Recent developments in fluorescent protein (FP) technology have made FPs essential as ‘smart labels’ for biosensing and advanced fluorescence imaging.\[1\] Photoswitchable and photoconvertible FPs for example play a crucial role in sub-diffraction microscopy, but are still limited in number and require optimization to access their entire potential. We aimed to develop a new reversibly switchable (RS) FP with improved effective brightness and similarly good photoswitching behavior based on rsEGFP.\[2\] In this work, we show that while simultaneous optimization of multiple fluorophore properties is a difficult task, a strategy wherein these are optimized separately and combined later yields great results.

rsEGFP was fused to a bait-peptide sequence, that interferes with the folding/maturation process,\[3\] and subjected to rational mutagenesis. A homebuilt \textit{E. coli} colony screening system was used to select a 20-fold brighter FP with slightly slower off-switching and reduced contrast. A single backmutation sufficed to increase the switching speed and contrast, but decreased the brightness to 10 times that of rsEGFP. Further random mutagenesis resulted in a series of RSFPs up to 30 times brighter than rsEGFP when expressed at 37°C, with similar or improved photoswitching speed and stability, and enhanced contrast. In \textit{vitro} protein expression revealed greatly enhanced maturation for all created RSFPs compared to rsEGFP, while all FPs exhibited similar spectroscopic properties. Improvements to photoswitching and structural stability were linked to structural information from x-ray crystallography. We used both the on- and the off-state crystal structure to determine key features of the photoswitching behavior. All mutants were shown to localize well in several fusion constructs and were used for superresolution microscopy using pcSOFI, which makes optimal use of the reversible photoswitching.\[4\]


