Design and implementation of 2-color illuminations to reduce fluorophore photobleaching and phototoxicity in live cell fluorescence imaging

Fluorescence imaging is widely used in life sciences to visualize and analyze biomolecules. The illumination used to excite the fluorophores however also leads to the formation of reactive oxygen species (ROS) by interaction of the excited-states of the fluorophores and endogenous chromophores with molecular oxygen.[1] ROS are harmful to cells and contribute to phototoxicity. In addition, their reaction with fluorophores leads to fluorescence losses (photobleaching).[2] Phototoxicity and photobleaching both hamper the long-term monitoring of biological dynamics in living samples. Solutions to reduce phototoxicity and photobleaching are currently limited. The light dose applied to the sample can be reduced, but at the expense of the quality of the images or the time resolution of the experiment. Other approaches rely on removing oxygen from the sample to prevent ROS formation, or on adding reducing agents to scavenge them.[3] These methods can however strongly affect cell physiology.

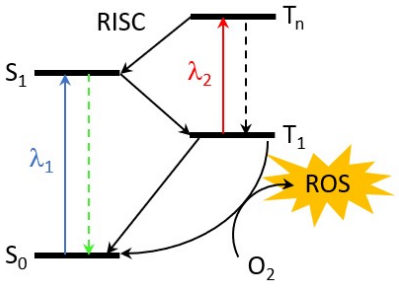

Fig. 1: Reverse intersystem crossing (RISC) under 2-color illumination (λ_1 and λ_2) to prevent ROS formation.

We recently introduced a novel method to reduce the photobleaching of fluorophores and the associated phototoxicity (i.e. the phototoxicity due to fluorophore excitation).[4] This method exploits the fact that some of the excited-states involved in ROS formation are triplet states, and that the relaxation of triplet states can in some cases be accelerated by light through a photophysical process called reverse intersystem crossing (RISC).[5] RISC is achieved by exciting the fluorophore from its lowest triplet state T_1 to a higher triplet state T_n, from which it transitions back to singlet excited-states and returns to ground-state S_0 (Fig. 1). This mechanism can be used to decrease the concentration of triplet states, and hence of ROS, in the samples. Our method therefore consists in submitting the samples to dual illuminations where the 1st illumination (λ_1 in Fig. 1) is applied according to the usual excitation conditions (wavelength, intensity) of the fluorophore and the 2nd illumination (λ_2) is tuned to the fluorophore's triplet absorption so as to trigger RISC. We validated this approach in wide-field fluorescence imaging for a series of green and yellow fluorescent proteins (FPs) expressed in live eukaryotic and prokaryotic cells.[6] Co-illumination at ~900 nm (absorption maximum of the triplet state of green and yellow FPs) allows a typical 4-fold reduction in photobleaching and suppresses the phototoxicity caused by FP excitation in growing bacteria (Fig. 2). The PhD project builds on these results and aims to further explore the potential of RISC and 2-color illuminations to reduce phototoxicity and photobleaching in live cell fluorescence imaging.

The first objective of the project is to **extend our method to other fluorophores**, in particular blue, cyan, orange, red and far-red FPs. Reducing the photobleaching and phototoxicity of FPs with different spectral characteristics would be particularly interesting for multi-color imaging.

The chromophores of orange/red and cyan/blue FPs are different from that of green and yellow FPs,[7] suggesting that they might have different photophysical behaviors, and their triplet properties are unknown (triplet absorption spectrum, triplet yield and lifetime, RISC yield). We will first select representative FPs of each color, which will be purified and studied by micro-millisecond transient absorption spectroscopy in ENS to determine their triplet spectra, triplet

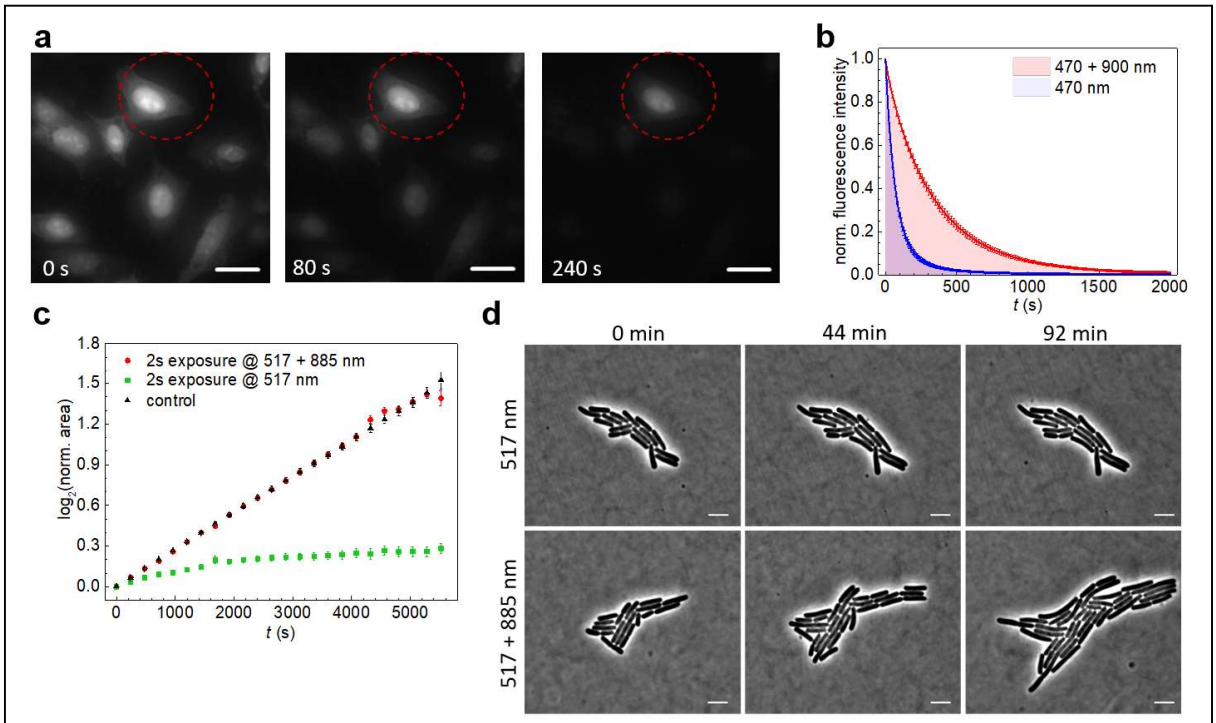

Fig. 2: Results on green and yellow FPs. a) Fluorescence images of fixed HeLa cells labelled with EGFP and illuminated at 470 nm (whole field) and 900 nm (red circle). b) Photobleaching kinetics extracted from these images. c) Time evolution of the area of bacterial colonies labelled with YPet growing on an agarose pad at 37°C in different illumination conditions: 517 nm alone (green), 517 nm and 885 nm (red) and no light (black). d) Phase-contrast images of the same bacteria showing the faster growth in presence of 885-nm light.

yields and triplet lifetimes. This will allow us to properly set the wavelength of the 2nd illumination. The effect of this 2nd illumination on the photobleaching kinetics will then be studied in vitro on a dedicated setup available in ENS. The 1st illumination will be provided by a LED or a laser and the 2nd illumination either by a tunable near-infrared laser (700-1000 nm range) or by a monochromatic visible laser (available wavelengths: 405 nm, 488 nm, 561 nm, 642 nm). The dependence on the intensities of the two illuminations will also be investigated. We will next evaluate the effect of the 2nd illumination on photobleaching and phototoxicity in live eukaryotic and prokaryotic cells. These experiments will be performed on the epifluorescence microscope of the Muse team (Micalis), which is equipped with temperature and CO_2 control, has been used for 2-color illumination of green and yellow FPs and will be adapted to provide an appropriate 2nd illumination for other FPs. The effect on long-term phototoxicity will be assessed by monitoring the growth of bacterial colonies, while that on short-term phototoxicity will be investigated using ROS probes. We will combine our experimental approach with simulations based on photophysical models to shed light on the underlying mechanisms.

The second objective of the PhD project is to **evaluate the possibility of exploiting RISC to reduce the phototoxicity due to endogenous chromophores**. Violet-blue light (under 500 nm) is known to be particularly toxic to cells, which can be problematic for the use of blue and even green FPs in live cell imaging. This toxicity has been suggested to be due to endogenous flavoproteins.[8] The absorption of oxidized flavins (the dominant redox form in vivo) increases in fact sharply below 500 nm. Although RISC has never been demonstrated in flavins, previous studies on flavin triplets[9,10] suggest that triplet populations comparable to those of green FPs could form under illumination. Flavin triplets have a very broad absorption

spectrum with a maximum around 650-700 nm.[10] We will first aim at observing RISC in isolated flavins such as FAD or riboflavin. For this purpose, we will exploit the fluorescence of flavins at ~520 nm to detect the possible effect of a co-illumination at 650-700 nm on their photobleaching kinetics. If flavins undergo RISC, we will then evaluate its impact on the phototoxicity induced in vivo by different wavelengths of primary illumination. Other endogenous chromophores such as porphyrins or NAD(P)H may similarly be investigated.

This project at the interface between Chemistry and Life Sciences will benefit from the skills of the CPBMV team of UMR PASTEUR in ENS in photochemistry and spectroscopy of fluorescent proteins and those of the Muse team of Micalis Institute in INRAE in microbiology and live cell microscopy. The student will perform in vitro experiments in PASTEUR (ENS) and in vivo experiments in Micalis (INRAE), thus benefiting from the expertise and equipment of both teams. The two teams have already established a successful collaboration on this topic,[4,6] ensuring a suitable work environment for the student.

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