

## Complementarity of time-resolved serial femtosecond crystallography & UV-Vis transient absorption spectroscopy to reveal the ultrafast photodynamics of photo-switchable fluorescent proteins.

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Reversibly photo-switchable fluorescent proteins find growing applications in cell biology, yet mechanistic details, in particular on the ultra-fast photochemical time scale, remain unclear. We choose to study rsEGFP2 (Fig. 1) which is the most common protein used in RESOLFT super-resolution microscopy.<sup>[1]</sup> We first employed time-resolved pump-probe absorption spectroscopy (TA) in solution to study photo-switching from the non-fluorescent (off) to the fluorescent (on) state. As reported for other photo-switchable fluorescent proteins<sup>[2,3,4]</sup> evidence is also provided here for the existence of several intermediate states ( $I_1$  and  $I_2$ ) on the pico- and microsecond time scales that are attributed to chromophore isomerization and proton transfer, respectively. Indeed isotopic experiments show clearly that *Off-to-on* photoswitching in rsEGFP2 involves excited-state isomerization and ground state proton transfer.

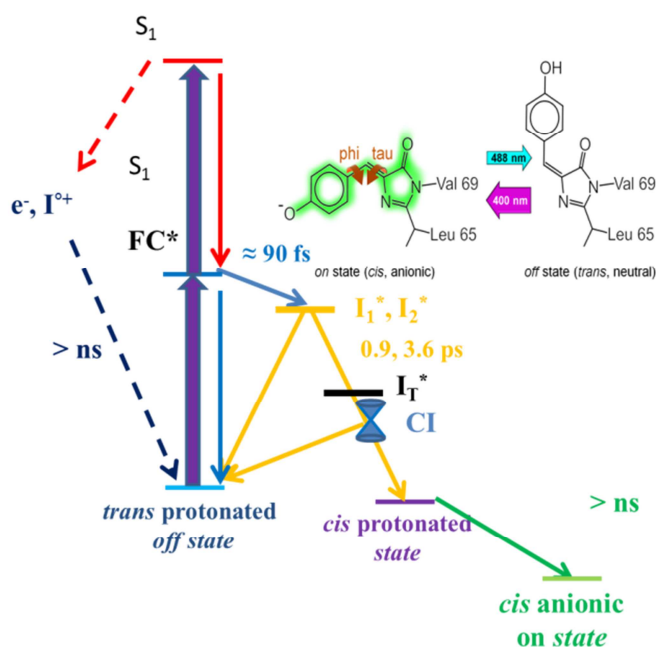


Figure 1. Model for the off-to-on photoswitching of rsEGFP2.  $I_1^*$ ,  $I_2^*$  are two excited state species identified by TA spectroscopy.  $I_T^*$  corresponds to the twisted intermediate (model T) determined in the 1 ps time-delay SFX data and not detected by TA spectroscopy. Chromophore isomers and protonation states of rsEGFP2 in the fluorescent *on* and the non-fluorescent *off* states.

Using an X-ray free-electron laser, we also performed time-resolved serial femtosecond crystallography (TR-SFX) and determine the structures of two excited states. In one of the excited states the hydroxybenzylidene imidazolinone chromophore assumes a near-canonical twisted configuration with the two cyclic moieties perpendicular (T). A model for the off-to-on photoswitching of rsEGFP2 can thus be drawn (Fig. 1), excitation of the *trans* protonated *off* state leads to a Franck-Condon state that relaxes in 90 fs to two excited state species  $I_1^*$ ,  $I_2^*$  seen by TA spectroscopy, one of which corresponds to a nearly planar conformation also detected in TR-SFX at 1 ps.  $I_1^*$ ,  $I_2^*$  decay in 0.9 and 3.6 ps back to the *trans* protonated *off* state and to a *cis* protonated state as evidenced by TR-SFX at 3 ps. The spectroscopically invisible twisted intermediate  $I_T^*$  observed in TR-SFX is close to the conical intersection (CI) and thus still electronically excited. Our result illustrates the complementarity of TA spectroscopy and TR-SFX, sensitive to electronic and structural changes, respectively.<sup>[5]</sup> In view of future experiments aiming at a structural characterization of photo-switchable protein using serial femtosecond crystallography (SFX) at an X-ray free electron laser, we will also discuss here the difference between TA and SFX, especially the high power density excitation which are used in the TR-SFX experiments leading to ionization or multi-photon absorption processes (Fig. 1). This research is carried out in collaboration with the Institut de Biologie Structurale in Grenoble (Adam, Bourgeois, Byrdin, Colletier, Coquelle, Feliks, Field, Fieschi, Guillon, Schirò, Thepaut, Weik, Woodhouse), Max-Planck-Institut für medizinische Forschung in Heidelberg (Barends, Doak, Foucar, Hilpert, Kovacsova, Nass, Roome, Schlichting, Shoeman), Departement of Physics in Rennes (Cammarata), and the SLAC National Accelerator Laboratory in Menlo Park (Aquila, Boutet, Hunter, Koglin, Liang, Robinson).

**Funding:** ANR-15-CE32-0004 BioXFEL.

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