Engineering red fluorescent proteins

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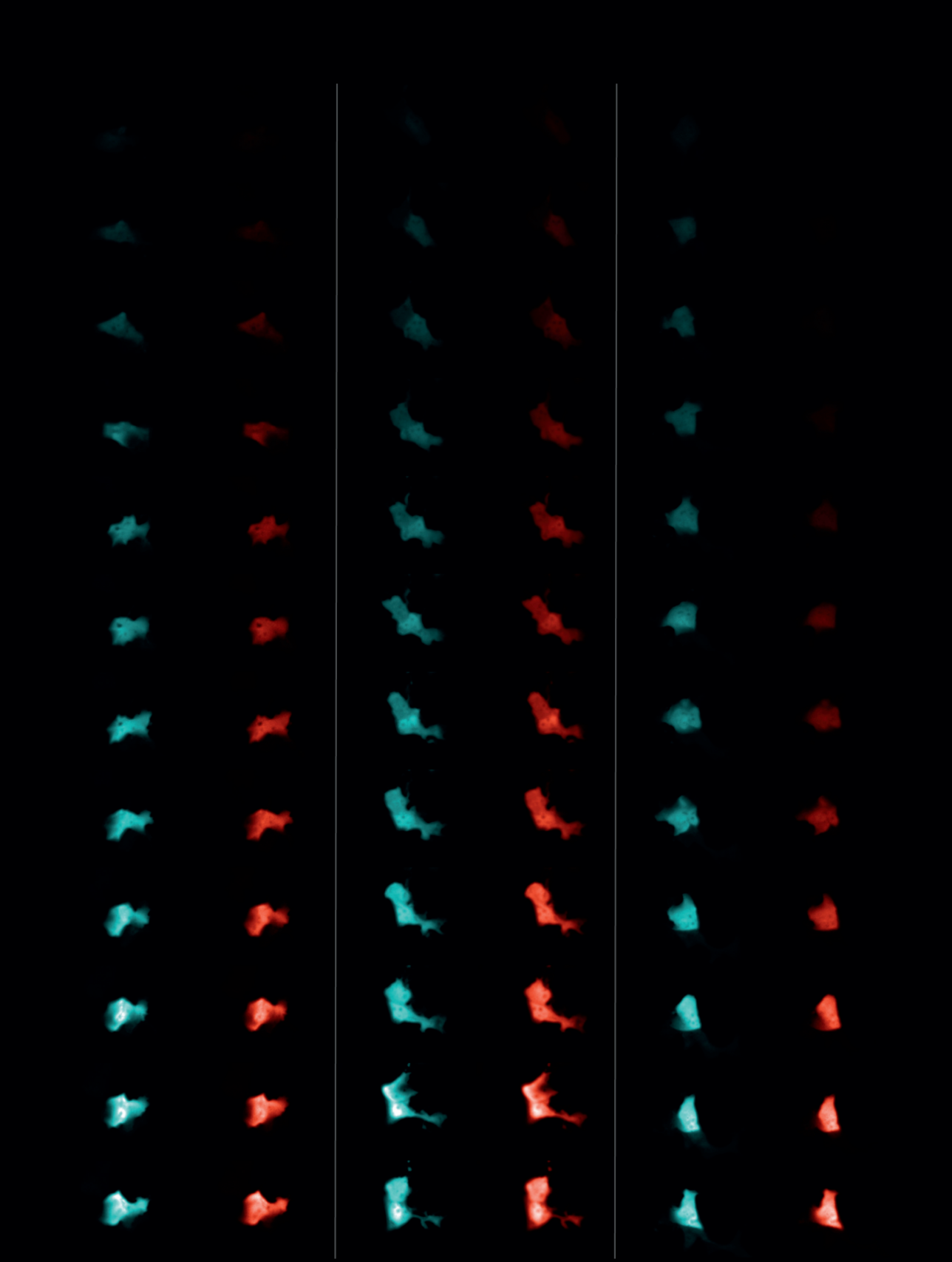

Red fluorescent protein maturation race

Summary

Engineering Red Fluorescent Proteins

An illuminating journey leading to the creation of the mScarlet family

Fluorescent proteins (FPs) are used as genetic labels to study processes in live cells by using fluorescence microscopy. Multiple processes can be observed simultaneously, when FPs in multiple colors are used. Currently, red fluorescent proteins (RFPs) are relatively dim, show poor maturation or have a residual tendency to dimerize, therefore currently available RFPs are not optimal for quantitative cellular imaging. The aim of the research presented in this thesis is to create an improved monomeric red fluorescent protein (mRFP). Initially, we applied established methods to the development of mRFPs. However, we found that the development of mRFPs was more complex and screening on merely one parameter is not sufficient. Therefore, we developed a multi-parameter screening method, as described in **Chapter 1**. First a mutant library is generated by polymerase chain reaction (PCR), after which the mutant library is expressed in bacteria. The bacteria are grown in Petri dishes and each colony expresses one new variant. The newly developed method contains two screening sections. First a primary screen for fluorescence lifetime and cellular brightness is performed on bacterial colonies and improved variants compared to its precursor are selected. During the second part, the selected variants are expressed in mammalian cell lines and screened for fluorescence lifetime, cellular brightness and photostability. Next, all these parameters are compared between the mutants and the best variant is selected. The development of this multi-parameter screening method coincided with the development of improved mRFPs and ultimately led to the mScarlet family, as described in **Chapter 2**. mScarlet is the brightest mRFP with a record quantum yield in its spectral class. Two other important variants are developed: mScarlet-I which shows enhanced maturation and mScarlet-H which displays extreme photostability. We quantitively compared the new mScarlets to existing RFPs *in vitro* and *in vivo*. All three mScarlets show great performance in protein fusions and in cellular functional imaging. The performance of the mScarlets is not complicated by undesired characteristics like photochromic behavior, cytotoxicity, unwanted residual dimerization in cells or incomplete maturation. Therefore, mScarlets are currently the preferred FPs in the RFP spectral class for live cell fluorescence microscopy. Photophysical properties of the mScarlet family and several well-known RFPs are studied by measuring the pH dependency, light dose responses and fluorescence fluctuations, as described in **Chapter 3**. The RFPs exhibit pH dependent states which correlate with the light dose response and fluorescence fluctuations. These observed states can be attributed to different protein conformations obtained from available X-ray crystallography data. Many photophysical properties critically depend on both pH and illumination and are different for each RFP variant. This makes it a major challenge to optimize and screen for RFPs with a particular combination of favorable photophysical properties. We demonstrated that the mScarlets are a valuable tool for biological research. So far > 2000 mScarlet plasmids have been requested by research groups all over the world and we have received positive responses from

scientists. This implies a major impact and contribution to the scientific community. In addition, we developed a multi-parameter screening method for RFP optimization, which can be applied to any color FP. The developed methods and new insights will assist future development of FPs of any color with high brightness, high photostability and fast maturation. Ultimately, mScarlet itself can serve as a new template for mRFP optimization.