

The fascinating possibility of studying and manipulating individual functional parts of matter has become a reality in recent decades. Nanoscience is emerging as a research field entailing different disciplines, from physics to chemistry, from materials science to molecular biology. One of the most exciting challenges in the last few years is the detection, monitoring, and control of single molecule.[1-2] From a fundamental point of view, manipulation and probing of single molecules can provide new and deeper insight into a host of phenomena. Actually, this new physical research fits well with the recent advances in molecular biology and genetic engineering, providing ideal strategies and tools for approaching some fascinating problems both in proteomics, like protein dynamics in living cells or protein-protein interaction, and in bioelectronics. In this framework, some biological systems such as the green fluorescent protein (GFP) class stand as naturally evolved optimized structures for fluorescent probing at single-molecule, single-event scale.

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GFP is a medium-sized, intrinsically fluorescent protein (238-amino acids) characterized by a cylindrical three-dimensional structure with a diameter of 24 Å and a height of 42 Å.[3] The chromophore, located at the center of the cylinder, is a photoexcitable green-light emitter autocatalytically generated by the post-translational modification of a 3-amino acid sequence (Ser65-Tyr66-Gly67),[4] and consists of the hydroxybenzyl side chain of Tyr66 and the imidazolinone ring formed by cyclization of the tripeptide (Figure 1).

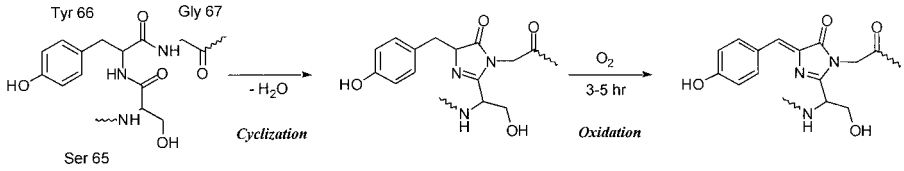


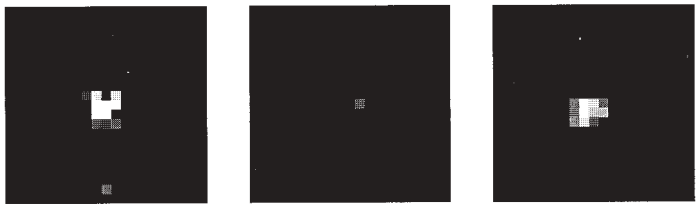
Fig. 1. Formation and structure of GFP chromophore.

The original demonstration that GFP can be functionally expressed by heterologous cells[5] paved the way to its use as *in vivo* fluorescent label of biologically relevant proteins. Indeed, GFP fusion protein technology has become the method of choice for the analysis of a number of biologically important phenomena.[6] The noticeable attention that the spectral characteristics of its emission and absorption bands received[4,7] allowed the design of several mutants of GFP displaying intensified fluorescence, enhanced brightness, photostability, high quantum yield, and other properties tailored to specific nano-applications.[8]

Design of GFP Mutants Displaying Photobleaching Reversal

The peculiar optical properties of GFP allowed its single-molecule detection, firstly demonstrated for the F64L/S65T mutant of wt-GFP (Enhanced GFP or EGFP).[9,10] EGFP intensity records at single-molecule level are characterized by "blinking transitions" (the fluorescence switches on and off sharply and quickly) terminated by an irreversible disappearance of fluorescence, a process called "photobleaching". Once photobleached, EGFP emission cannot be recovered by using optical irradiation. By means of molecular engineering techniques, photobleaching reversal of two yellow-shifted GFP mutants was firstly shown by Dickson in 1997 using Hg arc lamp illumination.[10] Starting from this result and on the basis of a theoretical analysis of the chromophore properties of EGFP, our group was able to demonstrate that one specific point mutation (T203Y, threonin into tyrosin at position 203 in

the amino-acid sequence) in the primary structure of EGFP yields easy and efficient optical control of the fluorescence down to the single-molecule level by means of two different laser lines.[11,12] More in details, our engineered mutant displays two absorption states A and B at 400 and 515 nm, associated to the protonated neutral and deprotonated anionic forms of the chromophore, respectively, whereas the fluorescent emission peak is centered at 523 nm. Photobleaching experiments of single molecules or clusters trapped into polyacrylamide gels showed the conversion into a long-lasting dark state (state C) upon few seconds of laser excitation at 476 nm;[11,12] however, photoconversion from dark state C back into state B is possible and can be driven by irradiation at 350 nm (Figures 2a-c, 3).



A B C

Fig. 2. (A) Image of a single E^2GFP molecule in a PAA gel; (B) the same molecule after photobleaching (2s at 476 nm); (C) the same molecule after reactivation (2s at 350 nm). Each frame size is 2 μm × 2 μm and the intensity of both laser sources was 0.2 kW/cm^2.

Noticeably, the gel preparation method provided pore sizes small enough for immobilization of proteins while maintaining their native conformation.[13] At sufficiently low protein concentration (≈ 5 ng/cm^2), optical emission from isolated single molecule was indeed observed (Figure 2).

Significantly, single EGFP molecules or clusters display photobleaching dynamics similar to E^2GFP, but they do not recover any fluorescence upon irradiation at 350 nm (Figure 3). Scheme 1 describes a working model of E^2GFP photophysics based on these results.[12]

Molecular Dynamics Characterization of E^2GFP

Although the nature of dark state C is still unclear, molecular modeling suggests a possible protein configuration compatible with experimental measurements. The key observation is that the excitation energy of the C state is blue shifted with respect to both A and B states. Following our analysis of how the chromophore excitation energy is affected by interactions with the surrounding protein matrix,[14] we infer that state C corresponds to a neutral

chromophore with less coordination to the surrounding residues. We thus investigated possible mechanisms of light-driven conformational changes. The natural candidate is *cis-trans* photoisomerization of the chromophore. Such photoreaction involves excited-state torsion around a double bond, and occurs in other biological chromophores, like for example rhodopsin and the photoactive yellow protein.

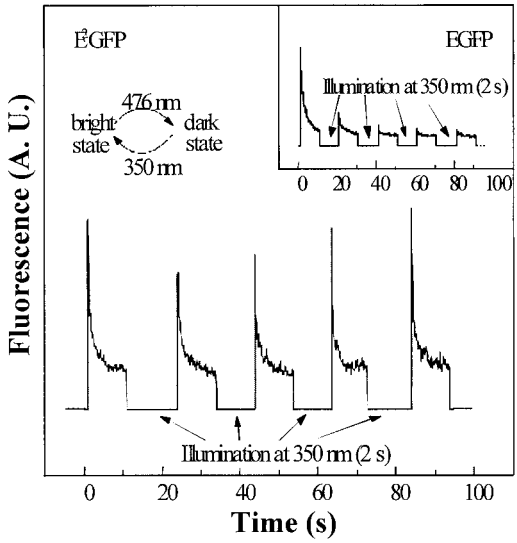
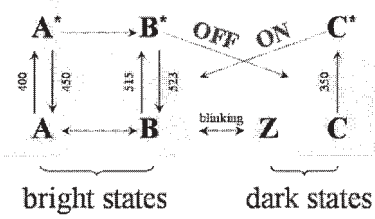


Fig. 3. Comparison of the photophysical characteristics of EGFP and E^2GFP highlighting the photoreversible conversion of E^2GFP between bright and dark states.



Scheme 1. Working model of E^2GFP photophysics.

The *trans* isomer of GFP chromophore, however, absorbs at the same wavelength as the normal *cis* one, so that *cis-trans* isomerization alone cannot explain the blue shift of the C state. Molecular dynamics simulations revealed that upon chromophore isomerization the protein environment undergoes configurational rearrangements,[12] shown in Figure 4, which

can account for the higher excitation energy of C. The *trans* chromophore configuration is dark because there is no gateway of communication with the fluorescent anionic chromophore state (the one present in state B): whereas the chromophore in the neutral *cis* configuration (that of state A) can exchange protons with the protein through a close water molecule, the *trans* chromophore has no proton-acceptor moiety in the vicinity of the hydroxyl group, so that it cannot interconvert to the anionic species.

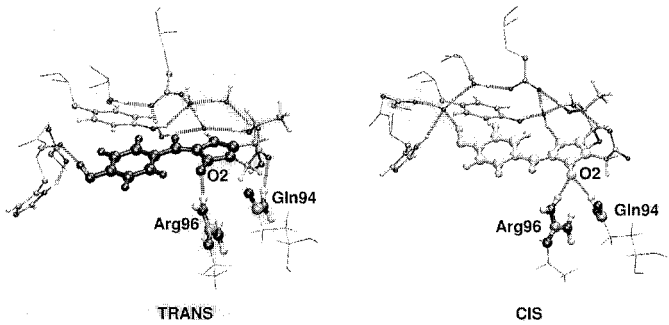


Fig. 4. Environmental changes upon chromophore *cis-trans* isomerization as monitored by molecular dynamics simulations.

E^2GFP as Optically Controllable Element for Biomolecular Devices

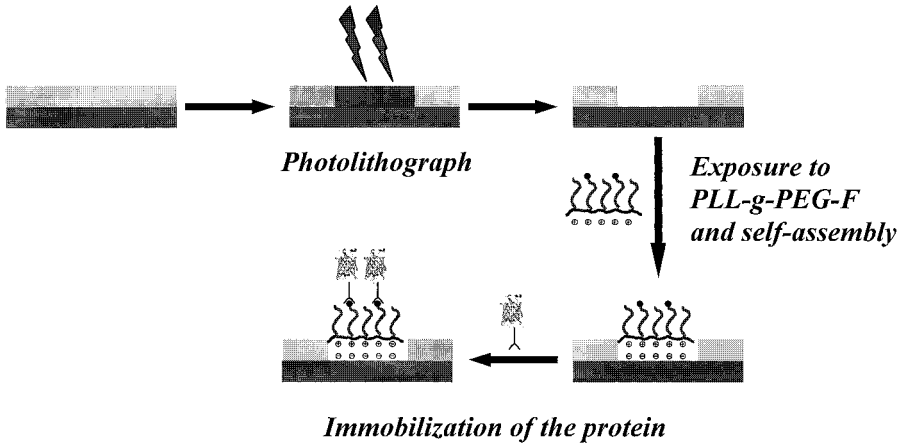
The availability of distinct ground states is a requirement for the development of high-density optical memories and switches, provided an efficient control of the photoconversion between these different states is achieved together with high spatial resolution. Since 1989, when volumetric optical storage by means of two photon absorption was firstly proposed,[15] many materials were suggested as components for optical memories and switches.[16,17] However, none of these materials could be possibly employed for obtaining rewritable optical-storage devices at the single-molecule level.

As seen previously, E^2GFP can effectively undergo a reversible photoconversion between a bright (B) and a dark (C) state. Therefore, states B and C encode a (0, 1) bit that can be stored and manipulated in the protein at the ultimate single-molecule limit. According to the rewritable storage-device paradigm, photoconversion from C to B with irradiation at 350 nm would represent the "WRITE" process, whereas fluorescence emission following weak excitation at 476 nm would represent the "READ" process, and photobleaching by intense or

prolonged excitation at 476 nm would represent the "ERASE" process.[11]

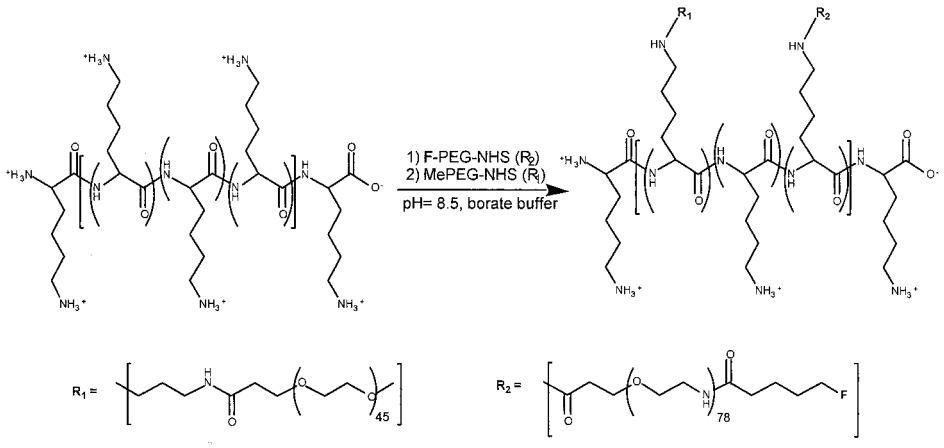
As a first step towards the implementation of dense volumetric E^2GFP-based optical memories, we set off for the realization of 2D patterned arrays of fluorescent proteins able to store optical information. Actually, several technologies are available for surface patterning and protein adsorption/binding to polymeric and/or metal substrates.[18-24] However, most methods do not fulfill the strict requirements essential for the realization of a protein-based 2D storage device, namely: 1) high yields of protein immobilization, 2) high selectivity towards the relevant biomolecule, 3) preservation of the protein optical activity (i.e. of the protein folding), 4) absence of lateral diffusion, and 5) preservation of the water environment of the protein. Indeed, lack of these characteristics would result in poor optical contrast, protein array inhomogeneity, and possibly loss of reversible photoconversion.

In this perspective, we are currently trying to address these problems by an adaptation of the selective molecular assembly patterning (SMAP) technique.[25] SMAP is based on the selective self-assembly of organic molecules, namely alkyl phosphates and poly(L-lysine-*g*-ethylene glycol) (PLL-*g*-PEG), on pre-patterned metal oxide surfaces such as TiO_2, SiO_2, and Nb_2O_5. Concerning PLL-g-PEG, it was established that the polycationic PLL backbone strongly interacts with the oxide surface whereas the PEG side chains extend towards the aqueous solution, thus yielding a bifunctional monolayer.[26] XPS and radiometry studies on PLL-g-PEG have shown that the monolayer thickness on TiO_2 is around 20 Å.[27] Since PEG is known to decrease strongly non-specific protein adsorption by means of steric stabilization and excluded volume effects,[26] the use of PLL-*g*-PEG-based polymers seems particularly interesting for highly selective protein immobilization on surfaces, provided the prepared polymers is supplied with a functional group capable to interact specifically with the protein of interest. Accordingly, we are currently dealing with the preparation of functionalized PLL-*g*-PEG copolymers where a fraction of the PEG chain ends bear bioaffinity groups (**F**) such as biotin, glutathione, etc., and their subsequent assembly onto lithographically pre-patterned TiO_2 or SiO_2 surfaces. Our strategy is described in Scheme 2. First, a patterned surface displaying regions of TiO_2 or SiO_2 is fabricated by conventional photolithographic methods. Then, we drive PLL-g-PEG-**F** self-assembly onto the oxide regions by carrying out the process at pH where the amino groups of the PLL backbone are protonated (and therefore positively charged) and the oxide regions are negatively charged. Eventually, the surface is put into contact with a solution of the relevant protein, to allow protein immobilization through the affinity bond.



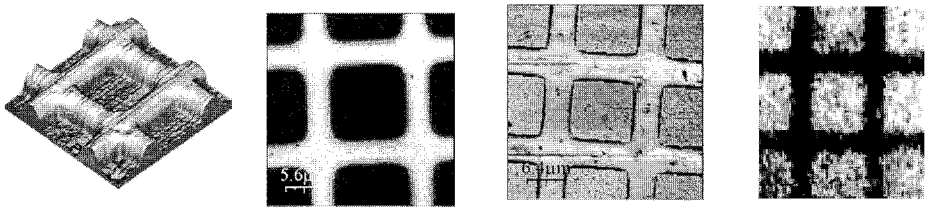
Scheme 2. Followed photolithographic-SMAP strategy to pattern relevant proteins onto surfaces.

The typical synthesis of PLL-g-PEG-**F**[28], where **F** is a functional group, is reported in Scheme 3. In this reaction, ε-amino groups of poly(L-lysine) are partially esterified with two carboxyl-activated NHS-PEG of different length, one bearing the **F**-group at the ω-terminus. The reaction is carried out in borate buffer at pH= 8.5 to ensure that a significant fraction of PLL ε-amino groups are in the deprotonated form, thus speeding up the grafting process.



Scheme 3. Synthesis of PLL-g-PEG-**F**.

A PLL-g-PEG-biotin (**F** = biotin) with grafting ratio around 4.1 and 25 % of PEG chains end-functionalized with biotin was employed for studies of protein immobilization onto patterned TiO_2 surfaces fabricated by e-beam lithography, according to the developed general strategy (Scheme 2). More in detail, a 100 nm-thick poly(methyl methacrylate) film was spun on top of a thermally-oxidized titanium film and square regions of different dimensions were exposed by electron beam lithography. After development, the patterned surface was placed in contact with PLL-g-PEG-biotin at pH 7.2, to allow the adsorption of the polymer to the irradiated regions. Contact-mode atomic force microscopy (AFM) was utilized to monitor the characteristics of the pattern and self-assembly process. Figure 5a shows a 3D topographic image detail of the e-beam patterned PMMA/TiO_2 surface, whereas Fig. 5b shows a lateral force image of the same patterned region (dark squares: TiO_2, bright lines: PMMA). Figure 5c shows the same detail of Fig. 5b in lateral force after the exposure of the surface to PLL-g-PEG-biotin: clearly, the TiO_2 regions were strongly modified upon the exposure, whereas minor changes occurred in the PMMA regions, thus suggesting a highly selective adsorption process. As final step of the protein immobilization process, the patterned surface enriched in biotin was exposed to a solution of fluorescent AlexaFluor-660-conjugated streptavidin (Molecular Probes), which was taken as a model of the engineered E^2GFP capable of biotin binding that is currently under preparation in our laboratory. Figure 5d represents a fluorescence micrograph of the patterned surfaces, wherein immobilization of the fluorescent protein is clearly evidenced (bright squares).

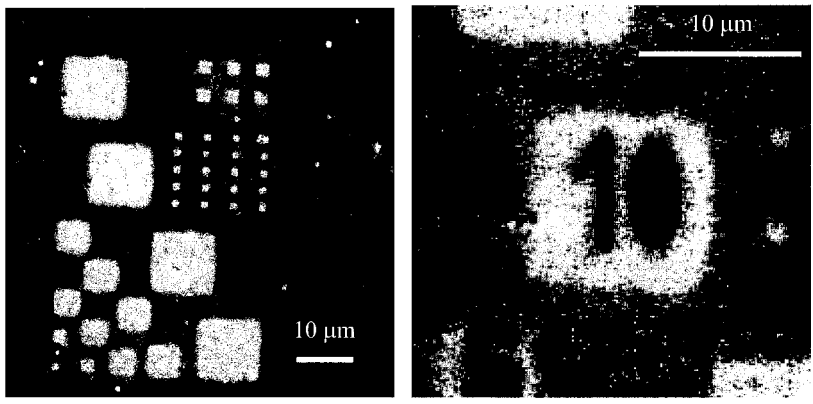


A B C D

Fig. 5. (A) AFM contact-mode topography of patterned PMMA/TiO_2 surface. AFM lateral force images before (B) and after (C) exposure to PLL-g-PEG-biotin. (D)Fluorescence micrograph of pattern (A-C) after immobilization of Streptavidin-Alexa Fluor 660.

Significantly, by this patterning procedure protein immobilization within features as small as 1 μm could be achieved and detected with high optical contrast (Figure 6A). Furthermore, the protein molecules bound to the larger pattern regions are amenable to photobleaching, either

completely or partially (Figure 6B). Indeed, permanent photobleaching demonstrates that the protein cannot diffuse out the immobilization region and provides a further way (optical) to protein micropatterning.



A B

Fig. 6. (A) Micrograph of fluorescent streptavidin on patterned TiO_2 surface: visible square sizes are 10, 5, 4 2, and 1 μm. (B) Photobleaching of the protein immobilized within the 10x10 μm square.

Conclusions

A single point mutation T203Y of EGFP was shown to yield photochromic behavior and an optically controlled molecular toggle switch by means of two focused laser beams at different wavelength. Current studies are focusing on the controlled realization by the selective molecular assembly patterning technique (SMAP) of patterned 2D arrays of engineered fluorescent proteins characterized by high optical contrast. Such biodevices are meant to provide the basis for the implementation of bio-optical memories or bioprobes for proteomics.

Acknowledgments

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