Engineering red fluorescent proteins

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Crystal structures of colorful fluorescent proteins
General Introduction
Fluorescent proteins
Almost 25 years ago, the first fluorescent protein (FP) was introduced as a tool to genetically label proteins of interest in a living cell. The gene encoding a green fluorescent protein (GFP) was isolated from the jellyfish *Aequorea victoria*\(^1\) and was used to visualize sensory neurons in a nematode\(^2\). The revolutionary aspect of FPs is that proteins and cellular processes can be visualized live in a non-invasive manner using fluorescence microscopy. In 2008 Osamu Shimomura, Martin Chalfie and Roger Y. Tsien received the Nobel Prize in chemistry 2008 for the discovery and development of GFP.

The gene encoding a fluorescent protein can be fused to a gene encoding a protein of interest. This recombinant DNA sequence can be introduced into a cell, after which the cell will synthesize this protein of interest covalently bound to a fluorescent protein. Using fluorescence microscopy, these cells can be imaged and the protein of interest can be studied live while perturbing the cells minimally, in contrast to e.g. immuno-labelling, for which cells need to be fixed, permeabilized and incubated with expensive and sometimes unspecific antibodies. The use of a genetic label like an FP ensures a hundred percent specificity, which is another major advantage.

For fluorescence microscopy there are several properties of FPs that are very important since these will affect the performance of an FP as a fluorescent label. Here, the most important properties are being discussed. First, it is highly desirable to use bright fluorescent labels. This will yield a high contrast between foreground and background, i.e. a bright FP will result in a high signal-to-noise ratio in fluorescence imaging. The brightness of an FP is often expressed as the product of the extinction coefficient and the quantum yield. The extinction coefficient is a measure for the number of absorbed photons (per molar concentration per cm) and is usually given at the wavelength of maximal absorption. The quantum yield is the ratio of emitted photons over absorbed photons. Second, the shape of the absorption and emission spectra are also important characteristics of an FP. Evidently, the wavelengths of the excitation light and detection window should match with the absorption and emission spectra of the FP. Moreover, when recording multiple FPs during one acquisition it is advantageous to use different FPs with minimal spectral overlap in order to minimize bleed through, i.e. FPs with narrow absorption and emission spectra are preferred. Third, photostable FPs allow prolonged acquisitions without loss of the fluorescence intensity over time (bleaching). Fourth, the maturation should be fast and complete in the model organism in which the FP is being expressed. Throughout this thesis, the maturation is defined as the process that occurs after translation until the moment the protein becomes fluorescent, this will be explained in more detail below. These four properties play a central role in FP engineering and are continuously modified and optimized when creating new improved fluorescent proteins for fluorescence microscopy.
The structure of GFP

The original wild-type GFP consists out of 238 amino acids that form a β-barrel structure (Fig. 1a). The β-barrel is formed by eleven anti-parallel β-strands. A chromophore is located at the heart of the β-barrel on a distorted α-helix that stretches through the β-barrel3 (Fig. 1b). A series of auto-catalytic reactions convert three sequential amino acids into a chromophore and requires only oxygen as an external factor4. For wild-type GFP, the chromophore is formed from the amino acid sequence SYG (one letter amino acid code) (Fig. 1c). In this the folding of the β-barrel and the formation of the chromophore is defined as maturation. The N- and C-termini are structured as random coils, which allows straightforward fusions to proteins of interest at the N- or C-termini.

Figure 1 | The structure of GFP. Cartoon representation5 of the crystal structure of GFP (PDB ID: 1EMA), the chromophore is depicted in green and other residues in grey (a) front view and (b) cross section. Water molecules are presented as red spheres, hydrogen bonds are shown in yellow. Amino acid residues that are in close proximity to the chromophore are shown as lines and sticks. (c) Chemical structure6 of the GFP chromophore shown in green, R1 represents the side chain of serine.

A chromophore alone will only absorb light and it will not emit photons, hence when it is dissolved in water it will absorb but not exhibit fluorescence itself7. However, inside the β-barrel the chromophore interacts with the interior amino acid residues through hydrogen bonds and van der Waals forces. These interactions can be direct between the chromophore and interior amino acid residues or indirect via a water molecule. Due to this complex network the FP as an entire unit can absorb and also emit photons, i.e. the FP is a fluorophore.

The size of the conjugated electron system of the chromophore is directly linked to the absorption cross section and thereby determines the absorption wavelength (the “color”). The rest group of R1 (Fig. 1c) directly affects the auto-catalytic formation of the chromophore and can result in a longer conjugated system8,9. Amino acids interacting with the chromophore can cause a blue (hypsochromic) or a red (bathochromic) shift of the “color”10. When the chromophore is rigidly constraint in the β-barrel, less energy is lost through non-radiant processes like vibrations and physical displacements, which results in a higher quantum yield.
Towards ‘sensing’ in living cells using FPs

Soon after the utilization of GFP, blue, cyan, and yellow color variants were engineered from GFP\(^8,11\). For blue and cyan FP (BFP and CFP), the chromophore itself was mutated, Y67H and Y67W for BFP and CFP, respectively. For yellow FP (YFP), 203Y is introduced and this residue interacts with the ‘green’ chromophore through π-stacking, thereby causing a red shift. In the meantime, other marine creatures were scrutinized for new templates. Many FPs were found in the class *Anthozoa*\(^4,12,13\), this class includes sea anemones, stony corals, and soft corals. Not only green FPs were found, but also blue, yellow, orange, red fluorescent proteins and several colors of chromoproteins\(^14,15\). Chromoproteins only absorb light and are not able to fluoresce. FPs and chromoproteins found in *Anthozoa* have a similar tertiary structure as green fluorescent protein from the jellyfish *Aequorea victoria*.

FP development started relatively straightforward by creating new colors and improving the brightness, but became more extensive due to higher requirements of fluorescent labels and new developed microscopy techniques. Early engineered color variants, such as EBFP\(^11\) and ECFP\(^11\), performed relatively mediocre, as these appeared dim resulting in a low signal-to-noise ratio in experiments. The next focus was to create brighter FPs, resulting in e.g. EBFP\(^2\)\(^16\) and Cerulean\(^17\). Wild-type FPs mature poorly in mammalian cell lines when cultured at 37 °C, since the FPs originate from sea organisms. Therefore improvements in maturation became a key objective as well and was the focus in the development of Venus\(^18\). In addition, the photostability got attention from the FP developers, the FPs SCFP3A, SGFP2 and SYFP2 were specially developed for optimal maturation in bacteria and mammalian cells with improved photostability relative to their precursors\(^19,20\). Currently, the above mentioned objectives are all combined in FP development, thus creating FPs with high brightness, high photostability and fast maturation which has led to mTurquoise2\(^21\) and mNeonGreen\(^22\).

Biosensors containing fluorescent proteins were developed to monitor small metabolites, signal molecules, protein states (i.e. phosphorylation), and protein interactions\(^23\). A general strategy to engineer a biosensor is to first create a ‘sensing’ domain by truncating a functional protein to a binding domain for a small molecule (that is desired to monitor) and upon binding a conformational change should take place. One option is to fuse the ‘sensing’ domain with one FP in such a way that the conformational change increases or decreases the fluorescence intensity. Monitoring this biosensor is biased to several artifacts that could change the detected fluorescence intensity (e.g. cell movement and bleaching), since only one color is recorded. A more robust method is to fuse the N- and C-termini of the ‘sensing’ domain each with an FP\(^24,25\). When the emission spectrum of one FP (donor) overlaps with absorption of the other FP (acceptor) an energy transfer process from an excited donor to an acceptor in the ground state can take place, this process is called Förster Resonance Energy Transfer (FRET). The distance and orientation between the FPs strongly affect the FRET efficiency, therefore the conformational change of the ‘sensing’ domain will alter the FRET efficiency.
Several research groups have developed a pair of FPs specially optimized as a FRET pair\textsuperscript{26-28}. The biomolecular development of FPs and biosensors coincided with improvements of fluorescence microscopy hardware. For example, microscopes with an elevated light transmittance and larger field of view were developed. Objective lenses that correct for chromatic aberrations were released. New cameras with increased quantum efficiency and larger image sensor chips were developed. In addition, microscope control became entirely automated, the XYZ positioning and also filter wheels and other components can be toggled on and off by software.

The improvements of fluorescent labels, biosensors and hardware enabled automated acquisitions of multiple fluorescent proteins (sequential or simultaneous) over a prolonged time. This means that several proteins of interest, small metabolites and signal molecules can be studied for hours up to days in a living cell or organism. Photostable FPs in multiple colors combined with improved fluorescent microscope techniques facilitate quantitative imaging, thereby enabling quantification of small biological changes. Unfortunately, the available red FPs (RFPs) are not optimal yet. The current available RFPs yield a low signal-to-noise ratio and thereby complicate quantifications. Biological research would truly benefit from an optimized red fluorescent protein, which led to the research project presented in this thesis.
Red fluorescent proteins

The development of red fluorescent proteins (RFPs) lagged behind the development of cyan, green and yellow fluorescent proteins and only started after the discovery of FPs in Anthozoa. Most popular RFPs are derived from only 3 natural templates: DsRed, eqFP578, and eqFP611. DsRed is cloned from disk coral Discosoma species and eqFP578 and eqFP611 are both cloned from anemone Anthmacea quadricolor, most likely from a different morph. The RFPs between the two species share about 50% homology and the two Anthmacea quadricolor RFPs share 76% homology among each other. The most remarkably difference between the two species is the amino acid sequence from which the chromophore is formed. The chromophore of DsRed is formed from the sequence QYG (amino acid 65-67) in contrast to the anemone RFPs of which the chromophore is formed from the amino acid sequence MYG (amino acid 62-64).

Figure 2 | Alignment of 3 parental Anthozoa RFPs: DsRed, eqFP611, and eqFP578. The chromophore is shown in black (65-67 DsRed numbering), conserved residues are colored according to the Clustal X color scheme. According to pairwise alignment algorithm, the sequences share 50%, 53%, 76% homology, for the sequence pairs DsRed and eqFP611, DsRed and eqFP578, and eqFP611 and eqFP578, respectively. The secondary structure (α-helix and β-strands) are also indicated.

All three RFPs have a similar GFP-like β-barrel structure, but they exist as oligomers (Fig 3a). DsRed and eqFP578 exist as a tetramer, while eqFP611 exists as a dimer. The oligomeric state depends on the local concentration and even larger aggregates could be formed. Compared to a ‘green’ chromophore, the ‘red’ chromophore is extended by an additional oxidized carbon bond (Fig. 3). In figure 3, two alkene bonds are indicated with 1 and 2, only alkene bond 1 is required for a ‘green’ chromophore (Fig. 1). The chromophore in DsRed adopts a cis-conformation relative to alkene bond 1 (Fig. 3b), in contrast to the trans-conformation relative to alkene bond 1 of the chromophores in eqFP578 and eqFP611 (Fig. 3c). The conformation of the chromophore is stabilized by the interactions of the chromophore with the residues inside the β-barrel.
The absorbance and emission maxima of DsRed are 558 nm and 583 nm, respectively\(^{32}\) (\textbf{Table 1}), hence the Stokes shift for DsRed is 25 nm. The extinction coefficient and quantum yield of DsRed are 75,000 M\(^{-1}\)cm\(^{-1}\) and 0.70, respectively\(^{32}\). DsRed and eqFP578 have similar spectroscopic properties, like brightness and Stokes shift. Although, eqFP578 is slightly blue shifted compared to DsRed (absorbance and emission maxima are 552 and 578 nm, respectively\(^{29}\)). EqFP578 has an elevated extinction coefficient\(^{29}\) (102,000 M\(^{-1}\)cm\(^{-1}\)), but a reduced quantum yield\(^{29}\) (0.54) compared to DsRed. EqFP611 has the largest Stokes shift of these natural FPs (52 nm), with an absorbance and emission maxima at 559 nm and 611 nm, respectively\(^{30}\). EqFP611 is the least bright of these three which has an extinction coefficient and quantum yield of 78,000 and 0.45, respectively\(^{30}\).
Table 1 | RFP characteristics

<table>
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<tr>
<th>RFP variant</th>
<th>Oligomeric state</th>
<th>Abs max (nm)</th>
<th>Em max (nm)</th>
<th>ε (10^3 M^-1 cm^-1)</th>
<th>ϕ</th>
<th>ε * ϕ (10^3 M^-1 cm^-1)</th>
<th>Stokes shift (nm)</th>
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<td>26</td>
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<td>611</td>
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*aOligomeric state: tetramer (t), dimer (d), and monomer (m). *Absorbance maximum. *Emission maximum.
*Extinction coefficient at maximum absorbance. *Quantum yield *Calculated brightness, product of extinction coefficient and quantum yield. Natural ancestor are shown in black (DsRed12,32, eqFP61130 and eqFP57829), important published intermediate variants are shown in grey (mRFP133, mOrange9, mRuby34, TurboRFP29, TagRFP29, Katushka15, and mKate35), and popular RFPs are shown in bold red (mCherry9, mApple36, dTomato9, mRuby227, TagRFP-T36 and mKate237), recent descents of popular RFPs are shown in italic red (mCherry238, mRuby338, and FusionRed39).

The spectral properties of these naturally occurring RFPs are adequate to be used as a fluorescent label for fluorescence microscopy, however the slow maturation at 37 ºC and oligomerization hinder the application of these RFPs for live cell imaging. In order to monomerize these natural RFPs, residues facing outward of the β-barrel that interact with residues from the other β-barrels are mutated. Unfortunately, monomerization leads to a severe loss of brightness. During the past two decades, multiple research groups focused on creating a monomeric bright RFP. In Figure 4 the evolution pathways of currently commonly used (popular) RFPs are shown and important characteristics of these RFPs can be found in Table 1. Figure 4 represents a selection of RFP development of the past decades. This selection includes the popular RFPs, shown in red bold, with their evolution pathway and most recent improved descendant, shown in red italic.
DsRed was first monomerized resulting in mRFP1\textsuperscript{33} at the cost of a severe loss in brightness \((\text{Table 1})\). Interestingly, mRFP1 displays a bathochromic shift compared to its parent DsRed. The monomerization strategy was re-evaluated and gave rise to the mFruit series\textsuperscript{9}. dTomato is a dimer RFP variant which has a similar brightness as its tetrameric precursor. The brightness of mRFP1 was improved and resulted in mCherry, with a significant increase of the extinction coefficient. Other spectral properties (absorbance and emission spectra and quantum yield) remained practically unaltered in the evolution of mRFP1 to mCherry. The nucleotide sequence for the first 7 and last 6 amino acids of GFP have been added to the RFP genes of the mFruit series to increase the fluorescence intensity in cells (amino acids sequence MVSKGEE- and -MDELYK for N- and C-terminus, respectively, \textbf{Fig. 5}). The mFruit series included several color variants ranging from orange (mOrange) to far-red (mPlum). Interestingly, mutagenesis applied to the orange variant mOrange resulted in mApple\textsuperscript{36}. mApple displays a bathochromic shift compared to its precursor mOrange and a hypsochromic shift compared to mCherry. mCherry displays a more cytotoxic phenotype compared to GFP, since cells transiently transfected with mCherry grow slower than cells transiently transfected with GFP. mCherry has been subjected to several rounds of mutagenesis to obtain a less cytotoxic variant, resulting in mCherry2\textsuperscript{38}. Spectroscopic properties of mCherry2 are very similar to its precursor.
Dimer eqFP611 was engineered into a monomer, resulting in mRuby\textsuperscript{34}. The first engineered monomers showed localization at peroxisomes, the C-terminus was altered to assure homogenous localization in cells of untargeted mRuby. The spectroscopic characteristics were slightly altered during the monomerization (Table 1). The high extinction coefficient of eqFP611 (146,000 M\textsuperscript{-1}cm\textsuperscript{-1}) is reduced to 112,000 M\textsuperscript{-1}cm\textsuperscript{-1} and the mediocre quantum yield of eqFP611 is slightly reduced from 0.45 to 0.35. Interestingly, the large Stokes shift of eqFP611 is maintained in mRuby. mRuby\textsuperscript{27} was engineered to function as an optimal acceptor in a green-red FRET pair (in this work Clover was optimized from GFP to function as an optimal donor). Lam \textit{et al.} reported an improved photostability and marginal improved brightness for mRuby2. In addition, similar to the mFruit series, the nucleotide sequence encoding GFP N- and C- termini were also included in the gene of mRuby2. The brightness of mRuby2 was slightly improved and gave rise to mRuby\textsuperscript{35}. Moreover, an improvement in photostability of mRuby3 compared to mRuby2 was reported.

The tetrameric eqFP578 was first dimerized, yielding TurboRFP\textsuperscript{29}. The maturation at 37 °C of TurboRFP was also improved relative to eqFP611. Surprisingly, even the brightness of TurboRFP increased when compared to its precursor (Table 1), the brightness of the first descents of DsRed and eqFP611 decreased when compared to the brightness of their parent. The dimer interface of TurboRFP is disrupted and resulted in TagRFP\textsuperscript{29}. TagRFP shows only a minor reduction in brightness compared to TurboRFP. The photostability was significantly improved by introducing a single point mutation resulting which gave rise to TagRFP-T\textsuperscript{36}. The nucleotide sequence encoding GFP N- and C- termini were also included in the gene of TagRFP-T.

From TurboRFP a red shifted dimer RFP with a large Stokes shift was created, named Katushka\textsuperscript{35}. Katushka has an absorbance maximum at 588 nm and emission maximum at 635 nm (Table 1) and was especially intended for whole-body imaging. Katushka was monomerized at a cost of a decrease in brightness due to a reduced extinction coefficient, resulting in mKate\textsuperscript{35}. After which, the brightness of mKate was rescued and yielded mKate\textsuperscript{27}. The extinction coefficient and quantum yield were both increased for mKate2 compared to mKate. The brightness of mKate2 is similar to the brightness of Katushka. Even after monomerization, mKate2 still shows oligomers and can form aggregates in cells. mKate2, even as other RFPs, displays a more cytotoxic phenotype than \textit{Aequorea victoria} derived FP variants. mKate2 is further engineered into a more monomeric version that shows a less cytotoxic phenotype than GFP and mCherry, resulting in FusionRed\textsuperscript{39}. The more monomeric and less cytotoxic phenotype came at the cost of the brightness, the brightness of FusionRed is only 75 % of the brightness of mKate2.

The amino acid alignment of popular RFPs is shown in figure 5. Interestingly, the shared amino acid sequence homology remained equal between the descendants of the natural RFPs. The DsRed descendants (dTomato, mCherry and mApple) share 55 % and 57 % pairwise
homology with eqFP578 descendants (TagRFP-T and mKate2). The eqFP578 descendants (TagRFP-T and mKate2) and eqFP611 (mRuby2 and mRuby3) descendants share 74% pairwise homology with each other. Although, all commonly used RFPs share the same chromophore formed from the same amino acid sequence (MYG).

The residues that interact with the chromophore are often targeted by directed mutagenesis. For example, position 164 (dTomato numbering) is in very close proximity to the phenol group of the chromophore for cis-configured chromophores (dTomato, mCherry, mApple and mKate2) and is a key position for the stabilization of the chromophore. The alignment shows that this residue is not conserved throughout the different variants. Conserved residues can imply a critical residue with a detrimental function. For example, E216 (dTomato numbering) plays a role in formation of the chromophore and is therefore conserved in all RFPs. However, some residues that do not play a critical role are left untouched by engineers and appear to be conserved.

Figure 5 | Amino acid sequence alignment of popular RFPs: dTomato, mCherry, mApple, TagRFP-T, mKate2, mRuby2, and mRuby3. The chromophore is shown in black (67-69 dTomato numbering), conserved residues are colored according to the CLustal X color scheme. The secondary structure (α-helix and β-strands) are also indicated.

Despite all efforts, the commonly used RFPs are still not ideal, since there is no monomeric RFP available with a quantum yield above 0.50. Even though mCherry is the least bright RFP of the popular RFPs, this protein is often the preferred RFP to be used in living cells. This is because the applicability of an RFP is affected by more characteristics than those...
summarized in Table 1. The properties of Table 1 are all determined in vitro, although we need parameters that reflect the applicability of RFPs for fluorescence microscopy in living cells.

From the published RFP data, it is not evident which RFP is the most suited for live cell fluorescence microscopy. None of the RFPs have been evaluated in live cells under the same conditions. The brightness in cells is a combination of the ‘intrinsic’ brightness (product of extinction coefficient and quantum yield) and the maturation. The brightness in cells also depends on the model organism and even on the cell type. The photostability of FPs is very difficult to compare when different excitation sources with different powers are used. The true applicability of a set of RFPs for live cell fluorescence microscopy can only be evaluated when important characteristics are monitored in live cells under equal conditions.

Current screening methods often include random mutagenesis, after which the mutated nucleotide sequences are expressed in bacteria that are grown on Petri dishes. From the Petri dishes, colonies are selected that appear bright. From these colonies, the new RFP variants are isolated and the parameters of Table 1 are assessed in vitro (oligomeric state, extinction coefficient and quantum yield). Next, monomeric variants with a high brightness are further assessed in (mammalian) cells. However, it would be more efficient to directly screen in cells for properties that affect the applicability of RFPs.

In the past, most attempts to create a monomeric bright RFP started with mutagenesis from one of the three oligomeric natural templates or their derivatives. So far, every monomerization attempt coincided with a loss in brightness. Therefore, the evolution strategy should be renewed. Since screening for a monomeric variant is difficult and labor intensive, it would be wise to start with a truly monomeric template.

So far, similar techniques and analyses which have been used to characterize GFPs have been applied to RFPs. However, the extended conjugated system of the ‘red’ chromophore brings more complications than have been observed for the ‘green’ chromophore FPs (and its derivatives). The maturation of the ‘red’ chromophore occurs in two steps that even need to occur in the correct order. In addition, the ‘red’ chromophore seems to have different protonation states with different brightness levels and spectral shifts, then merely a ‘on’ and ‘off’ state as is observed with GFP variants. A more comprehensive understanding of the ‘red’ chromophore would aid the development of improved monomeric RFPs.

In this thesis we considered all the critical parameters mentioned to develop a new bright red fluorescent protein that outperforms current RFPs in live cell microscopy experiments.
References

22. Shaner, N. C. *et al.* A bright monomeric green fluorescent protein derived from Branchiostoma


