Fluorescent proteins for multiphoton and super-resolution imaging

Progress in fluorescence imaging is enabling the study of biological events at unprecedented detail, thanks to novel microscopy techniques that provide imaging with nanoscale resolution. 3D imaging of living cells within intact tissues, organs, and whole animals, is accessible through multiphoton excitation. The remarkably rich photophysics of Fluorescent Proteins (FPs) makes them flexible enough to be used in conjunction with these advanced spectroscopic approaches. Here we report on our progress in understanding FPs multiphoton excitation spectra and in unraveling the photophysics behind photoactivation/deactivation of some FPs, a property enabling their use as fluorophores for nano-resolution imaging. This analysis allows us to develop new reversibly switchable FP mutants.

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Photoswitching and cis-trans photoisomerization

Recently, a novel approach to *in vivo* imaging has emerged with the development of new FPs that can be reversibly or irreversibly photoconverted between two optical states, adding a new temporal dimension to imaging of proteins at intracellular level. The most impressive results are obtained by using *reversibly* switchable FPs (RSFPs) [1,2], as they allow repeated activation events, thus prolonging the observation of biological dynamics, and the photolabeling of several subcellular regions one after the other. Furthermore, RSFPs stand as excellent fluorophores for novel nano-resolution imaging techniques based on the regioselective activation/deactivation of emissive states at the nanoscale.

Based on computational modeling, we previously linked the photoswitching behavior of certain FPs to chromophore *cis-trans* photoisomerization [3]. As a further step to understanding the photophysics of photoswitching, we investigated the spectral and structural modification of synthetic chromophore analogues upon irradiation (Fig. 1). We found that *cis-trans* photoisomerization can be induced in all the chromophores and determined for the first time the optical, NMR and Raman spectra of neutral *trans* isomers (Fig1a-b, Fig2c), along with photoconversion quantum yields φ_c by steady-state absorption measurements and by nanosecond laser-flash photolysis. Surprisingly, φ_c ranged from 0.1 to 1.0, demonstrating that photoconversion is a general and very efficient intrinsic photophysical mechanism of FP chromophores (Fig. 1c), whose efficiency is modulated by the mutant-specific protein environment [1,2].

Pre-resonant Raman experiments are able to determine the vibrational features of the chromophore also when embedded in the

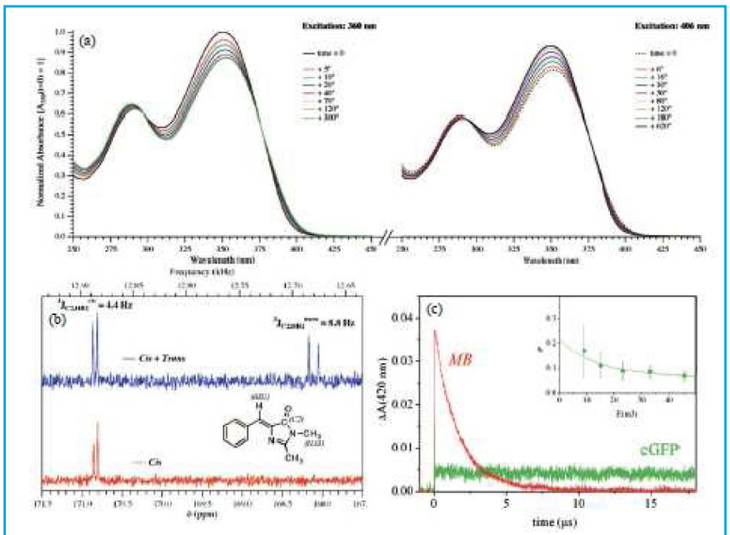

Fig. 1
The photochromic characteristics of chromophores are exemplified by the optical behavior of cBFPF upon irradiation. (a) Continuous irradiation at 360 nm leads to the time-dependent decrease and red-shift of the absorption spectrum due to the formation of a photoproduct (identified with the *trans* form tBFPF by NMR); (a, right panel) constant irradiation of photoconverted cBFPF at 406 nm, leads to the reversion of the absorption spectrum. (b) ^{13}C-NMR spectra of native and photoconverted cBFPF while adopting selective decoupling between the methyl protons HA3 and C2: the detected $^3J_{C2,HB2}$ values in the photoproduct correspond to those expected for the *cis* and *trans* isomers. (c) Single high-energy pulse irradiation at 355 nm of cGFP in methanol (OD=0.6) leads to a fast and irreversible absorption change (< 5 ns) detected at 420 nm (green line); methylene blue in water (OD=0.67) irradiated at the same wavelength shows a fast conversion to triplet state followed by slower (few μs) decay to the ground singlet state because of quenching by molecular oxygen (red line). (inset) By using methylene blue as an actinometer, the comparative method yields φ_c as a function of laser-pulse energy: extrapolation to zero energy gives the actual φ_c value.

proteins (Fig. 2). We studied the Raman signatures of different chromophore states in GFP mutants and compared them to those of the isolated chromophores. Our experiments allowed us to demonstrate that the *cis-trans* isomerization is responsible for the photoswitching behaviour of at least two RSFPs, namely EYQ1 (a newly developed GFP variant with T203Y E222Q) and BFPF (Y66F) [2]. In particular, for the case of EYQ1 we showed that at pH=8 the chromophore is anionic in the native form and neutral *trans* in the photoconverted form; it is neutral *cis* in the native form at lower pH. Our investigation highlights the relevance of Raman spectroscopy for the study of ground and metastable states of optically-active portions of proteins.

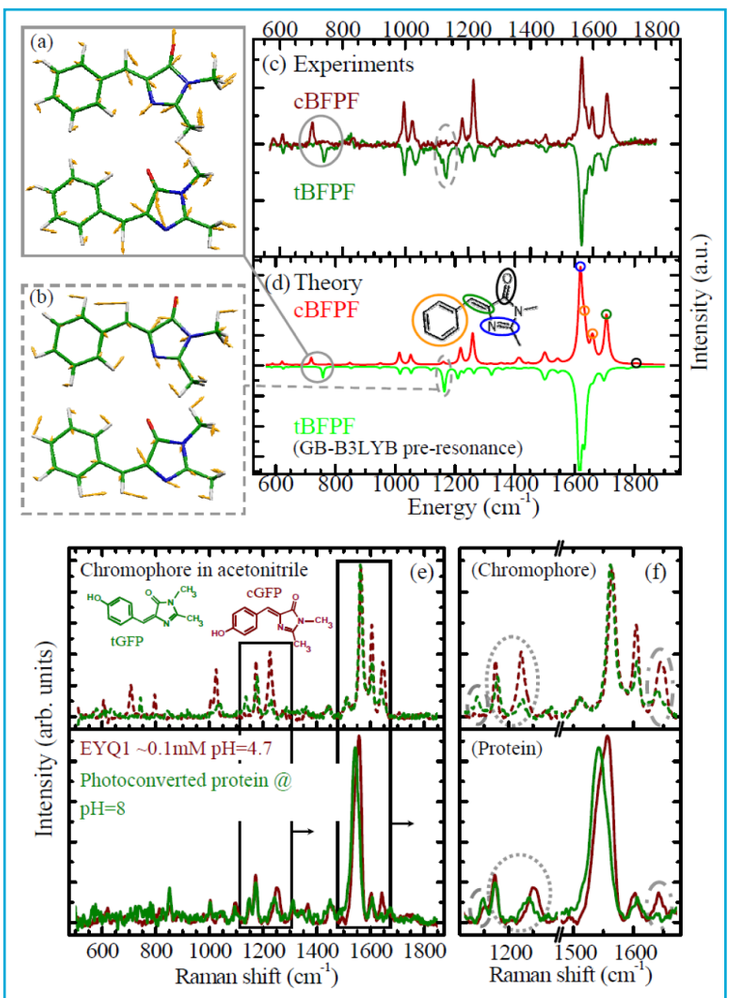

Fig. 2
Top: Experimental (c) and calculated in vacuo (d) pre-resonant Raman spectra of the isolated chromophore of BFPF in *cis* and *trans* forms (cBFPF, tBFPF); the spectra of the photoconverted form are shown inverted around the x-axis. Calculations are based on the B3LYP functional with gaussian bases, considering an excitation at 514.5 nm. Solid and dashed gray ellipses highlight two of the modes that change significantly in the cis-trans transition, and that are described in panels (a) and (b). Small circles emphasize stretching modes localized on double bonds in the chromophore region, highlighted in the inset of panel (d) with corresponding color: black, C=O; green, C=C on the bridge; orange, C=C on the phenylic ring; blue, C=N. Bottom: The dark red solid curves represent the Raman spectrum of native EYQ1 at pH=4.7 after the subtraction of the baseline, and the dark green solid curves represent the spectrum of the photoconverted form at pH=8. For comparison are reported the Raman spectra of its neutral chromophores in *cis* and *trans* forms (cGFP and tGFP, dark red and dark green dashed curves, respectively). Panel (f) contains magnifications of the highlighted regions in panel (e); the ellipses emphasize peaks with similar behavior for isolated and protein-embedded chromophores.

In order to develop more efficient RSFPs, we started by addressing those residues adjacent to the chromophore that can hinder its photochromic behaviour. Remarkably, we demonstrated that E222Q replacement induces reversible photoswitching in four GFPs otherwise differing in the mutation pattern, with switching 2-3 orders of magnitude faster than in any other reported photoswitchable GFPs.

We assessed the utility of the new RSFPs for intracellular studies by Fluorescence Recovery After Photobleaching to the -1, a technique that relies on the localized photoactivation of a fluorophore and the real-time monitoring of its subsequent dynamics. EYQ2 (S65T/T203Y/E222Q) was conjugated to a short peptide sequence that acts as a nuclear localization signal (NLS), i.e. it is recognized by the cellular importin system and actively transported to the nucleus [4]. Then, the dynamics of the nucleocytoplasmic shuttling of NLS-EYQ2 was determined by FRAP^{-1} (Fig. 2d). EYQ2 allowed for the long-term repetition of the experiment in the same cell, thus increasing the accuracy of kinetic measurements and ideally providing the correlation of transport dynamics with different cell states. FRAP^{-1} on NLS-EYQ2 yielded a shuttling time-constant (t=117±3 s) in excellent agreement with the value measured by conventional FRAP on NLS-EGFP.

Non-linear spectroscopy

We investigated the two-photon absorption (TPA) properties of various fluorescent proteins (BFP, CFP, GFP,

DsRed, mOrange, zFP and Kaede), and compared them with one-photon properties, using computational methods based on Density Functional Theory. This technique allowed us to calculate excitation wavelengths and cross sections for various model chromophores. A general relationship between excitation wavelength and structure was extracted, based on the variation of electric dipole upon excitation, and ultimately connected with the extension of the π-conjugated system [5]. The two-photon calculations shed light on the peculiar TPA features of DsRed (Fig. 3a) [6], and predicted the presence of high-energy (500-700 nm) TPA bands in several other FPs [7]. These excitation bands, later confirmed

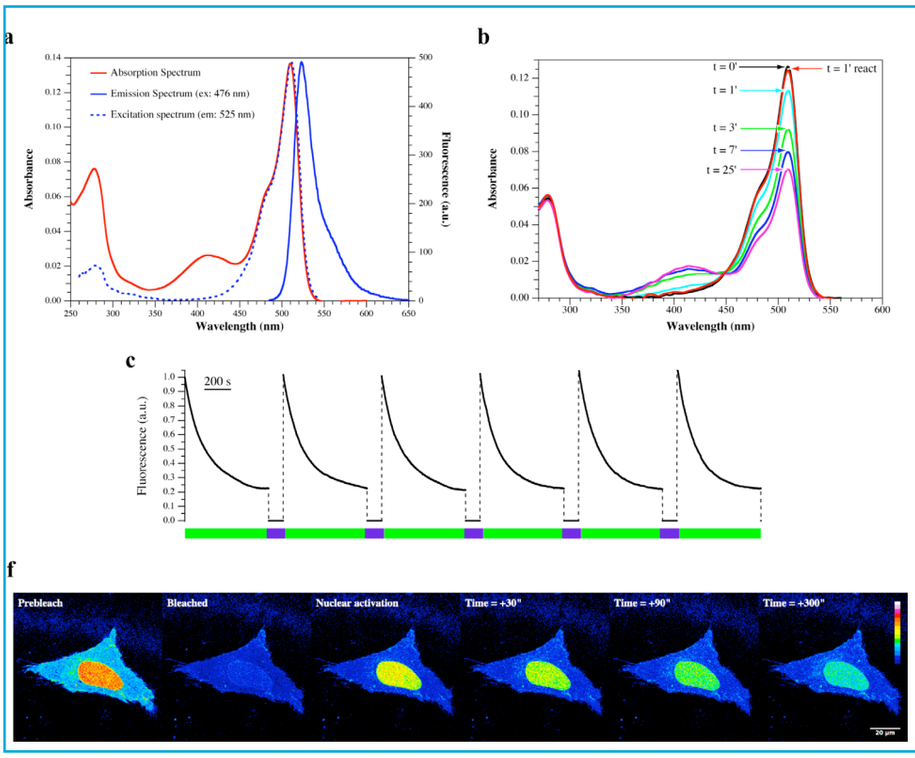

Fig. 3
(a) Absorption (red line), emission (solid blue line), and excitation (dashed blue line) of EYQ1 (T203Y/E222Q) at pH 7.2; (b) Absorption spectrum photoconversion of EYQ1 at pH 8.7: following 514 nm-illumination (0.5 W/cm^2) the anionic chromophore band (510 nm) decreases its intensity and the dark state band at 410 emerges (t=0′ to 25′); 405 nm-illumination (0.06 W/cm^2) for 1′ is sufficient to restore the original absorption (t=1′ react); (c) photoswiching on/off cycles of EYQ1 in transfected HeLa cells by means of 514-nm (*bleaching cycle: green abscissa*) and 403-nm (*reactivation step: violet abscissa*) laser light; (d) FRAP-1 of NLS-EYQ2 in one HeLa cell: initially fluorescence is bleached off cell-wide by 514 nm scan-irradiation; then, fluorescence is reactivated only in the nucleus by means of short (1s) 405-nm point-irradiation and the cell is imaged by using low power 514 scan-excitation.

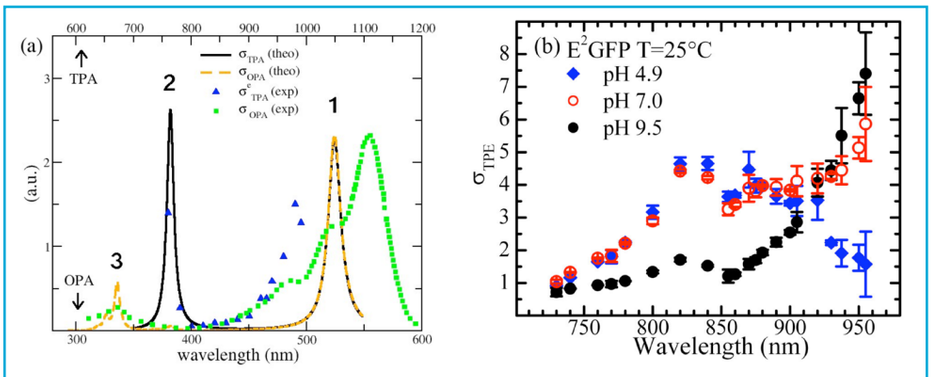

Fig. 4
(a): Comparison between experimental and theoretical spectra of DsRed. The two-photon measured cross section (blue triangles) displays a strong increase below 800 nm (band 2), whereas the one photon spectrum (green squares) is rather featureless in that region. The calculated TPA cross section (black solid line) reproduces this band, as due to excitation at a higher excited state. The blue shift between the theoretical and experimental 1 band is due to having considered the isolated model chromophore instead of the entire protein, to intrinsic errors of the theory, and to neglect of vibronic features. (b): 2-photon-action cross-sections σ_{TPE} in GM units (1 GM=10^{-50} cm^4 s/photon) of E^{2}GFP (at pH = 4.9, 7.0 and 9.5) over the 730–960 nm excitation range, with fluorescence emission in the range 450–650 nm. Error bars are estimated as standard deviations of the results of 4 to 10 measurements, often at different excitation intensities.

by experiments, can provide researchers with useful, and yet unexploited, spectral windows for two-photon imaging.

We are currently performing detailed measurements of two-photon excitation and photoconversion spectra in selected proteins. Preliminary results demonstrated the suitability of E^2GFP (F64L S65T T203Y) as a two-photon pH ratiometric indicator (Fig. 3b) [8].

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