Green fluorescent proteins as optically-controllable elements in bioelectronics

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A single-biomolecule optical toggle-switch is demonstrated based on a mutated green fluorescent protein (GFP). We have exploited molecular biology techniques to tailor GFP molecular structure and photophysical properties and give it optically-controlled bistability between two distinct states. We present optical control of the fluorescence dynamics with two laser beams at 476 and 350 nm down to the ultimate limit of single molecules. These results indicate that GFP-class fluorophores are promising candidates for the realization of biomolecular devices such as volumetric optical memories and optical switches.

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The optical control of the molecular structure of materials is an attractive way to store and manipulate data at high spatial resolution [1]. Promising candidates for this application are photochromic molecules that exhibit photoinducible isomerization between forms with different optical properties. The potential of photochromism has generated a large interest in the synthesis of new photosensitive materials and in the development of new techniques for their use [2]. In this framework, some biological systems, like bacteriorhodopsin and the green fluorescent protein (GFP) of the *Aequorea victoria* jellyfish, are offering naturally-evolved optimized structures with unique properties that can be further tailored for specific applications by genetic engineering [3,4]. While bacteriorhodopsin has already been the subject of an intense research effort and was proposed for a broad range of bioelectronic applications [5], GFP potential for bioelectronics is still largely unexplored in spite of the advantage of a very efficient fluorescence emission that allows detection down to the single-molecule level [4,6,7].

GFP has emerged in recent years as a unique fluorescent marker in molecular and cell biology [8]. Molecular engineering is playing a fundamental role in designing GFP mutants with modified spectral characteristics, enhanced brightness, photostability, quantum yield, and other properties tailored for different applications. GFP fluorescence dynamics is characterized by transitions between bright and dark states which, at the single-molecule level, leads to reversible turning on and off (blinking) and ultimate switching off (photobleaching) of the emission [4,6,7].

The availability of distinct ground states is a requirement for the development of high-density volumetric optical memories and optical switches. Other important issues, however, must be addressed such as efficient control of the photoconversion between these different states and achievement of high spatial resolution. A first important step in this direction was the observation of photobleaching reversal in two yellow-shifted GFP mutants by means of prolonged (5 min) irradiation at 405 nm by a Hg arc lamp [4]. Control of photoconversion between dark and bright states is not achievable with other GFP mutants like the F64L/S65T GFP (enhanced GFP, EGFP), one of the most common optical markers in biology [6].

In this letter we demonstrate that one specific point mutation (T203Y, threonine into tyrosine at position 203 in the amino-acid sequence) enables the optical control of transitions between two distinct states down to the ultimate limit of single molecules. We show that it is possible to induce unlimited optically-controllable cycles between fluorescent and dark configurations by means of two laser beams at the wavelengths of 476 and 350 nm. Additionally a model for the observed photophysics is suggested based on the selectivity of photoconversion on the excitation wavelength.

EGFP and T203Y EGFP (E^2GFP in the following) molecules [9] were obtained as recombinant proteins and studied both in solution and trapped in polyacrylamide (PAA) gel films ($\approx$10 μm thick) [10]. Gel preparation provided pore sizes small enough for immobilization of proteins while maintaining their native conformation [6]. At sufficiently low fluorophore concentration (around 5 ng/cm^2), optical emission from isolated single molecules and cluster could be observed. Clusters originate from unavoidable inhomogeneities of the PAA gel that act as localization centers for the fluorophores [6]. By comparing fluorescence intensities of single molecules (see below) and clusters, we could establish that clusters are typically composed of approximately one hundred molecules. For both molecules, room-temperature fluorescence was excited by an Argon-ion laser at 476 nm. Radiation at 406 nm and 350 nm was provided by an Argon/Krypton-ion laser. The two lasers were focused onto a circular field of view of $\sim$10 μm^2 in PAA gels by a 100$\times$ 1.3 N.A. oil-immersion objective and GFP molecules were imaged by an intensified CCD camera (0.1 s integration time).

E^2GFP absorption measurements were performed on 300 μl solution (70 μM) prepared from PBS (phospate buffered saline) at pH=7.0 and display an equilibrium between two states (see Fig. 1 upper panel, dotted line) analogous to that of wild-type GFP [11]. The absorption peaks at 400 and 515 nm suggest that these states (A and B) are associated to the protonated neutral and deprotonated anionic form of the chromophore, respectively [12]. On the contrary, EGFP shows a very different equilibrium strongly favoring state B (see Fig.1 lower panel, dotted line). Fluorescence after excitation of state B is shown in Fig.1 for both mutants (solid lines). In the case of E^2GFP, fluorescence peaks at 523 nm and is red-shifted with respect to EGFP (512 nm). Excitation of state A in both molecules produces a similar but weaker fluorescence signal which was interpreted in wild-type GFP in terms of excited-state photoconversion from A to B through a proton-transfer process [14].

Single-molecule studies were performed in gel-trapped samples. We observed blinking and photobleaching into a long-lasting dark state (state C in the following) within few seconds after excitation at 476 nm. The top histogram of Fig. 2, however, shows that photoconversion from state C back into state B of E^2GFP is possible and can be driven by irradiation at 350 nm. The histogram reports the fluorescence intensity of an E^2GFP cluster in PAA gel composed of approximately 50 molecules after alternate 10 s-long excitation at 476 nm and 2 s at 350 nm (0.2 kW/cm^2 at both wavelengths). The measurements are an extract of an arbitrarily-extendable experiment and show photobleaching by excitation at 476 nm and efficient photoconversion from C to B by irradiation at 350 nm. In order to rule out possible changes in the ground-state configuration after excitation in the UV, we also analyzed the E^2GFP emission lineshapes after photoconversion. The lineshapes (data not shown) are identical to that reported in Fig.1 confirming that the 350 nm irradiation indeed converts the protein back to the original state B. In addition, we noted selectivity of photoconversion on wavelength: radiation at 406 nm (from 0.1 to 10 kW/cm^2) instead of 350 nm favored E^2GFP

photobleaching indicating that state C is distinct from state A. The bottom histogram shown in Fig. 2 presents the analogous experiment for an EGFP cluster. Following the initial photobleaching, no significant recovery is observed. This shows unambiguously that photoconversion originates from the single point-mutation T203Y.

States B and C thus encode a (0, 1) bit that can be stored and manipulated in the protein. However, in a memory device we must be able to write, read and erase data. These operations can be accomplished with E^2GFP. One possible implementation is to exploit photoconversion from C to B with irradiation at 350 nm (WRITE), fluorescence emission following weak excitation at 476 nm (READ) and photobleaching (B to C) (ERASE). The last process can be induced by intense or prolonged excitation at 476 nm.

One important issue for advanced bioelectronic applications concerns the possibility to address the protein state at the single-molecule level. To this end we investigated the limit for the observation of E^2GFP optical switching presented above. Individual E^2GFP molecules in state B were imaged as bright spots at the resolution limit of the optical setup ($\sim$0.3 μm). The fluorescence intensity of single spots ($\sim$300 CCD counts) was similar to EGFP and displayed the characteristic signatures of single-molecule emission. Most of the molecules, in fact, blinked and then photobleached within 10 s under excitation at 476 nm. However, compared to EGFP, we typically observed longer duration of E^2GFP emission. Blinking events were detected at longer times (up to $\sim$1 min). Figure 3 shows that controlled photoconversion is achieved both on E^2GFP clusters and E^2GFP single molecules. In this latter case, the same molecule (three lower images) was repeatedly photobleached by excitation at 476 nm (ERASE), photoconverted with 350 nm laser irradiation (WRITE) and its fluorescent emission detected with 100 ms integration time (READ). The three upper images of the figure show efficient photoconversion for a cluster of around one hundred molecules and confirm the behavior of the histograms shown in the top part of Fig. 2.

In order to rationalize the observed differences between E^2GFP and EGFP, we performed molecular dynamics simulations of the whole proteins using the AMBER suite of programs [15]. Figure 1 shows our results for anionic states B in the two mutants (chromophores and their immediate environment). Similarly to other T203Y mutants, tyrosine at site 203 interacts with the chromophore through π-stacking [13]. In E^2GFP, this π-stacking and the peculiar hydrogen bonding around the chromophore phenolic ring cause the red-shift and destabilize state B in favor of state A as observed in the absorption and emission spectra reported in Fig. 1 [13]. These simulations suggest that the peculiar hydrogen-bond network resulting from the point mutation at position 203 is responsible for the photoconvertibility between bright (B) and dark (C) states. Photobleaching and photoconversion most probably involve a change in the protonation state of the chromophore, i.e. a proton transfer. The inset in the top panel of Fig.2 describes a working model of E^2GFP photophysics based on these results. The equilibrium between states A and B as schematically indicated by the arrows follows the results of Ref. [11]. Our data indicate that we must include two additional distinct states responsible for blinking and photobleaching. The zwitterion was indicated as the chromophore form associated to the blinking dark state Z [16]. The latter reference also discusses the equilibrium between Z and B states. Additionally our experiments show photoconvertibility of E^2GFP from C to B following absorption at 350 nm (i.e. from the C* excited state to B) and from B to C following absorption at 476 nm (i.e. from the B* excited state to C). We should like to underline that this situation differs from what observed in yellow-shifted GFP mutants [4,16]. In the latter case photoconversion was reported between states A and B without invoking an additional chromophore state. Further analysis based on ab-initio calculations is required to identify the nature of C and the precise mechanism of conversion among the different configurations. We do expect that this analysis will make it possible to suggest new mutations able to further tune the potential barriers separating different states.

In conclusion, we demonstrated that a single point mutation T203Y of the enhanced green fluorescent protein yields a photochromic behavior and an optically controlled molecular toggle-switch by means of two focused laser beams at different wavelengths. We showed that controlled and efficient photoconversion between two distinct ground states is possible down to the ultimate limit of the single molecule. We argue that this process can represent the basis for the implementation of dense volumetric GFP-based optical memories that exploit the fluorescent properties of single proteins. Finally we note that the photoconversion of E^2GFP opens the way to advanced biological applications where prolonged monitoring of GFP-tagged molecules in live cells is required. This may lead to a new level of resolution in the study of biomolecular processes.

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FIG. 1. Normalized absorption (dotted lines) and emission (following excitation at 476 nm, solid lines) spectra of E^2GFP (upper panel) and EGFP (lower panel). Structures of the chromophore environment of E^2GFP (top) and EGFP (bottom) in the anionic B state following $\sim$1 ns molecular dynamics simulations are also reported. Dashed lines indicate hydrogen bonds. The hydrogen atoms are hidden and the water molecules are shown as balls. The picture was produced by Insight2000 (Molecular Simulations Inc.).

FIG. 2. Fluorescence intensities of E^2GFP (first histogram) and EGFP (second histogram) clusters in PAA gels after alternate 10 s-long excitation at 476 nm and 2 s at 350 nm (0.2 kW/cm^2 at both wavelengths). Horizontal dots are guides to the eye at the same background level. Model for E^2GFP photophysics is described in the top panel. On the left, the E^2GFP bright states A and B (associated to the neutral and the anionic chromophore forms, respectively) and their excited states A* and B* are indicated together with their excitation and emission wavelengths in nm. On the right, E^2GFP dark states Z and C are reported. Transitions between bright and dark states in blinking, photobleaching, and photoconversion are shown. Excitation from C to C* at 350 nm is deduced from the selectivity of photoconversion on the wavelength.

FIG. 3. Typical images (from left to right) of an E^2GFP cluster (upper row) and a single E^2GFP molecule (lower row) in PAA gels after alternate photobleaching by 10 s excitation at 476 nm and photoconversion by 2 s at 350 nm (0.2 kW/cm^2 at both wavelengths). Each frame size is 2 μm $\times$ 2 μm and integration time 100 ms

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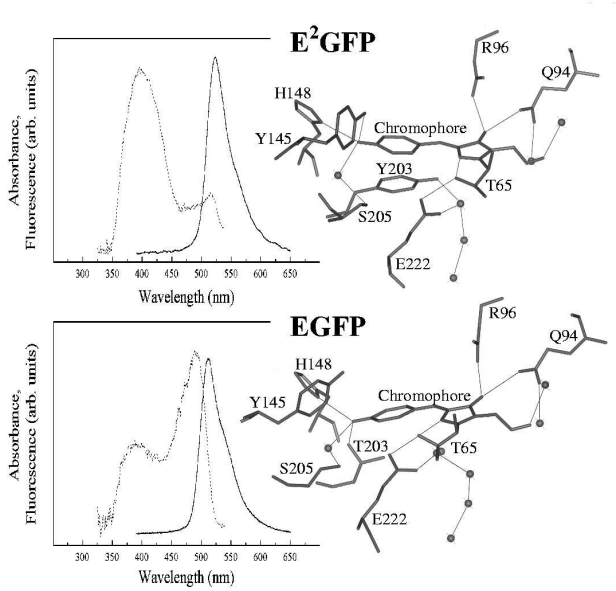

Figure 1 Cinelli et al.

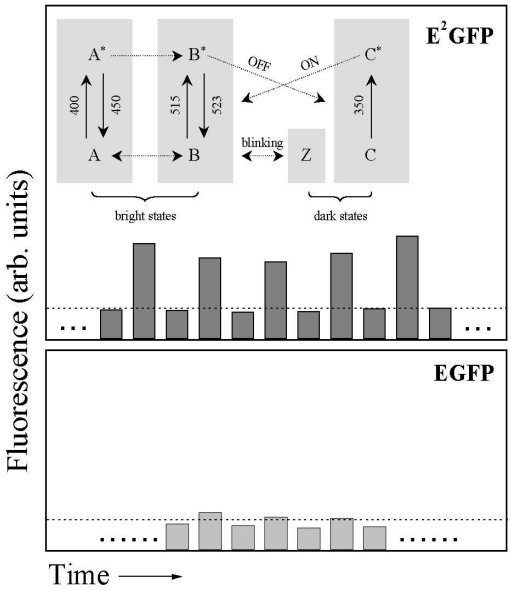

Figure 2 Cinelli et al.

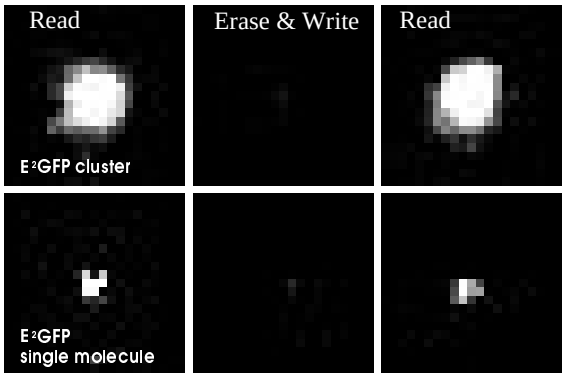

Figure 3 Cinelli et al.