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Engineering red fluorescent proteins

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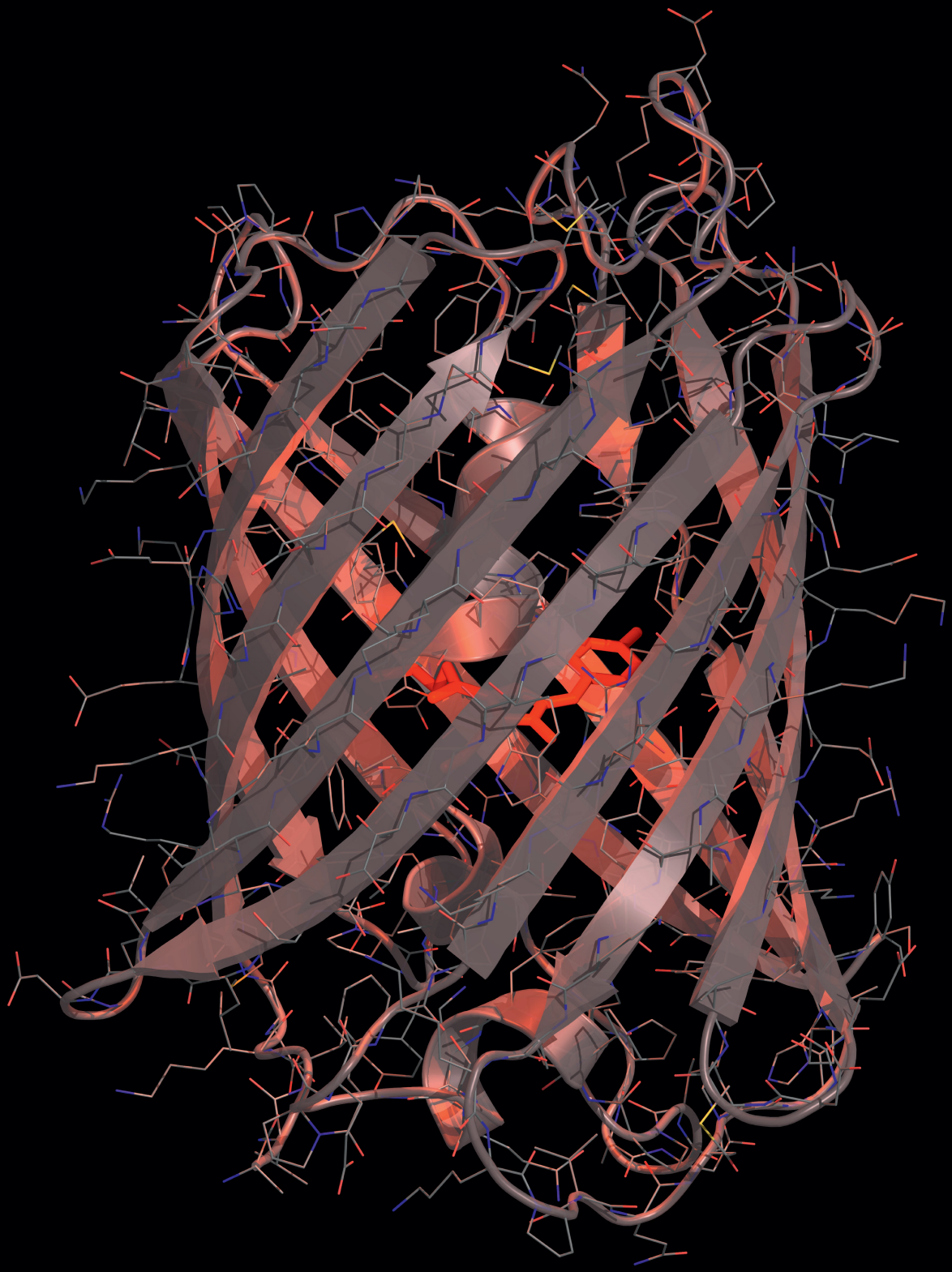

mScarlet in detail

General Discussion

The general approach to engineer improved red fluorescent proteins (RFPs) is as an iterative cycle that starts with mutagenesis and primary screening. New site-directed mutations are inserted by polymerase chain reaction (PCR), resulting in a library with mutations only at desired positions. The mutated genes are expressed in bacteria which are grown on Petri dishes and each colony synthesizes one new variant. The fluorescence intensity of each colony depends on the brightness of the new mutant, but also on colony size and thickness. To prevent this bias, the Petri dishes are imaged by fluorescence lifetime imaging microscopy (FLIM). Previously screenings were performed on either fluorescence intensity or fluorescence lifetime. However, we found that the optimization of red RFPs is more complex and demanded a novel thorough approach.

We selected five critical parameters that affect the performance of an FP including, oligomeric state, fluorescence lifetime, cellular brightness, maturation and photostability. We often found anti-correlations between the parameters. For example, a variant that displays a high cellular brightness often displays a low fluorescence lifetime and a variant with a high photostability often displays a low cellular brightness. We developed a multi-parameter screening method to select improved RFPs with respect to the fluorescence lifetime, cellular brightness, maturation and photostability, as described in **Chapter 1**. We started from a truly monomeric synthetic RFP (mRed7), therefore it was not necessary to subsequently screen for oligomeric state. The screening procedure contains a primary screen on bacterial colonies grown in Petri dishes and each colony expresses a new variant. Colonies with increased fluorescence lifetime and cellular brightness compared to its precursor are selected. The selected variants are expressed in mammalian cells grown in a 96 well plate and the fluorescence lifetime, cellular brightness and photostability are monitored. We constructed a dual expression screening plasmid for bacteria and mammalian cells to accelerate this workflow. For each assay of the secondary screen, we developed an automated multi-position acquisition protocol with automated analysis, yielding detailed information per well and an overview of the whole 96-well plate. A GUI has been developed in which data of all parameters is combined and the best mutant with respect to all parameters can be selected. This multi-parameter screening method will expedite future development of FPs with high brightness, high photostability and fast maturation.

The development of the multiparameter screening method coincided with the development of monomeric bright RFPs, as described in **Chapter 2**. We applied a new evolution strategy and started from a synthetic gene mRed7. The amino acid sequence of mRed7 is inspired by published RFPs, monomeric FPs and fast folding chromoproteins. Thereby, we increase the evolutionary distance with respect to the three natural RFPs (DsRed, eqFP578 and eqFP611) and start fresh using a new template. The most important characteristic of mRed7 is that this RFP is monomeric and screening for oligomeric state is redundant. Unfortunately, mRed7 is very dim compared to published RFPs and required serious brightness improvements. We then applied different mutagenesis methods: rational designed mutant libraries in which up to 5 positions were mutated and random mutagenesis.

We screened the mutant libraries for high fluorescence lifetime, high cellular brightness, fast maturation and high photostability using the methods described in **Chapter 1**.

Finally, this resulted in a novel monomeric RFP series: mScarlet, mScarlet-I, and mScarlet-H. We quantitatively compared the mScarlets to existing RFPs, all RFPs have been measured under the same conditions and have been compared to the same controls. We performed extensive analysis *in vitro* for extinction coefficient, quantum yield, fluorescence lifetime, and pH dependence. In addition, we conducted a thorough characterization *in vivo* for cellular brightness, photostability, photochromicity, maturation, oligomeric state and cytotoxicity. Moreover, we assessed their performance as a fusion protein and as FRET acceptors *in vivo*. In conclusion, mScarlet is the brightest monomeric RFP with record quantum yield in its spectral class. The other two variants: mScarlet-I shows enhanced maturation and mScarlet-H displays improved photostability. The applicability of mScarlets is not hindered by complications due to photochromicity, cytotoxicity, unwanted residual dimerization in cells or incomplete maturation. All three mScarlets show great performance in protein fusions and in cellular functional imaging. Therefore, mScarlets are the preferred FPs in the RFP spectral class for live cell fluorescence microscopy.

We studied the photophysical properties of the mScarlet family and other well-known RFPs to gain a more detailed understanding of their fluorescent mechanisms, as described in **Chapter 3**. The pH dependency was studied in detail by measuring the absorbance and emission spectra, and fluorescence lifetimes at pH levels from 3 – 11. The RFPs display multiple pH dependent red fluorescent states as well as dark states. The different states have a distinct absorbance and emission spectra as well as distinct fluorescence lifetimes and apparent quantum yields (i.e. brightness). In addition, the light dose responses of a large selection of RFPs expressed in cells was measured using different types of illumination, as well as the fluorescence fluctuations of a small set of RFPs in solution with physiological pH using a wide range of excitation intensities.

The studied RFPs can be categorized in roughly three groups. Group 1 shows at least three different protonated states from low pH towards high pH: a blue absorbing state, a dim red fluorescent state and a bright red fluorescent state. The RFP variants mScarlet, mScarlet-I, mScarlet Y84F, mApple, mCherry and dTomato belong to this group. The light dose response curve measured in live cells for RFP variants of group 1 show a multi-exponential decay if the variant is predominantly in its bright red fluorescent state at physiological pH level and a mono-exponential decay if the variant is predominantly in its dim red fluorescent state at physiologic pH level. We observed a mono-exponential decay for mCherry and a multi-exponential decay for all other RFPs of group 1. Group 2 also shows at least three different states, but with inverted brightness, from low pH towards high pH: a blue absorbing state, a bright red fluorescent state and a dim red fluorescent state. Group 2 comprises of mScarlet-H, mScarlet-H Y84F and mKate2. The light dose response curve measured in live cells for the RFP variants of group 2 show first an increase in fluorescence signal, after which the signal eventually also declines. Group 3 also shows a blue absorbing

state at low pH, but displays multiple red fluorescent states with similar molecular brightness. The RFP variants mRuby2 and TagRFP-T are classified as group 3. The light dose response curves for RFP variants belonging to group 3 show a mono-exponential course as well. The relation between the pH dependent states and the light dose response curves implies that the different red fluorescent states are not only connected through protonation, but are connected through their excited states as well. This is corroborated by the fluorescence fluctuations data. The different states can be associated with different protein conformations obtained from available RFP crystal structures. **Chapter 3** provides a qualitative description of photophysical behavior of RFPs. A quantitative description that includes every state and the conversion rates between the states could be provided with a more extensive data set at a higher resolution with a larger range and more variables, including temperature.

The results from **Chapters 2 and 3** imply relations between properties of FPs that were initially assumed to be isolated. Early in this thesis research, we observed an anti-correlation between photostability and cellular brightness (i.e. maturation). As example, two RFP variants show this anti-correlation: TagRFP-T and mScarlet. Cells expressing TagRFP-T result in a low recorded intensity, although TagRFP-T displays a high intrinsic brightness. In addition, TagRFP-T is photostable when it is not activated with blue light. Cells expressing mScarlet result in a high recorded intensity, this agrees with mScarlet's high intrinsic brightness. Moreover, mScarlet has a reduced photostability compared to TagRFP-T, but mScarlet is not activatable with blue light. Now, we can finally speculate about the anti-correlation between high cellular brightness (i.e. maturation) and photostability.

Chapter 3 implies that the photostability of TagRFP-T is a pseudo-photostability. When TagRFP-T is excited it continuously converts between multiple states. In addition, its photochromic behavior implies that a fraction is in a dark state which only converts to a bright state with blue light. This results in a low cellular brightness and is classified as 'bad' maturation. Even though, TagRFP-T maturates at the same rate as mScarlet. mScarlet only displays one bright state at physiological pH, intense excitation could convert the bright state to a dim state after which it converts to a permanently off state (i.e. irreversible bleaching). mScarlet is therefore present in one bright state in cells and displays a high cellular brightness which is classified as 'good' maturation. mScarlet's record quantum yield is caused by stabilizing the bright red fluorescent state, all rational designed mutant libraries were entirely focused on stabilizing the chromophore from every side. As a consequence, the chromophore is not able to adopt another conformation, and cannot convert to another bright state. This confirms the correlation between 'photostability' and low cellular brightness (e.g. 'bad' maturation). According to this reasoning, the photostability of mScarlet-H should also be referred to as pseudo-photostability.

The 'red' chromophore is a long conjugated system that could bend and twist at different positions. It is complex to stabilize the 'red' chromophore in a bright state, e.g. to hold it rigidly in a planar configuration, but we succeeded which resulted in mScarlet.

Stabilizing the chromophore also led to a pH stable RFP where the fluorescence lifetime is stable from pH 11 to pH 7 and then converts into only one other red fluorescent state. The next step would be to create a bright monomeric RFP that is 'truly' photostable. With our current knowledge, that should be an RFP which is present in only one bright red fluorescent state that has a small to nihil chance of bleaching. But we need more comprehensive understanding of the concept bleaching and the entire photocycle. Moreover, these concepts need to be linked to the conformational changes and irreversible reactions that occur inside the β-barrel. The results of **Chapter 3** give a clue towards understanding the photocycle, this research should be continued to obtain a complete understanding of the photophysical mechanism of RFPs.

One might think that pursuing a 'truly' photostable RFP is needless and that we suffice with a bright pseudo-photostable RFP, however the pseudo-photostability of TagRFP-T in fact coincides with complex photochromic behavior. This complicates multi-color acquisitions and TagRFP-T is thereby inadequate for usage in applications like FRET. In addition, the pseudo-photostability will also come at the cost of the cellular brightness. Moreover, the pseudo-photostability is a result of multiple bright states which are easily converted, this could mean that the energy is lost to a conformational change instead of emitting a photon which reflects in a lower quantum yield. Therefore, it might not be possible to obtain a pseudo-photostable RFP with a quantum yield above 0.5.

As mentioned above, we did succeed in stabilizing the 'red' chromophore compared to currently existing monomeric RFPs. However, the quantum yield is 'only' 0.70 and the theoretical limit is 1. This means that there is still a major improvement possible. So far, the emphasis was to create a planar chromophore with respect to the imidazoline and phenolate rings of the chromophore. This approach is inherited from the 'green' chromophore optimization, but the conjugated system of the 'red' chromophore is extended with four bonds compared to the 'green' chromophore. We should focus on creating an entire planar 'red' chromophore to further elevate the quantum yield.

Another intriguing aspect is the structure of the β-barrel of RFPs. We noticed that the cross-section of the β-barrel of RFPs is more oval compared to the round cross-section of the β-barrel of GFPs. This could be due to the fact that the RFP β-barrel needs to accommodate a longer chromophore. In addition, the 'gap' between sheet 5 and 6 (**Chapter 3 Supplementary Fig. 1**) seems larger in RFPs and might cause instability by possible flow through of small molecules and giving solvent molecules access to the phenolate moiety of the chromophore. It would be better to completely shield the chromophore by 'fixing' the β-barrel (although oxygen still needs to be able to access for the chromophore maturation). This could be done by adding 1 β-sheet. This would have a few advantages. The entire β-barrel then consist out of 12 β-sheets which can then be perfectly aligned in a non-parallel fashion. The stress on the β-barrel might be relieved and can be round with enough space to accommodate the long RFP chromophore while protecting the chromophore from every side.

Recent results from a de novo synthesis of a β-barrel[1] could aid the re-design of an RFP β-barrel.

Another notable observation is that mScarlet exhibitis punctate structures in neurons and in common cultured cell lines (e.g. U2-OS) after a week of transfection. It still remains unclear what these punctate structures exactly are, but they are undesired and might affect the role of RFPs as inert label. Other RFP variants like mCherry show similar punctate structures, but dTomato does not. In addition, the precursor of mRuby, eqFP611, displayed a dominant localization in the lysozymes, while transfecting with a plasmid encoding a 'free' diffusing eqFP611. Engineering the C-terminus of eqFP611 led to a gradient of confined and 'free' localization and ultimately resulted in a free diffusing mRuby, this property is inherited by mRuby2. I suspect that mScarlet has inherited a weak signal peptide sequence from existing RFPs, most likely mCherry, and that this signal peptide is shielded or 'tugged in' for dTomato. Finding and removing this signal peptide would further expand the applicability of mScarlet in neurosciences, stable cell lines and in vivo.

At the moment of this writting, the mScarlets have been requested for > 2000 times by research groups all over the world and we have received positive responses from scientists. The number of requested plasmids imply a major impact and contribution to the scientific community from a genetically encoded probe perspective. We developed new screening methods for RFP development, which can be applied to any color FP. In addition, we obtained a more comprehensive understanding of RFPs and are now able to connect different aspects such as maturation and photostability, and photostability and pH dependence. The continuation of this research will lead to a holistic understanding of FPs, using this knowledge we can combine all enhanced properties in one RFP. To complete the optimization cycle, mScarlet itself can serve as a new template for RFP optimization.

References

1. Dou, J. et al. De novo design of a fluorescence-activating β-barrel. Nature 561, 485–491 (2018).