PAFPs, a new generation of fluorescent proteins: the example of EosFP

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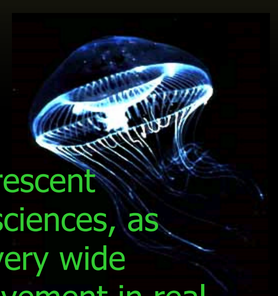
1. CONTEXT



Since the GFP (Green Fluorescent Protein) was cloned in 1992, fluorescent proteins (FPs, **Fig. 1**) have taken a considerable importance in life sciences, as being biological markers. Indeed, those proteins can be fused to a very wide choice of interest targets, allowing to track their localisation and movement in real time within a living cell, in function of environment conditions or under the action of therapeutic drugs.
Those last years, novel FPs were identified in anthozoan species (e.g. corals that form the reefs in tropical waters), that possess remarkable photoconversion properties (**Fig. 2**). Those proteins, called PAFPs (photoactivatable fluorescent proteins), form new tools to track objects in living cells and let hope revolutionary applications in biotechnology such as the conception of data storage memories with unequalled power.

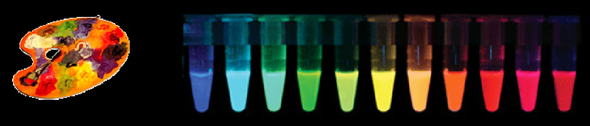


Fig. 1 – The fluorescent proteins palette

2. OBJECTIVES

Despite their considerable interest, FPs present weaknesses. For example they tend to form oligomers, which renders difficult the labelling of some proteins. They also "flicker", unceasingly blinking between fluorescent and non-fluorescent states.
Even worse, FPs are rapidly and irreversibly destroyed (photobleached) under laser illumination, which limits the available time for imaging experiments. These intrinsic properties and the photoconversion mechanisms that were newly discovered are still bad known.

Considering the fast development of new fluorescence imaging techniques and especially for single molecule imaging, it is essential to design powerful, monomeric and stable FPs.
The goal of this project is to discover on a structural point of view the mechanisms through which FPs emit fluorescence, change their fluorescence properties or cease their fluorescence. Drawing advantage from a better fundamental knowledge of those mechanisms, rational design of novel FPs will be envisaged then tested (Fig. 8)

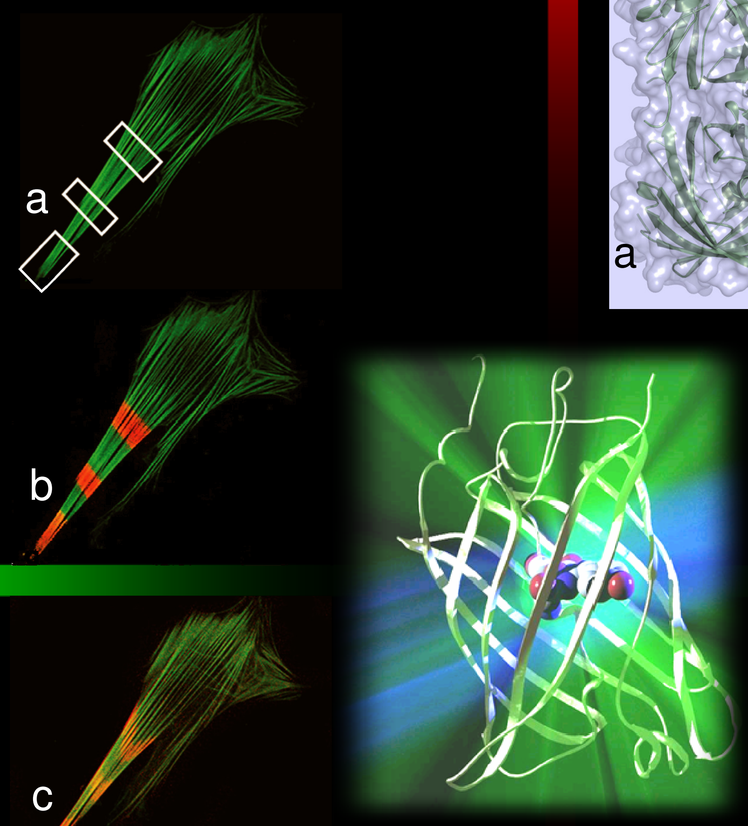


Fig. 2 – Interest of a photoactivatable fluorescent protein (PAFP): actin filaments labelled *in vivo* with EosFP and which green fluorescence is visualised with an illumination at 488 nm (a); After 10 seconds of irradiation at 400 nm on three areas, the red fluorescence of the photoconverted areas is visualised with an illumination at 502 nm (b); After 100 mn, one can see how the filaments grow thanks to the displacement of the red areas (c)

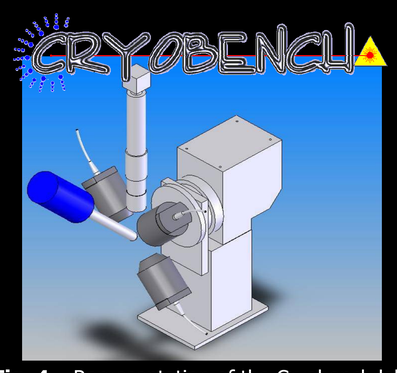


Fig. 4 – Representation of the Cryobench lab microspectrophotometer (ESRF)

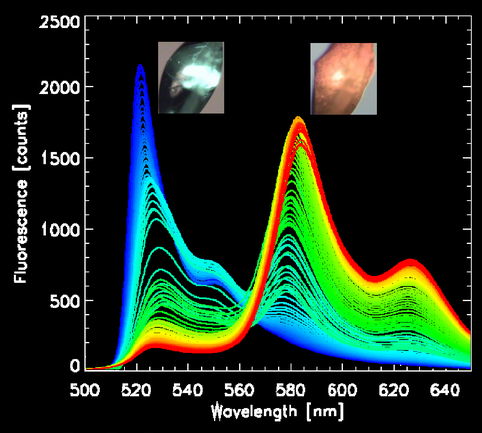


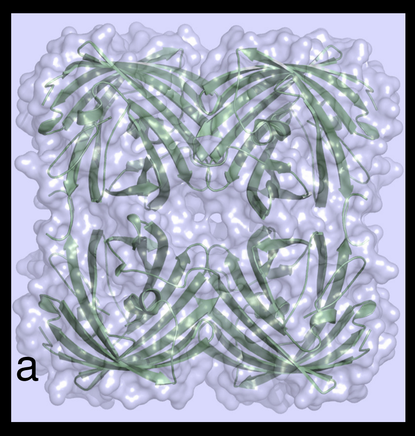
Fig. 5 – Fluorescence spectra recorded at the Cryobench lab on a crystal of EosFP during its photoconversion from 516 nm to 581 nm. Inset: photographs of the crystal at the initial and final states of the reaction

3.METHODS

Structural biology currently privileges a "dynamic" approach of living systems, in which several complementary techniques are used in parallel on a same system. To study at the atomic scale the fluorescence mechanisms of FPs, we trigger *in situ* the fluorescence cycle within a fluorescent protein crystal. A succession of crystallographic structures (**Fig. 3**) allows to reconstitute a "movie" of events [1]. In parallel, the reaction is followed by "in crystallo" spectroscopy. To this aim, we have the Cryobench laboratory that we developed at the ESRF and that allows to measure absorption, fluorescence and Raman spectrometry in crystals (**Figs. 4, 5**).
Moreover, we are using quantum mechanics and molecular mechanics (QM/MM) methods (modelisation) to create a link between the structures and the spectroscopic properties (**Fig. 6**).
Finally, the molecular biology will allow to rationally design mutants having enhanced properties (**Fig. 8**).

4. EXAMPLE: THE Eos FLUORESCENT PROTEIN

EosFP is a fluorescent protein from the stony coral *Lobophyllia hemprichii* that can be subject to a photoconversion phenomenon from green to red under UV-light illumination [2]. The X-ray structures both in green and red states [3] confirmed that the photoconversion process results from a clear break in the protein mainchain (**Fig. 3**). We are trying to follow in detail the photoconversion process "in crystallo" by using in a combined way microspectrophotometry (**Fig. 5**), X-ray crystallography and quantum mechanics modelisations (**Fig. 6**). The proposed mechanism (**Fig. 7**) would involve the passage through an unstable biprotonated histidine 62 which relaxation to a non-charged state would be accompanied by a β-elimination and a concerted cleavage of a peptide bond close to the chromophore. The resulting chromophore delocalises the electrons on a wider conjugated system which shifts the fluorescence emission toward red wavelengths.



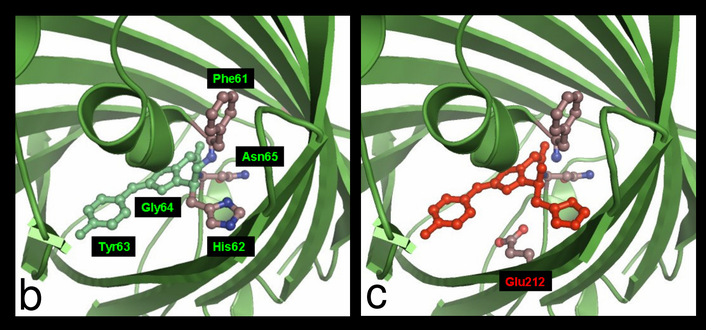


Fig. 3 – (a) Overview structure of the tetrameric wild-type EosFP obtained by X-ray crystallography. (b, c) Structure details of EosFP chromophore in its green form (b) and in its red form after cleavage of the peptide bond between Phe61 et His62 (b). The β-elimination process would involve the capture of a proton from His62 by Glu212

We are also working on several other fluorescent proteins : ECFP, Cerulean, Dendra, eqFP583…

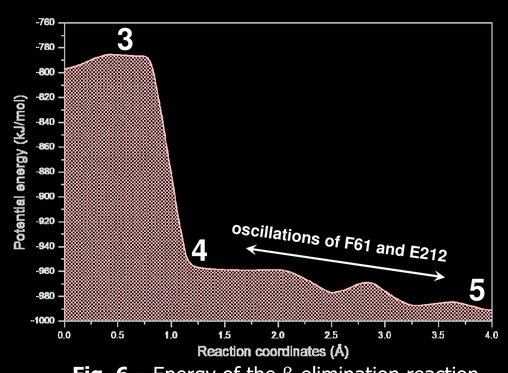


Fig. 6 – Energy of the β-elimination reaction in EosFP chromophore calculated by QM/MM

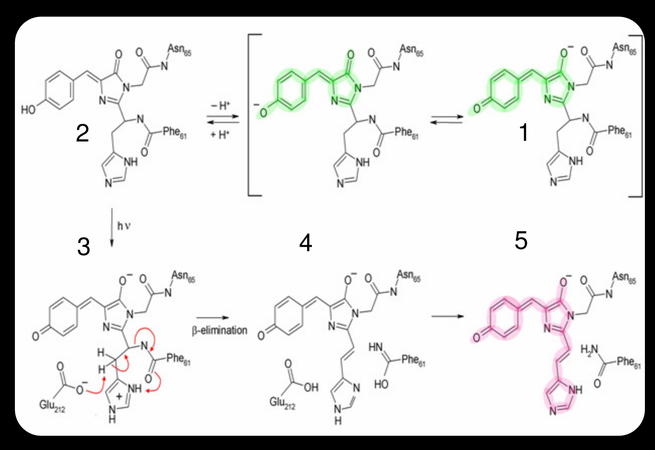


Fig. 7 – Proposed mechanism for the photoconversion green → red of EosFP

5. PERSPECTIVES

This study, initiated in 2005 has three main goals:
1/ a fundamental knowledge of fluorescent proteins photophysics
2/ methodological and instrumental developments
3/ Engineering of powerful biological markers for cell imaging and nanosciences

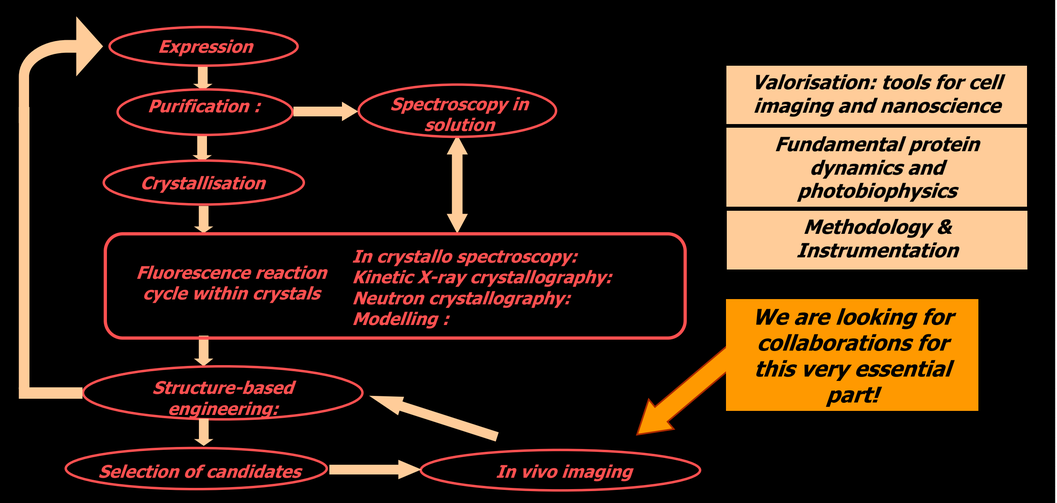


Fig. 8 – Proposed iterative approach for the study and the engineering of novel fluorescent proteins

References
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