Molecular modeling and spectroscopy of fluorescent proteins

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The activity in this research line evolved in understanding the photophysics of E^{2}GFP, with emphasis on its peculiar photochromic behavior. We also investigated possible strategies of protein immobilization, as a first step towards the implementation of an E^{2}GFP-based optical memory device.

In collaboration with the group of G. Chirico at the University of Milano Bicocca, the photochromism of E^{2}GFP was investigated using two-photon spectroscopy. Despite the need of a more intense laser power, multiphoton spectroscopy has some key advantages for fluorescence cell microscopy, such as increased resolution a and decreased fluorophore photobleaching.

As in the one-photon case, two-photon irradiation leads to photobleaching of single E^{2}GFP proteins, and excitation with 720nm light (to be compared with the 350nm wavelength in the one-photon case) restores the fluorescent state, with up to 100% efficiency at the suitable irradiation intensity [1].

In general, two-photon photoconversion could be demonstrated with shorter irradiation times, down to few milliseconds. In addition, the use of two-photon irradiation allowed to observe some interesting features of this photoconversion process (on/off switching, hereafter) that could not be detected in the one-photon case. Because of their low quantum yield, proteins in the A state (with a neutral chromophore) are almost invisible in one-photon single-molecule experiments, so that only proteins in the B state (with an anionic chromophore) can be efficiently imaged. By contrast, thanks to the enhanced two-photon cross section of state A, single E^{2}GFP proteins can be separately visualized in both states. Surprisingly, no transition between A and B was observed in a single molecule, indicating that the conformational barrier between the two states is higher than previously expected. (Alternatively, this separation could be attributed to local inhomogeneities of the support -i.e. the gel in which the proteins are entrapped- favoring one or the other protonation state of the chromophore). Both A and B populations are switched on and off at wavelengths corresponding to the ones used in one-photon experiments. As shown in Fig. 1, the excitation spectrum for switching on for the B state is remarkably narrow and peaked at 720nm. At such wavelength, photoconversion is 100% efficient for a 16.5 ms-long irradiation at a power of 5.7 mW. Two independent on/off switching pathways are observed for each state, i.e. E^{2}GFP proteins in the B state are photoconverted back to the same initial

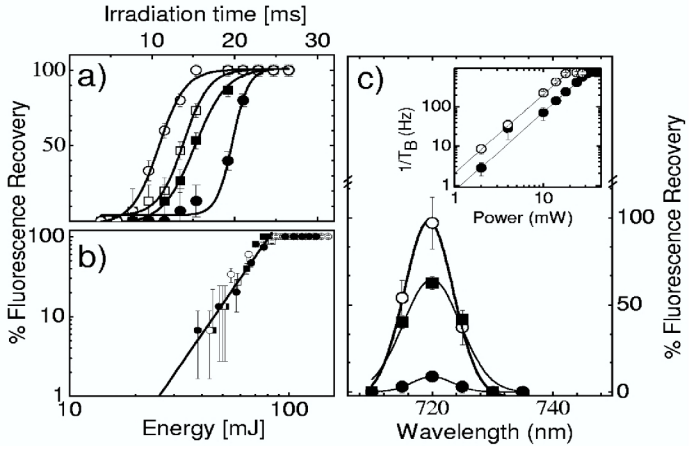

Fig. 1
(a): Percentage of single-molecule fluorescence recovery versus the illumination time at 720 nm for different excitation powers: 3.34 mW (solid circles), 4.34 mW (solid squares), 5 mW (open squares) and 5.7 mW (open circles). The solid lines are best fit to a sigmoidal function.
(b): Recovery efficiency with laser excitation at 720 nm versus the product of the illumination time with excitation power: 3.34 mW (solid circles), 4.34 mW (solid squares), 5 mW (open squares) and 5.7 mW (open circles). The solid line is the best-fit power law with exponent 3.8 ± 0.2.
(c): Recovery efficiency at fixed illumination time of 16.5 ms versus the excitation wavelength at 5.7 mW (open circles), 4.34 mW (filled squares) and 3.34 mW (filled circles). The inset shows the photobleaching rate $1/T_B$ versus excitation power for the two states (A, open squares; B filled squares).

state, and the same is valid for proteins in state A.

These observations challenge the photophysical model that we proposed to explain one-photon photoconversion experiments. In particular, two distinct dark states should be included, while the *cis-trans* photoisomerization model[2,3] accounts for a single dark state (i.e. neutral chromophore in the trans configuration). How to include the two-photon experiments in a consistent model will be the objective of future research. This task will benefit from our theoretical analysis of how the excitation energy of the protein is determined by the configuration of the chromophore and the surrounding amino acids [4].

Another remarkable observation allowed by two-photon experiments is that blinking (*spontaneous* on/off turning of fluorescence in the second-millisecond timescale) happens only for proteins in the B state. Based on theoretical studies, some authors proposed that blinking stems from transitions to a zwitterionic protonation state of the chromophore [5]. More recently, blinking was attributed to anionic - neutral state transitions [6]. Nonetheless, the zwitterionic state could still play some important role in GFP photophysics as a gateway to dark states. By molecular dynamics simulations, both force-field based (FF) and *ab initio*, we investigated how the structure of EGFP (a mutant differing from E^2GFP by a single amino acid substitution) is affected by transition from anionic to zwitterionic chromophore [7]. The hydrogen bond network around the zwitterionic chromophore (Fig. 2) shows intermediate features between that

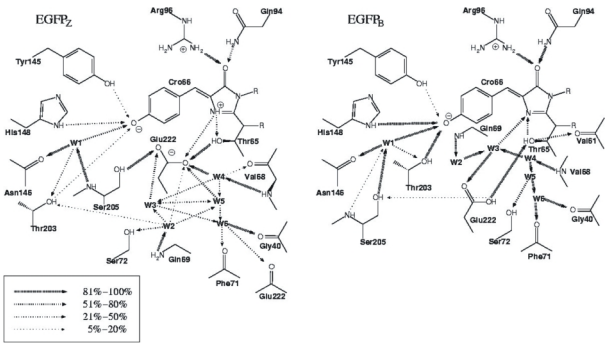

Fig. 2
Hydrogen-bond network around the chromophore of EGFP in the zwitterionic state (left) and anionic state (right) from molecular dynamics simulations. The different style of lines indicates the degree of occupancy of the H-bond during the molecular dynamics simulation. A hydrogen bond is considered as occupied when the donor-acceptor distance is shorter than 3.5Å and the donor-hydrogen atom-acceptor angle is less than 40°.

of the A and B states, and overall the chromophore is less well coordinated with the protein matrix. This suggest a reduced stability of the zwitterionic state with respect to the equilibrium neutral and anionic states and provides a possible explanation for its absence in normal conditions. We then used *ab initio* molecular dynamics to investigate possible proton transfers leading to a zwitterionic state. It was found that the simple transfer of proton from the near Glu222 to the chromophore imidazolidinone ring leads to an unstable state, whereas the thermalized configuration (taken from FF molecular dynamics simulation) is stable in the picosecond timescale. Further investigation is needed to unambiguously determine the molecular mechanism of blinking.

We are currently evaluating the possible use of photochromic E^2GFP as switching elements of bio-optical memory devices. As a first step towards the implementation of dense volumetric E^2GFP-based memories, we carried out studies on the immobilization of model fluorescent proteins onto two-dimensional surfaces. In a first approach, we combined

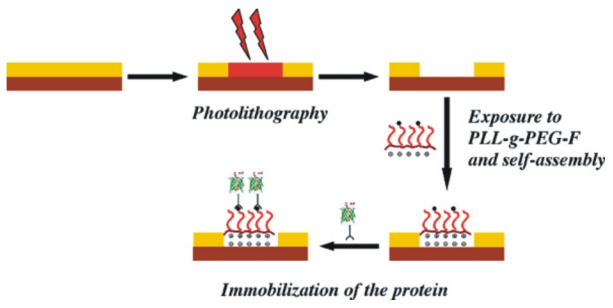

Fig. 3
Followed photolithographic-SMAP strategy to pattern the fluorescent protein onto a surface.

the so-called selective molecular assembly patterning technique (SMAP) [8] with a more conventional photolithographic technique to obtain patterned samples of protein with resolution ranging between 700 nm and 10 mm [9]. In more detail, a photoresist pattern was created by e-beam lithography onto a TiO_2 surface; then, a poly(L-lysine)-grafted-poly(ethylene glycol) polymer supplied with biotin end-chain functionalities was allowed to self assemble onto the free TiO_2 areas in the pattern (Fig. 3). Eventually, fluorescent streptavidin was bound to the biotin groups, resulting in the protein pattern shown in Fig. 4. In a second approach, carried out in collaboration with the group of Prof. Cingolani, University of Lecce, a polyacrylamide gel containing wtGFP was directly patterned (2mm resolution) by an adapted micromolding technique (Fig. 5). Both approaches were developed in order to create a strongly hydrophilic environment onto the surface and thus preserving the integrity of the protein structure and optical functionality.

Fig. 4
Micrograph of fluorescent streptavidin on patterned TiO_2 surface according to the photolithographic-SMAP method.

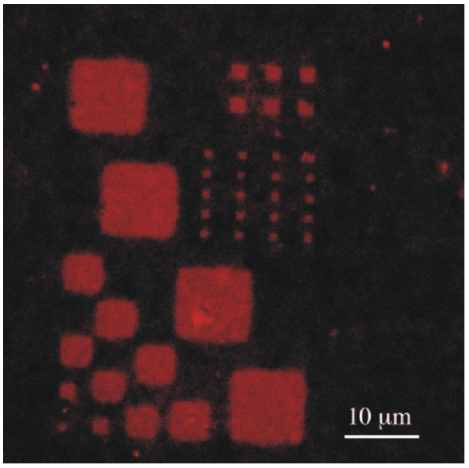

Fig. 4

Fig. 5
Micrograph of wtGFP on patterned poly(acrylamide) gel according to the micromolding technique.

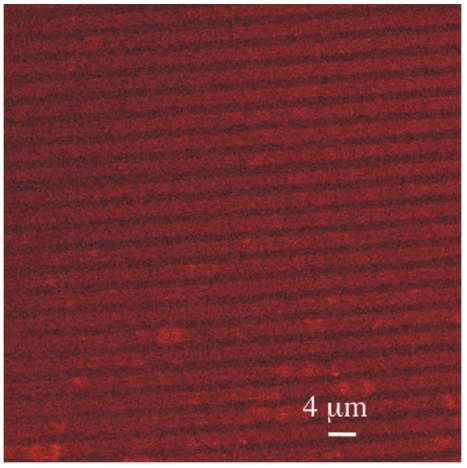

Fig. 5

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