

Quantitative Assessment of the Phototoxicity of Red Fluorescent Proteins

The internship will take place at the Chemistry Department of ENS (24 rue Lhomond - 75005 Paris). It will be co-supervised by Agathe Espagne (CNRS researcher, agathe.espagne@ens.psl.eu) and Théo Béguin (postdoctoral researcher, theo.beguिन@ens.psl.eu).

Context:

Fluorescent proteins (FPs) of the GFP family are ubiquitous tools in biology laboratories. Their fluorescence is entirely genetically encoded, which allows them to be selectively targeted to any protein or structure of interest for live-cell imaging. However, as with any fluorophore, the light excitation required to produce fluorescence also leads to the generation of reactive oxygen species (ROS). These ROS are formed through interaction between the fluorophore's triplet excited state (T_1) and the oxygen present in the samples (Fig. 1a). They degrade the fluorophore by oxidizing it (photobleaching) and trigger unwanted cellular stress responses (phototoxicity), both of which are highly disruptive during an imaging experiment.

The photobleaching of FPs has been the subject of several comparative studies aimed at helping users select the most photostable fluorophore of a given color.^{1,2} Its mechanisms have also been characterized at the fundamental level for several FPs.³⁻⁵ In contrast, the phototoxicity of FPs has hardly ever been examined to date. This is due in particular to the lack of standard protocols for quantifying it. The relationship between photobleaching and phototoxicity is also poorly understood. The two phenomena are often conflated, since they are both caused by the photoproduction of ROS by the fluorophore. However, there are examples of FPs that are both highly phototoxic and highly photostable.⁶

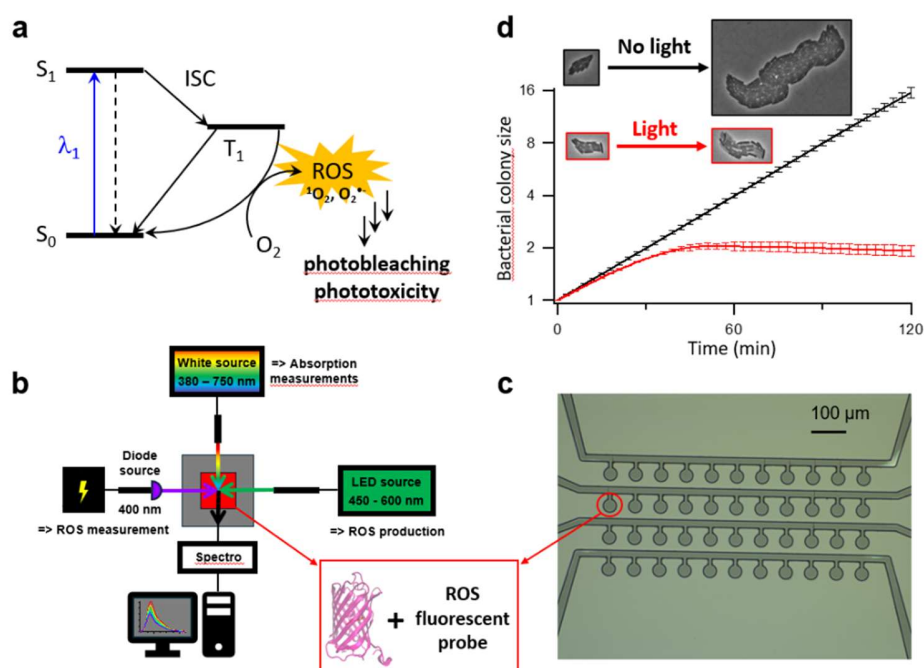


Figure 1: a) Photophysical scheme illustrating the formation of reactive oxygen species (ROS) upon excitation of a fluorophore. b) Optical setup for illuminating fluorescent proteins (FPs) in a cuvette and measuring ROS. c) Microfluidic chip for the parallel measurement of ROS under a microscope. d) Effect of phototoxicity caused by fluorophore illumination on the growth of bacterial colonies expressing FPs.

Objectives:

The objectives of the internship are:

- to develop protocols for measuring the phototoxicity of FPs that are quantitative, reproducible and, if possible, compatible with the screening of dozens of FP variants;
- to apply these protocols to a library of red FPs in order to identify the least phototoxic variants for sensitive imaging experiments;
- to examine the relationship between photobleaching and phototoxicity for the red FPs under study.

Techniques:

We plan to measure phototoxicity both in vitro (quantification of ROS formation) and in vivo (impact on cell division).

The in vitro experiments will involve:

- the expression of FPs in *E. coli* and their purification;
- the development of an optical setup to illuminate FP solutions in cuvettes and monitor their absorption and fluorescence emission spectra in real time⁴ (Fig. 1b);
- quantification of the ROS formed (notably singlet oxygen $^1\text{O}_2$ and superoxide $\text{O}_2^{\bullet-}$) using dedicated fluorogenic probes, first in cuvettes;
- depending on the time available, adaptation of the cuvette protocols to parallel ROS measurements under a microscope, using microfluidic chips (Fig. 1c).

The in vivo experiments, meanwhile, will aim to measure the impact of light excitation on the growth of *E. coli* colonies. We will test two approaches:

- illuminating a small volume of a bacterial suspension in a cuvette or under a microscope, followed by spreading it onto an agar plate and counting the colonies after one night;
- real-time monitoring under a microscope of bacterial colony growth under illumination (Fig. 1d).

The intern will also be required to analyze data from these experiments (microscopy images and kinetics of photobleaching or ROS production) using standard software or custom Python code.

This work could be extended to other fluorophores as part of a PhD thesis.

References:

1. Shaner et al., Nat. Methods 2005, 2, 905 [[Link](#)]
2. Cranfill et al., Nat. Methods 2016, 13, 557 [[Link](#)]
3. Duan et al., J. Am. Chem. Soc. 2013, 135, 15841 [[Link](#)]
4. Béguin et al., ACS Sensors 2026, 11, 7196 [[Link](#)]
5. Ludvikova et al., Nat. Biotechnol. 2024, 42, 872 [[Link](#)]
6. Bulina et al., Nat. Biotechnol. 2006, 24, 95 [[Link](#)]