# Structural evidence for the bleaching caused by oxygen in rsCherry

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Fluorescent proteins (FPs) play an indispensable role in advanced imaging techniques. Such proteins are considered as "smart labels" allowing scientists to overcome the diffraction barrier of conventional light microscopy to visualize subcellular events. Since the first discovery of GFPs in the 1960s [1], numerous studies have been conducted to design new fluorophores not only covering the whole visible light from cyan to far-red region but also displaying improved photochemical performances. Furthermore, the FP technology gains remarkable achievements by developing successfully special classes of FPs which exhibit photo-transformable properties including photoactivation (PA), irreversible photo-conversion (PC) and reversible photo-switching (RS) [2]. Currently, irreversible photoconvertible and reversible photo-switchable FPs attract wide interest of scientists due to their potential of converting from an emissive state to another emissive state or switching between a fluorescent and a non-fluorescent state, respectively. The combination of reversibly switchable behavior and spectrally different emission has enabled application of multicolored super-resolution microscopy techniques in live-cell imaging. However, various drawbacks of currently used reversibly switchable red FPs (rsRFPs) have limited their application greatly and made them still being the least used in GFP-like proteins family. Moreover, the structure-function relationship and the mechanism controlling photo-switching behavior of rsRFPs have not been understood completely. Therefore, structural studies are essential to provide valuable information for the rational design of improved rsRFPs which fit better to experimental requirements.

The rsCherry protein was the first reported reversibly switchable red FP which was developed from mCherry – a good label in imaging techniques [3]. However, due to the non-optimal properties of rsCherry such as limited brightness, poor photostability and low contrast between *on* and *off* states [4] its application in super-resolution microscopy was not very widespread. Our current study has shown that rsCherry lost its maximum absorption at 572 nm as well as fluorescence when it aged, despite being well protected from light, making studying its molecular structure and photo-mechanisms challenging. We were able to identify that the time-dependent bleaching in rsCherry is related to chromophore modifications and proposed that oxygen, a critical external reagent in the maturation process of FPs, is involved in unexpected chemical reactions of the chromophore. Spectroscopic data, native MS results and mutagenesis analysis, and especially structural studies of rsCherry crystallized in strictly anaerobic conditions strongly confirm our hypothesis that oxygen diminishes the rsCherry fluorescence through modifying its chromophore. These findings can help to develop improved red fluorescent proteins suitable for specific advanced imaging techniques.

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**Keywords: macromolecular crystallography; fluorescent proteins; reversibly switchable**.

*Acknowledgements: T.Y.H.B. thanks the Vietnamese government (Project 911) and Schlumberger Foundation Fellowship for a doctoral grant.*