Spectroscopic Studies and Applications of Novel Fluorescent Proteins

Dissertation

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# List of Abbreviations

## Fluorescent Proteins

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<tr>
<td>CFP</td>
<td>cyan fluorescent protein</td>
</tr>
<tr>
<td>BFP</td>
<td>blue fluorescent protein</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>PA-GFP</td>
<td>photoactive green fluorescent protein</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
</tr>
<tr>
<td>OFP</td>
<td>orange fluorescent protein</td>
</tr>
<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
</tr>
<tr>
<td>KFP</td>
<td>kindling fluorescent protein</td>
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## General Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACF</td>
<td>autocorrelation function</td>
</tr>
<tr>
<td>APD</td>
<td>avalanche photodiode</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptors</td>
</tr>
<tr>
<td>cw</td>
<td>continuous wave</td>
</tr>
<tr>
<td>CARS</td>
<td>coherent anti-Stokes Raman scattering</td>
</tr>
<tr>
<td>FCA</td>
<td>fluorescence cumulant analysis</td>
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<tr>
<td>FIDA</td>
<td>fluorescence intensity distribution analysis</td>
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<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
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<tr>
<td>FWHM</td>
<td>full width at half maximum</td>
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<tr>
<td>HEK293</td>
<td>human embryonic kidney cells</td>
</tr>
<tr>
<td>HO</td>
<td>harmonic oscillator</td>
</tr>
<tr>
<td>INM</td>
<td>instantaneous normal mode</td>
</tr>
<tr>
<td>TCSPC</td>
<td>time-correlated single-photon counting</td>
</tr>
<tr>
<td>IRF</td>
<td>instrumental response function</td>
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<tr>
<td>MD</td>
<td>molecular dynamics</td>
</tr>
<tr>
<td>MLS</td>
<td>mitochondrial localization signal</td>
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<tr>
<td>OPE</td>
<td>one-photon excitation</td>
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<tr>
<td>PDB</td>
<td>protein databank</td>
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<tr>
<td>PMT</td>
<td>photomultiplier tube</td>
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<tr>
<td>PSF</td>
<td>point spread function</td>
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<tr>
<td>QD</td>
<td>quantum dot</td>
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<tr>
<td>SAM</td>
<td>self-assembled monolayer</td>
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<td>SERRS</td>
<td>surface enhanced resonance Raman spectroscopy</td>
</tr>
<tr>
<td>SHG</td>
<td>second harmonic generation</td>
</tr>
<tr>
<td>SHM</td>
<td>somatic hypermutation</td>
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<tr>
<td>TIPHC0</td>
<td>time and photon counting</td>
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<tr>
<td>TPE</td>
<td>two-photon excitation</td>
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<tr>
<td>ZPL</td>
<td>zero-phonon line</td>
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### Prefixes

- **av-** *Aequorea victoria*
- **ds-** *Discosoma* species
- **lh-** *Lobophyllia hemprichii*
- **t-** tandem
- **wt-** wild type

### Chemicals

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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>NATA</td>
<td>N-acetyl-L-tryptophanamide</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PMMA</td>
<td>polymethyl methacrylate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PYP</td>
<td>photoactive yellow protein</td>
</tr>
<tr>
<td>SSE</td>
<td>sulfosuccinimidyl ester</td>
</tr>
<tr>
<td>TCEP</td>
<td>tris(2-carboxyethyl)phosphine hydrochloride</td>
</tr>
<tr>
<td>TS</td>
<td>trehalose/sucrose mixture</td>
</tr>
</tbody>
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### Standard Nomenclature in Equations

- **c** light velocity
- **d** dipole moment
- **h** Planck’s constant
- **h_0** = \( h/2\pi \)
- **λ** wavelength
- **ω** circular frequency
- **v_0** frequency in wavenumbers
- **ε** relative permittivity
- **ε** extinction coefficient
- **N_A** Avogadro’s number
- **Φ_F** quantum yield for fluorescence
- **Φ_B** quantum yield for bleaching
Introduction

Why Fluorescent Proteins?

Fluorescence microscopy is one of the most important non-invasive tools in studies of vital processes. Fluorescent dyes and their derivatives generally allow specific staining of different cellular compartments. In many cases, however, a common staining procedure can not be performed. In 1962 Shimomura and co-workers [1] found that a jellyfish *Aequorea victoria* contains a green fluorescent protein (avGFP). Only in 1992 Prasher [2] and later in 1994 Chalfie [3] developed avGFP into a cellular marker. Fluorescent proteins (FPs) do not require any co-factors to become fluorescent, and can be fused to many proteins of interest on the genetic level without disturbing protein function. Generally, FPs have been utilized not only for studies of intracellular traffic, but also as markers of gene expression [4], calcium [5, 6] and pH indicators [7]; they are enrolled in studies of protein-protein and protein-DNA interactions and even as voltage sensors [8]. FPs are being intensively exploited by the pharmaceutical industry (e.g. in high throughput screening). An example of dual-color cell labeling is presented in figure 0.1. Correlated movement of the cytoskeleton and mitochondria in this example helps to establish the character of interactions between the two cellular networks.

Specific research tasks pose specific FP challenges: for studies of protein-protein interactions, the main requirement is the monomeric state of the protein, whereas for cancer research red-shifted emission is of higher priority. Some properties of GFPs e.g., blue light excitation, are undesirable, as blue light is considered to be more harmful and less penetrating than red light inside living tissues [9]. A growing amount of experiments on single-particle tracking requires bright and highly photo stable fluorescent markers. The discovery of photoactivatable FPs increased labeling specificity [10] and even introduced new imaging techniques allowing resolution on a subwavelength level [11]. In spite of a broad range of applications involving fluorescent proteins, their true biological function remains unclear. Understanding the relation between the protein structure and its properties is important for the development of novel fluorescent proteins.

Thesis Outline

The scope of this thesis is to explore spectroscopic properties of the two fluorescent proteins eqFP611, EosFP and their derivatives, to determine the relation between the properties and protein structure and to test these fluorescent proteins in a number of applications.
Introduction

Figure 0.1: Fluorescent proteins in life science research: visualization of different compartments of HeLa cells by fusions with green (EGFP, cytoskeleton) and red (eqFP611 variant, mitochondrial network) fluorescent proteins. Photo courtesy of Simone Kredel, Dr. Franz Oswald and Dr. Jörg Wiedenmann.

The presented thesis contains two parts. Part I provides the necessary background information on fluorescent proteins and gives a short description of spectroscopic theory and techniques.

Chapter 1 introduces the structural and spectroscopic aspects of fluorescent proteins and explains the biochemical synthesis of the chromophore. This chapter also reviews the present knowledge on photoinduced processes that occur in fluorescent proteins.

Chapter 2 provides the theoretical basis for the description of spectral lineshapes. We also consider two-photon excitation processes and its symmetry relations.

Chapter 3 gives an overview of techniques being used in confocal microscopy and introduces the spectroscopic properties of modern fluorescent labels. Furthermore, the chapter describes the principle of two-photon microscopy and its applications. A brief description of methods of fluctuation spectroscopy (FCS and FCA) is also provided.

Part II presents experiments on fluorescent proteins and describes the experimental apparatus and procedures.

Chapter 4 shows the experimental setup and explains sample preparation, data correction and analysis.

Chapter 5 presents spectroscopic studies of the red fluorescent protein eqFP611 on the bulk and single-molecule levels. Experiments on single molecules help us to define the bleaching yield and to explore the on-off fluorescence dynamics of the protein. We show cryogenic studies of eqFP611 and describe the spectral broadening within a
harmonic model below the glass transition point of the cryosolvent. The fluorescence quantum yield is investigated in this chapter as a function of temperature. The temperature-dependent behavior of absorption and emission spectra are discussed at the end of this chapter. Spectroscopic studies of fluorescent derivatives of eqFP611 reveal a broad variety of photoinduced effects. Some of them are explained in the discussion section. These variants are characterized with respect to quantum yield, extinction coefficient and molecular brightness.

Chapter 6 presents bulk and single-molecule studies of the photoconvertible fluorescent protein EosFP. Extinction coefficients and quantum yields are determined for a number of EosFP derivatives. Absorption, emission and photoconversion properties are studied at various pH. Single-molecule experiments demonstrate fluorescence resonance energy transfer between the two fluorescent forms of EosFP and also allow us to determine the bleaching yield for monomeric variants. Two-photon excitation and conversion properties are studied in detail. Multiple examples of EosFP application are shown in this chapter.
Introduction
Part I

Theoretical Basis
Chapter 1

Introduction to Fluorescent Proteins

1.1 Green Fluorescent Proteins

The discovery of a green fluorescent protein avGFP in the jellyfish Aequorea victoria, shown in figure 1.1 (A), has offered a great tool for monitoring of cellular activity. The avGFP primary structure contains 238 amino acids, which build up 11 β-strands and a central helix with a chromophore in the middle. The final tertiary structure is the typical barrel fold, which is common to all of the fluorescent proteins known to date (figure 1.2). This barrel is approximately 40 Å long and 30 Å wide. Although the typical structures of GFPs are all very similar, their folding rates can be very different [12]. The quaternary structure denotes the oligomerization state of the protein. The original avGFP, for example, has a weak dimerization tendency [13]. On the other hand, many anthozoan FPs form tetrameric structures [14]. It is necessary to distinguish between oligomerization and aggregation of fluorescent proteins. Oligomerization is a specific process, whereas aggregation can be the subject of either specific or nonspecific interactions [15].

The chromophore is formed in an autocatalytic reaction for which no additional cofactors except for oxygen are required. The formation of a GFP chromophore from an amino acid sequence is called maturation. This reaction, which is shown in figure 1.3 (A), can be divided in three steps and takes about 1 or 2 hours [13, 16]. The order of the steps 2 and 3 (namely dehydration followed by oxidation) can be reversed. In this reaction, the tripeptide Ser-Tyr-Gly (residues 65-67) forms an additional loop creating p-hydroxybenzylideneimidazolinone - the green-absorbing chromophore, which is only fluorescent when inside a protein or when embedded in a rigid glass matrix [17, 18, 19]. This additional backbone cyclization is catalyzed by the positively charged Arg96 [16]. The autocatalytic cyclization of the peptide backbone occurs in many enzymes, however, these mechanisms cannot be directly related to the formation of the GFP chromophore [20].

The absorbance spectrum of wild-type avGFP features two spectrally different forms [13], representing the neutral A ($\lambda_{ex}^{max}$ =378 nm) and the anionic B ($\lambda_{ex}^{max}$ =478 nm) states of the chromophore (≡ states of the phenol oxygen, see figure 1.6). Surprisingly, a stable ratio between the two protonation states is maintained over a broad range of pH [13]. Excitation
Chapter 1. Introduction to Fluorescent Proteins

Figure 1.1: Native sources of fluorescent proteins: (A) The jellyfish *Aequorea victoria*, the origin of the first fluorescent protein, avGFP; (B) the sea anemone *Entacmaea quadricolor*, the origin of the red fluorescent protein eqFP611; (C) *Discosoma sp.*, the native source of the red FP dsRed; (D) the origin of the photoconvertible protein EosFP, *Lobophyllia hemprichii*. The pictures are courtesy of Dr. Jörg Wiedenmann.

of the A and B forms leads to emission peaked at 508 and 503 nm, respectively. As the emission maxima of the two forms are remarkably similar, an excited-state deprotonation reaction has been proposed. The excited-state proton transfer was supported by time-resolved experiments [21, 22, 23, 24, 25] and by computer simulations [26, 27, 28]. In the proposed mechanism, Glu222 is transiently protonated through Ser205 and a bridging water molecule (figure 1.2). If deprotonation of the chromophore is hindered in the excited state, excitation of the A-state results in weak blue emission at $\approx 460$ nm, which decays on the subnanosecond timescale [24, 21]. Among the two states, state A is less sensitive to the environment than state B [29].

It has been possible to stabilize the anionic form of the chromophore by introducing two mutations (F64L and S65T) next to it [13]. This protein was named “enhanced GFP” or EGFP for short. The pKa of the EGFP chromophore was determined to be 5.8 [30]. At the same time, the pKa of the phenolic oxygen for the denatured wild type GFP was found to be 8.1 [31], which is very close to the value 8.2 for a model chromophore in solution [29]. These experiments show significant impact of the chromophore surrounding on the protonation state. By reducing the pH below 1.8 the imidazolinone ring nitrogen of this model chromophore (figure 1.3) becomes protonated [29].

Within the protein, the GFP chromophore is present in two resonance structures: benzenoidal and quinonoidal (see figure 1.4) [29]. Although the benzenoidal structure is most frequently used to depict GFP chromophores, Arg96, which is highly conserved among FPs, is expected to shift the balance towards the quinonoidal structure. Besides the role
1.1. Green Fluorescent Proteins

Figure 1.2: The structure of avGFP (left) and its chromophore surrounding (right). The dotted lines show hydrogen bonds. Structure data: Protein Data Bank code 1EMA.

of this highly conserved residue as a catalyst in chromophore biosynthesis, Arg96 forms a hydrogen bond to the imidazolinone carbonyl group, providing a pull of electron density from the phenolic ring of the chromophore. GFP mutant R96C has the emission shifted from 489 nm to 472 nm [13], the substitution of Arg96 by methionine in GFPsol (a solubility-enhanced EGFP variant [16]) creates a 15-nm blue shift of emission. Redistribution of electron density away from the phenolic ring has been proposed to induce a red shift of the emission [13, 18]. The impact of Arg96 on the electron density distribution of the chromophore, however, could not be confirmed by quantum mechanical calculations within the precision of the method [33].

To derive a red-shifted FP, modifications of the chromophore environment, such as the hydrogen-bonding network around the chromophore or the phenolate anion $\pi$-stacking interaction (with His203, Tyr203 or Phe203) has been performed [13]. These perturbations of the protein structure brought the excitation (488 nm) and emission (509 nm) maxima of EGFP to 515 and 528 nm, respectively, and yielded the yellow fluorescent protein YFP. Zacharias et al. found that YFP (like GFP itself) tend to form dimers with dissociation constant $K_d = 0.11$ mM [34]. The authors were able to increase the $K_d$ value by at least two orders of magnitude. Almost at the same time, Nagai and coworkers developed fluorescent variants with greater stability and brightness [12].

In general, the X-Tyr-Gly motif is common to the chromophores of all fluorescent proteins. However, there are a few exceptions. The substitution of Tyr by His or Trp leads to a blue shift of the excitation and emission spectra [13]. These derivatives are called blue fluorescent protein (BFP) ($\lambda_{em}^{max} = 448$ nm) and cyan fluorescent protein (CFP) ($\lambda_{em}^{max} = 485$ nm), respectively. Introduction of unnatural amino acids can in principle expand the variety of accessible colors. However, in the recent experiments, where the
Figure 1.3: Chromophore formation of (A) GFP and (B) dsRed. Maturation of dsRed goes through a green-emitting stage. (C) The chromophore of eqFP611 is in trans configuration as compared to dsRed. (D) The chromophore of the red form of EosFP is different from all of the above. Formation of this chromophore is discussed later in chapter 6. (E) The chromophore of a photochromic protein KFP is formed after termination of the backbone. (F) The chromophore of zFP538 uses a part of its backbone to form a six-membered ring. The schemes (A) and (B) are adopted from [32].
1.2. Red Fluorescent Proteins

Intracellular scattering and low transparency at shorter wavelengths make the development of new red-shifted markers one of the most urgent tasks. Modification of the protein environment alone, however, does not yield a large red shift of the emission. A leap towards the red edge of the spectrum was made by the discovery of red fluorescent proteins (RFPs) in various anthozoa species. The most famous RFP was found in the Discosoma coral (figure 1.1 (C)). The protein christened dsRed is most effectively excited at 558 nm; its emission spectrum is peaked at 583 nm [36].

Interestingly, the formation of the red chromophore (Gln66-Tyr67-Gly68) always goes through the GFP chromophore stage and requires oxygen [37]. This process is called the maturation of RFP. It usually takes more than several hours, which is very slow if compared to the formation of the green chromophore (this process is depicted in figure 1.3 (B)). dsRed is known to have a fraction of green species, which does not mature at all. Although this property can be used for some kinds of applications like optical labeling [38], slow maturation is in general considered as a drawback of RFPs, which prevents their use in a number of applications.

A mechanism of the autocatalytic maturation of dsRed suggested by Yarbrough et al. [39] is shown in figure 1.5. In this mechanism a crucial roles are played by residues Glu215 and Ser69, the latter of which abstracts a proton from the Cα atom of Gln66. Lys70 is believed to stabilize the negative charge of Glu215.

In 2000 Gross and coworkers [37] suggested, based on theoretical calculations, that the conjugated π-system making up the chromophore does not couple well to the terminal oxygen of the acylimine (see figure 1.3 (B)). The structural analysis made by Yarbrough et al. [39] supports the results of the theoretical predictions, because the carbonyl oxygen

Figure 1.4: Resonance forms of the GFP chromophore: benzenoidal (left) and quinonoidal (right).
of Phe65 is almost 90° out of the plane of the chromophore. Spectroscopic support for this statement comes from studies of the kindling fluorescent protein (KFP) [40]. The chromophore of this protein is shown in figure 1.3 (E). The conjugated $\pi$-electron system is obviously terminated directly after the nitrogen atom of Met63. Nevertheless, this protein shows far-red emission peaking at 600 nm [40].

An additional isomerization of the Phe65-Gln66 peptide bond occurs upon formation of the red chromophore. This trans-cis isomerization is caused by the transition from sp$^3$ to sp$^2$ hybridization of the Gln66 C$\alpha$ atom [39, 41]. Currently, the majority of RFPs feature this type of chromophore. Only a few deviations from the GFP and RFP chromophore structures have been found in nature so far. These are the red form of Kaede and EosFP [42, 10], KFP and the yellow-emitting zFP538 [43] (see figure 1.3 (D,E,F)).

It has also been shown that in alkaline and acidic solutions, the C$\alpha$ =N bond (figure 1.3 (B)) can be hydrolyzed, converting the red chromophore into the ”green” one [37]. Alkaline denaturation of RFPs helps to determine their extinction coefficients, as the spectrum of the hydrolyzed chromophore is always the same [37].

In 2002, a red fluorescent protein eqFP611 was discovered by Wiedenmann and co-workers, a promising red FP with advantageous properties over dsRed, such as more red-shifted emission ($\lambda_{\text{max}} = 611$ nm), faster maturation and reduced oligomerization tendency [44].

Recently, a weakly fluorescent RFP with maximum emission wavelength at 663 nm was derived from the non-fluorescent chromoprotein acCP597 [45]. This protein, currently the most redshifted RFP, spontaneously reverses its maturation and turns into a green fluorescent protein. The common backbone cleavage (figure 1.3 (B), last reaction) does not occur in this case. The exact mechanism of this reaction remains to be clarified.

Figure 1.5: Proposed mechanism of dsRed maturation. The figure was adopted from [39].

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Chapter 1. Introduction to Fluorescent Proteins
1.3 Brightness, Stability and Oligomerization of RFPs

Low molecular brightness is the problem which all of the far-red FPs have in common. Starting with dsRed, the molecular brightness of FPs decreases while the fluorescence spectrum shifts further to the red. A complication that became apparent after discovery of FPs in anthozoa species (dsRed is one of them) is the formation of a tetrameric ternary structure [14]. It is clear that this property can be a source of undesired interactions and may cause malfunction of fusion proteins. Reduction of the oligomeric state, however, often results in weakly fluorescent molecules, having folding problems at physiological temperatures.

Disruption of a tetramer into dimers appears much easier than splitting a dimer into monomers. In order to circumvent problems associated with monomerization, especially the reduced brightness and lower stability, the two individual subunits of a dimer can be genetically merged into a single protein, called a tandem. This approach was introduced by Campbell and co-workers [46]. Initially, it had not been clear whether the monomeric version of dsRed could be generated at all. From the crystal structure, it was concluded that, in order to maintain the dimeric structure, the two proteins in the dimer should be separated by a 10-20 amino acids long polypeptide linker.

Fradkov et al. [47] published the tandem variant of the far-red emitting dimeric hcRed1 (t-hcRed1). This protein has very low quantum yield of 0.04 and an extinction coefficient of 80,000 cm$^{-1}$M$^{-1}$ at 590 nm. When the two proteins are brought together to form a tandem, the extinction coefficient becomes 160,000 cm$^{-1}$M$^{-1}$. In contrast to dsRed tandems, best results for t-hcRed1 were achieved with a four amino acid linker.

Campbell and co-workers [46] performed random and site-directed mutagenesis of ds-Red. Altogether 33 mutations were made to obtain the first monomeric RFP, mRFP1. This mutant, however, exhibited a lower stability and a lower fluorescence quantum yield as compared to the wild type dsRed. Later on, a more advanced technique, somatic hypermutation (SHM), was used [48, 49]. This method provides much higher mutation rates than the conventional random mutagenesis. By means of this method, monomeric variants of various hues have been developed. Among these variants (named after fruit coloring), mPlum appeared the most red-shifted FP ($\lambda_{\text{em}}^\text{max} = 649$ nm, [49]). This protein showed a surprisingly large Stokes shift of 59 nm and low quantum yield of 0.1.

1.4 Optical Highlighters

Exploration of a whole variety of fluorescent proteins can sometimes reveal unexpected and at the same time useful properties of FPs. In this section, we will discuss photochromic properties of fluorescent proteins.

Changes of spectral properties upon illumination have been reported for many dyes. Reversible photoswitching was found for diarylethene derivatives [50] and carbocyanine dyes [51]. Many FPs exhibit these photoswitching properties, thereby making them targets for intracellular optical labeling.

Optical highlighters are a class of FPs which are able to change their emission properties
Figure 1.6: Fluorescence excitation and emission spectra (solid and dashed lines, respectively) for different protonation states and excited-state proton transfer of the avGFP chromophore. The GFPs depicted are (A) wild-type, (B) Emerald (stabilized B-state of the chromophore), (C) H9-40 (stabilized A-state of the chromophore). Spectra and the corresponding structure are shown together. The figure was adopted from [13].

upon illumination at certain wavelengths. These proteins enormously expand the number of applications as compared to the standard fluorescence recovery after photobleaching (FRAP), replacing it with more elegant optical labeling.

avGFP (section 1.1) can be ranked among the photochromic proteins. Upon intense blue light illumination around 400 nm, the population of the A state irreversibly converts to the anionic B state [52]. The photochemistry of this process is associated with decarboxylation of Glu222 and rotation of Thr203.

An intermediate "I" form of GFP creates an alternative relaxation pathway for the excited A and B forms. Existence of this form has been confirmed by low temperature [53, 54] and room-temperature experiments [25]. In addition, Lill and Helms [26] performed molecular dynamics (MD) simulations and proposed a relation between the spectroscopic I form and protonation of Glu222 in syn position. Thr203 does not form a hydrogen bond with the phenolate oxygen of the chromophore, formation of this bond creates the B state. Small displacement of His148 might also be involved in a complex protonation mechanisms in GFP. The pH-dependent conformations of Thr203 and His148 were confirmed by x-ray studies [55]. Lill and coworkers [26] have also suggested that the I form may not be unique and, therefore, the exact assignment of the I forms in different experiments may be difficult.

The substitution T203H in avGFP produced photactive GFP (PA-GFP) [56], which becomes fluorescent after activation with blue light (413 nm). Similar properties were observed for the yellow-shifted variant E2GFP (F64L, S65T, T203Y), which can be optically switched between an emitting and a dark state. Recently, two-photon activation of PA-GFP has been reported. By two-photon activation it is possible to precisely locate the activated region in 3D because the extension of the photoactivation region along the opti-
1.4. Optical Highlighters

cal axis is significantly reduced as compared to the case of one-photon activation [57]. This happens because activation by scattered light can be strongly reduced in the two-photon case, as only the tightly focused illumination is able to cause nonlinear photoactivation (section 3.2).

Lukyanov and coworkers, who successfully transformed a set of chromoproteins (CP) into fluorescent markers, have found one of their CPs exhibiting photochromic properties [40]. The protein called aCP can be reversibly turned into an FP by illumination with green light at ≈550 nm. Subsequent illumination with blue light (≈450 nm) quenches the fluorescence and pushes this kindling fluorescent protein (KFP) into a non-radiative state. The short-lived photoactivated state (τ ≈100 s at room temperature) has been significantly extended by introducing the single mutation A143G. In combination with mutations that prevent aggregation, the resulting protein was termed KFP1 [58].

The x-ray structure of KFP has shown that the chromophore is formed via an alternative reaction, which involves interruption of the peptide backbone (figure 1.3 (E)). The structure demonstrates that the chromophore undergoes trans-cis isomerization upon photoactivation [59]. Despite its unique properties, applicability of the protein to intracellular studies is, however, limited by its poor brightness (here we define brightness as the product of the extinction coefficient and the quantum yield of fluorescence). The fluorescence quantum yield of only 0.07 is extremely small as compared to its competitors [58]. The low brightness is mainly a result of non-planarity of the chromophore, which creates efficient pathways for non-radiative decay [59].

Recently, a photosensitive protein called Dronpa, with a high fluorescence quantum efficiency of 0.85 and an extinction coefficient ε = 95,000 M⁻¹cm⁻¹ at 503 nm, was discovered [60, 61]. These properties, in combination with its high-fidelity reversible 400-nm light photoactivation, make Dronpa attractive for studies of fast cellular dynamics.

Surprisingly, one of the dsRed derivatives, mRFP1, can be turned into a photoactive probe by substitutions at positions 146, 161 and 197 [62]. It seems, though, that the low brightness of this protein can be a serious drawback for cellular applications.

However, the fast quenching of Dronpa and similar proteins by the excitation light can be disadvantageous when slow cellular dynamics is being explored. In this case, irreversible photoconversion, taking place in PS-CFP (photoswitchable CFP) [63] can serve as an effective optical highlighter. This protein has the excitation maximum at 402 nm, indicating neutral chromophore, and produces weak emission peaking at 468 nm. Upon intense illumination at 405 nm, the protein emits intense green fluorescence with λmax = 511 nm. The mechanism of photoconversion is thought to be similar to the decarboxylation reaction in GFP.

Other proteins, including Kaede [42], Dend FP [64] and EosFP [10], are also capable of irreversible switching, and thanks to their longer wavelength emission, they are very attractive for optical labeling in vivo. Kaede can be stably converted from a green-emitting state (λmax = 518 nm) to a red-emitting state (λmax = 582 nm) by irradiation with blue light (≈400 nm). This conversion is associated with a break of the peptide backbone next to the chromophore [32, 10]. The properties of EosFP will be discussed later in section 6.

A quite exotic application exploiting the tetrameric nature of dsRed as a marker for
optical labeling was proposed by Marchant et al. [65]. The large fraction of the immature green species (up to 25%) [66] allowed to restore the green emission of dsRed by selectively bleaching the acceptor (matured dsRed) and, therefore, disrupting fluorescence resonance energy transfer (FRET) within the tetrameric complex.

Generally, all fluorescent proteins can exhibit spectral changes under intense illumination. As a result, different fluorescent forms may appear [66, 67, 68, 69]. GFP and YFP were found to switch between multiple fluorescent forms [68]. Three fluorescent states were detected for dsRed in bulk [67, 69] and on the single-molecule level [66]. Some of the states were also found to be similar to those of YFP/GFP [68].

The photochemical mechanisms of these processes are still a mystery. These effects are, however, of extremely low yield, which makes it problematic to study and utilize them. A possible conversion scenario for dsRed was proposed by Habuchi et al. [70], where decarboxylation of Glu215 and isomerization of the chromophore were proposed. In our work we found many FPs exhibiting pronounced photochromic properties including photoactivation, quenching and photoconversion.

1.5 Molecular Basis of Emission Fluctuations of Fluorescent Proteins

Blinking (transitions between radiative and non-radiative states) of individual GFP molecules has first been reported in 1997 [71]. In order to explain this switching, studies of the protonation-deprotonation dynamics of EGFP were performed [30]. Fluorescence correlation spectroscopy (FCS) revealed pH-dependent flickering on the time scale of hundreds of microseconds ([30], see figure 1.7). These experiments have shown that flickering can indeed be caused by proton transfer in the excited state. This finding has also been verified by MD simulations [26]. The proton traveling pathway (the so-called proton wire) was extensively modeled by Lill and Helms [26] and later by Vendrell et al. [28]. The latter group employed quantum chemistry calculations to show that it is energetically most favorable to transfer first a proton from Ser205 to Glu222, then a proton from water to Ser205, and finally a proton from the chromophore to the water molecule. This pathway has been confirmed spectroscopically by Thor et al. [25]. Leiderman and coworkers have predicted two other proton migration pathways [27]. They determined experimentally that at room temperature the proton shuttling has three-dimensional character, while at temperatures below 230 K shuttling occurs in one dimension.

Single-molecule surface enhanced resonance Raman spectroscopy (SERRS) experiments on EGFP [72] have shown protonation-deprotonation dynamics on the seconds timescale, which can in principle explain the blinking behavior of GFP at these times. In support of this idea, Habuchi et al. [61] performed a single-molecule study of Dronpa (this photochromic GFP is mentioned in section 1.4). They proved that protonation of the chromophore is responsible for the long-term switching behavior of this protein.

Although proton hopping is responsible for the flickering of GFP, it is not the only process that can contribute to flickering of FPs. Processes such as rotation of the methine group connecting phenolate and imidazolinone rings of the chromophore [73, 24] and
1.5. Molecular Basis of Emission Fluctuations of Fluorescent Proteins

![Figure 1.7: pH-dependence of GFP flickering dynamics. The figure is adopted from [30].](image)

Conformational rearrangements of the surrounding side chains can quench the fluorescence as well. Quenching of KFP, for instance, is driven by the former process [40, 59]. This type of quenching is also observed for the FP chromophore in solution [18, 74, 75]. It is assumed that conformational activity of the chromophore in solution is the main source of non-radiative relaxation. At temperatures below the glass transition of the solvent, the fluorescence quantum efficiency of the chromophore is significantly increased [18]. Increase in solvent viscosity also causes an increase in the quantum yield of the model chromophore in solution [75, 18]. However in the case of protic compounds such as water and glycerol, specific interactions with the chromophore reduce the quantum yield [18]. Reducing the conformational freedom of CFP by tightening up the chromophore pocket in high-pressure experiments Mauring and coworkers [76] have demonstrated a 75% increase in fluorescence intensity at the maximum attainable pressure of 570 MPa, which corresponds to a 9% reduction of the protein volume [77]. Vibronic structure became better resolved as well. Flickering is a general property of FPs, and, in contrast to many dyes, fluorescence quenching via the triplet state is not the main source of the intensity fluctuations.

Single-molecule unfolding studies of a GFP mutant with enhanced folding properties GFPmut2 [78] revealed a new type of emission dynamics [79]. It was found that adding a denaturant caused highly periodic blue bursts, coming from the protonated form of the chromophore. This oscillatory effect can be resonantly enhanced by applying external acoustic and voltage pulses matching the burst frequency.
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Chapter 2

Principles of Absorption and Fluorescence Spectroscopy

2.1 The Golden Rule

In this section we will focus on the molecular basis of light-matter interaction. The behavior of an atom in an electromagnetic field can be described via the Schrödinger equation,

\[
(i\hbar\partial/\partial t - H)\Psi(r, t) = 0.
\]

We can use the perturbation theory approach, assuming that the hamiltonian \( H \) consists of a stationary part \( H_0 \), and a perturbation term \( V(t) \),

\[
H(t) = H_0 + V(t).
\]

The wave function \( \Psi(r, t) \) can be expressed in terms of the eigenfunctions \( \phi_n(r) \) of the stationary-state hamiltonian \( H_0 \),

\[
\Psi(r, t) = \sum_n c_n(t)\phi_n(r) \exp(-iE_n t/\hbar),
\]

where the index \( n \) designates the number of the energy state. In the stationary case, the coefficients \( c_n(t) \) do not depend on time. Their squared magnitudes define the relative populations of the energy states \( E_n \). Substitution of \( \Psi(r, t) \) into the Schrödinger equation gives

\[
\text{i}\hbar \frac{dc_n}{dt} = \sum_n V_{mn}c_n \exp(i\omega_{mn}t).
\] (2.1)

Here \( V_{mn} \) and \( \omega_{mn} \) are defined as

\[
V_{mn} \equiv \int \phi^*_m V \phi_n dr, \quad \omega_{mn} \equiv \omega_m - \omega_n = (E_m - E_n)/\hbar.
\]

The equation (2.1) can not be solved in general but with the help of decomposition \( c_n = \sum_k c_n^{(k)} \). After this substitution, the equation 2.1 transforms into a system of equations:

\[
\text{i}\hbar \frac{dc_n^{(k)}}{dt} = \sum_n V_{mn} \exp(i\omega_{mn}t)c_n^{(k-1)}/\hbar.
\] (2.2)
Chapter 2. Principles of Absorption and Fluorescence Spectroscopy

The zeroth-order equation $\frac{dc_{m}^{(0)}}{dt} = 0$ sets the starting conditions. By letting $c_{n}^{(0)} = \delta_{n1}$ (system with definite energy at $t = 0$), we can disentangle this system and equation 2.1 transforms then into

$$\frac{dc_{m}^{(1)}}{dt} = \mathcal{V}_{m1} \exp(i\omega_{m1}t)/i\hbar.$$  \hspace{1cm} (2.3)

If the start level is different from 1, $c_{m} \mapsto c_{mn}$. Let $n$ be the starting level. The solution of this equation helps us to define the probability of a transition between levels $n$ and $m$.

$$P_{mn}^{(1)}(t) = h^{-2} |c_{mn}(t)|^2 = h^{-2} \left| \int_{t_0}^{t} \mathcal{V}_{mn} \exp(i\omega_{mn}t') dt' \right|^2.$$  \hspace{1cm} (2.4)

Let us assume $\mathcal{V}_{mn}(t) = -d_{mn} \cdot E = -d \cdot (E_0 e^{-i\omega t} + E_0^* e^{i\omega t})$, where $d_{mn}$ is the transition dipole and $E$ is the electric field. We evaluate the expression 2.4 and define the transition rate $W_{mn} = P_{mn}/t = 2\pi |d_{mn} \cdot E_0/2\hbar|^2 \delta(\varpi)$, where $\varpi \equiv \omega - |\omega_{mn}|$. In the case of a finite level width, the delta function turns into the spectral shape function $g(\varpi)$:

$$W_{mn} = 2\pi |d_{mn} \cdot E_0/2\hbar|^2 g(\varpi),$$  \hspace{1cm} (2.5)

which has different shapes for different broadening mechanisms. The transition rate can be simply related to the absorption cross-section, $\sigma = W/F$, where $F$ is the photon flux, i.e.

$$\sigma_{mn} = (4\pi^2/\hbar c) \omega g(\varpi) |d_{mn}|^2,$$  \hspace{1cm} (2.6)

where $d_{mn}$ is the projection of $d_{mn}$ on the field direction.

2.2 Mechanisms of Spectral Broadening

An exponential decay of the excited state with lifetime $\tau$ leads to a lorentzian spectral shape,

$$g_{L}(\varpi) = \frac{\Delta \omega}{\pi \Delta \omega^2 + \varpi^2},$$

which represents the natural linewidth $\Delta \omega = 2\pi/\tau$. This spectral shape represents a non-interacting two-level system. At room temperature, linewidths close to the natural one are only observable for gases at low pressures and rare-earth atoms. In solids, it can be also seen for impurity centers at low temperatures. Otherwise, atomic systems participate in a variety of interactions, which cause an additional broadening of the spectrum. Collisional and Doppler broadening mechanisms are irrelevant in the case of a chromophore in a solid phase. Instead, coupling to vibrational modes takes place. Coupling to low-frequency (acoustic) modes ($\nu \ll kT/\hbar$, where $T$ is the maximum temperature used in the experiments) causes line broadening, whereas coupling to high-frequency (optical phonons) modes appears as sharp absorption/emission spectral features at low temperatures and in Raman spectra. Coupling of the electronic state to optical phonons is illustrated in an energy level diagram (figure 2.1). The ground ($S_0$) and first excited electronic state ($S_1$) are split by electron-phonon interaction. After transition from the lowest vibrational level of the ground state to the excited states, vibrational relaxation occurs within the excited
2.2. Mechanisms of Spectral Broadening

Figure 2.1: Ground ($S_0$) and first excited electronic state ($S_1$) potential surfaces of a molecule. Blue arrows represent the absorbed and red arrows the emitted photons; dotted lines indicate non-radiative relaxation; E is the energy and q is the molecular coordinate scale.

state. After that, a radiative or non-radiative transition to the electronic ground state, again followed by vibrational dissipation, closes the excitation-relaxation cycle.

Movements of the nuclei of the matrix can be very well modeled within a harmonic potential, assuming that the Born-Oppenheimer (adiabatic) approximation holds [80]. By considering $N$ vibrational modes of the host we can express the energies of the ground $E_g$ and excited $E_e$ state as [81]:

$$E_g = E_{0g} + \frac{1}{2} \hbar^2 \sum_{i=1}^{N} \nu_{q_i}^2 q_i^2,$$

$$E_e = E_{0e} + \frac{1}{2} \hbar^2 \sum_{i=1}^{N} \nu_{q_i}^2 Q_i^2,$$  \hspace{1cm} (2.7)

where $q$ and $Q$ are the normal nuclear coordinates in the ground and the excited states, $\nu_i$ are the frequencies of the normal modes, $E_0$ is the energy of an electronic state, when all the nuclei are at their equilibrium positions ($q, Q = 0$). Equations 2.7 allow us to define the Huang-Rhys coupling factors [81, 82]:

$$S_i \equiv \frac{\hbar}{2} \nu_{q_i} (Q_i - q_i)^2,$$  \hspace{1cm} (2.8)

which serve as a non-dimensional measure of the linear electron-phonon interaction. Additionally, a quadratic coupling constant can be defined as follows:

$$R_i \equiv \nu_{q_i}^2 / \nu_{q_i}^2.$$  \hspace{1cm} (2.9)

In the case of linear coupling to high-frequency phonons, the shape of the absorption spectrum can be modeled by a Franck-Condon progression [81],

$$g' (\Omega) = \sum_{f=0}^{\infty} \frac{S f}{f!} e^{-S} g(\Omega).$$  \hspace{1cm} (2.10)
Chapter 2. Principles of Absorption and Fluorescence Spectroscopy

It is possible to show that coupling of the chromophore to over-damped soft modes of the host matrix introduces a convolution of the Lorentzian lineshape with a Gaussian function [81, 82], thereby producing a Voigt function. Frequently the Gaussian dominates the spectral width and the Lorentzian can be treated as a delta-function in the convolution, which results in a Gaussian lineshape:

\[
g(\omega) = \frac{1}{\sqrt{2\pi\Delta\omega}} e^{-\frac{\omega^2}{2\Delta\omega^2}}.
\]

Hence, the final spectral shape is determined by equation 2.10, where \(g(\omega)\) is a Gaussian function. For practical purposes, we now express the Gaussian width in wavenumbers (\(\omega \rightarrow 2\pi\tilde{\nu}/c\)). After that, the temperature dependence for the width \(\Delta\tilde{\nu}\) and the maximum position \(X_C\) can be expressed as follows [81, 82]:

\[
\Delta\tilde{\nu}(T) = \langle \tilde{\nu} \rangle \left[ S \cdot \coth \left( \frac{h\epsilon(\tilde{\nu})}{2kT} \right) \right]^{1/2} + C_1,
\]

\[
X_C(T) = \tilde{\nu}_0 - \frac{1}{4} \langle \tilde{\nu} \rangle (1 - R) \cdot \coth \left( \frac{h\epsilon(\tilde{\nu})}{2kT} \right) + C_2,
\]

where \(\langle \tilde{\nu} \rangle\) is the effective phonon mode frequency, \(\tilde{\nu}_0\) is the position of the maximum at low temperatures, \(C_1\) and \(C_2\) account for deviations from the harmonic model.

When an impurity center (chromophore) is embedded in a crystal, it creates certain defects in the crystal structure, thereby changing locally the crystal’s phonon spectrum and creating a limited number of local (localized) or quasilocal (delocalized) vibrational modes. These modes often exhibit a high coupling efficiency to either electronic transitions or vibrational modes of the chromophore. In amorphous solids (glasses) numerous defects produce quasilocal modes. They are characterized by a large vibrational amplitude of some atomic groups [83] and can be described as low-frequency harmonic oscillators (HO), which couple to the acoustic modes. Gurevich et al. [83] have shown that interaction between the HOs creates the so-called Boson peak, a vibronic band known for all glasses. In order to decouple a chromophore from the host matrix, a proper combination of both can be found. For example, Shpolskii (normal alkane) matrices are known to interact weakly with aromatic chromophores [84]. In the case of biomolecules, the choice of the host is limited to those matrices that do not interfere with the biological function.

2.3 Solvent Effects

Upon transition to the excited state, several relaxation processes occur. These are the dissipation of the vibrational energy, charge transfer and the rearrangement of the solvent dipoles around the chromophore. For most chromophores, the dipole moment is higher in the excited state, which therefore increases dipole-dipole interactions with the environment. Relaxation processes lower the energy of the excited state and introduce a shift between the maxima of excitation and emission spectra, which is called the Stokes shift.

The interaction of the solvent and chromophore can be specific or non-specific. The former case can be realized, e.g., via hydrogen bonding between the chromophore and the
2.3. Solvent Effects

solvent; the latter is considered more general, as it excludes chemical interaction between the solvent and the solute. The Stokes shift in the case of non-specific interaction can be described by the Lippert equation [85]:

\[
\delta \omega = \frac{2}{\hbar c} \left( \frac{\epsilon(0) - 1}{2\epsilon(0) + 1} - \frac{\epsilon(\omega) - 1}{2\epsilon(\omega) + 1} \right) \frac{(d_e - d_g)^2}{a^3} + \text{const},
\]

where \(\epsilon(0)\) is the static relative permittivity of the solvent and \(\epsilon(\omega)\) is high frequency relative permittivity, which is simply the squared refractive index \(n^2(\omega)\) at the transition frequency \(\omega\). The parameter \(a\) is the radius of the cavity in which the chromophore resides, \(d_g\) and \(d_e\) are the dipole moments in the ground and excited states, respectively.

Biomolecules interact with a highly polar environment and frequently exhibit specific interaction with the solvent, which may limit applicability of equation 2.13. Moreover, the Lippert equation assumes that solvent relaxation occurs much faster than the decay of the excited state, which may not be true, especially when the fluorophores are studied at low temperatures [85]. If solvent relaxation is so slow that it becomes comparable to the fluorescence lifetime, a dynamic Stokes shift can be observed. Solvent relaxation can be described by either a continuous or a discontinuous model. In the former case, the emission maximum exponentially shifts to lower frequencies with the solvent relaxation time \(\tau_s\) [85]:

\[
\delta \omega_s(t) = \omega_{\infty} + (\omega_0 - \omega_{\infty})e^{-t/\tau_s},
\]

where \(\omega_0\) and \(\omega_{\infty}\) are the emission maxima in the initial and (solvent) relaxed excited states, respectively. In the discontinuous model, a finite number of intermediate states is present on the way from the initially excited to the relaxed state.

In 1972, Johari [86] proposed that two relaxation processes occur in molecular glasses. The process of \(\alpha\)-relaxation can be observed down to the glass transition temperature \(T_g\) of the solvent, while the process of \(\beta\)-relaxation should exist below and above \(T_g\). Relaxation processes in liquids extend over many orders of magnitude on the timescale. While ultrafast (tens of femtoseconds) processes are considered uncorrelated, slower ones (hundreds of femtoseconds) can be attributed to collective vibrational modes of the solvent molecules, longer processes are diffusive and are temperature- and viscosity-dependent [87]. Uncorrelated and correlated motions can be described in terms of normal modes, as instantaneous normal mode (INM) analysis suggests [88]. Relaxation dynamics of water around proteins is different from bulk water, the influence of protein on surrounding water dynamics extend up to 7 Å from the protein surface (serine endopeptidase [89, 90]). Near the surface, the slowing down of fast (180 fs) and slow (1.1 ps) dynamics to 800 fs and 38 ps, respectively, occurs. Solvation processes were a subject of detailed analysis in time-resolved studies of photoactive yellow protein (PYP). Stokkum and coworkers investigated a "locked" PYP variant, where photoizomerization is precluded [91]. They found several relaxation processes with different time constants and concluded that 0.1-ps relaxation stems mainly from the internal vibrational relaxation, while a 3.5-ps decay was ascribed to the internal diffusive solvation process.

In general, solvent relaxation can produce a shape of the spectrum that changes from gaussian to lorentzian with increasing fluctuation rate of the solvent [92].
Figure 2.2: A two-photon excitation process requires simultaneous absorption of two photons. This transition involves a virtual level (dotted line); $n$, $m$ and $l$ are the ground, excited and intermediate energy states, respectively. Red arrows represent the absorbed and green arrows the emitted photons; the dotted arrows show non-radiative relaxation. $\hbar(\omega_m - \omega)$ is the frequency detuning between excitation photon energy and level $l$.

2.4 Two-Photon Excitation Process

The description of two-photon absorption requires absorption of two light quanta within a short period of time ($\tau \approx 10^{-15}$ s). This process is illustrated in figure 2.2.

The process of two-photon absorption can be described analogously to the one-photon case (section 2.1) by taking into account the next order of the perturbation theory in the system of equations 2.2. It results in the following expression for the transition rate:

$$W_{mn} = \frac{\pi}{8\hbar^2} \sum_l |V_{ml}V_{ln}|^2 \frac{1}{(\omega - \omega_l)\tau} g(\omega);$$  \hspace{1cm} (2.15)

where $\omega$ is the light frequency, $n$, $m$ and $l$ are, respectively, the ground, excited and intermediate states. From expression 2.15 follows that the presence of a level $l$ close to the incident frequency $\omega$, increases the transition rate $W_{mn}$ dramatically. As in the linear case, we can present $V = \mathbf{d} \cdot \mathbf{E}$, which leads to a quadratic dependence of $W_{mn}$ on excitation intensity (fourth power of the electric field).

Molecular symmetry plays an important role in electronic transitions. The symmetry of the ground electronic state of most molecules matches the full symmetry of the molecular point group, meaning that the wave function has the same sign throughout the molecule [93]. One-photon excitation requires the ground and excited states to have opposite parity. This follows from the parity conservation principle and the fact that the parity of a linearly polarized photon is odd. In the case of two-photon absorption, two photons of odd (ungerade) symmetry produce even (gerade) symmetry, therefore, transitions between the states of the same parity are allowed.

The excited molecule usually relaxes to the lowest excited state, which is usually the same as in the one-photon case. Hence, the fluorescence spectra and lifetimes of the excited state usually coincide in the OPE and TPE cases (figure 2.2). Up to now, only a few
exceptions of this rule have been observed for synthetic dyes [94]. All emission spectra of fluorescent proteins published to date are in agreement with this rule.
Chapter 3

Techniques of Confocal Microscopy

3.1 Conceptual Basis and Applications

In November 1957 Marvin Minsky submitted a patent for a stage-scanning optical system [95]. The system featuring a confocal objective lens arrangement and a pinhole was able to discriminate out-of-focus light very effectively, thereby reducing the detection volume and increasing the signal-to-background ratio. This property was found to be important not only for high-resolution imaging and optical sectioning, but also for a number of fluorescence fluctuation spectroscopy methods. The principle of confocal detection is illustrated in figure 3.1. Light from a point source is reflected by a dichroic mirror and focused by a lens onto a specimen. However, luminescence is excited not only in the focal plane, but also between planes a and c, which define the sample borders (upper and lower pictures). In order to collect the luminescence signal exclusively from the focal plane b, a pinhole must be inserted in the detection path to suppress emission coming from other parts of the sample. In figure 3.1, efficient detection is only observed for the middle panel.

3.2 Two-Photon Microscopy

Two-photon microscopy is a particular case of multiphoton microscopy, which employs nonlinear processes such as simultaneous absorption of multiple light quanta. Utilization of nonlinear effects in microscopy requires powerful light sources, objectives with high numerical apertures and fluorophores with reasonably high two-photon excitation cross-section. In spite of technical complications, two-photon techniques extend the imaging depth from \( \approx 100 \mu m \) in the linear case to up to 1 mm in the case of two-photon microscopy [96]. This advantage arises from the higher permeability of living tissues for infrared radiation [9].

For the process of two-photon excitation (TPE) the fluorescence intensity is proportional to the square of the excitation power (see section 2.4):

\[
I_f = Kl \Phi^2 c \delta I_{ex}^2.
\]

Here, \( I_f \) is the emission intensity, \( \Phi \) is the fluorescence quantum yield, which is divided
Chapter 3. Techniques of Confocal Microscopy

Figure 3.1: Principle of a confocal system, consisting of a point excitation source, dichroic mirror, objective lens, detection pinhole and detector.

by 2, as twice as many absorbed photons are required to produce one emitted photon as compared to OPE, thereby effectively "Reducing" the quantum yield, $c$ is the concentration of the fluorescent molecules, $I_{ex}$ is the excitation intensity, $K$ is the dimensionless volume parameter and $l$ is the interaction length. This expression is especially useful for estimations of the two-photon absorption cross-section $\delta$, which is usually measured in G"opper-Mayer units ($10^{-50}\text{cm}^4\text{s photon}^{-1}$). When relating to a reference, $K$ and $l$ can be easily eliminated [97]:

$$\delta_1 = \frac{I_{f1}c_2\Phi_2\Delta_2}{I_{f2}c_1\Phi_1\Delta_1} \cdot \delta_2.$$  \hspace{1cm} (3.1)

Here, the index 1 corresponds to the sample of interest, the index 2 refers to the reference and $\Delta$ quantifies the spectral detection window of the system.

The quadratic dependence of the emission on excitation intensity helps to achieve fluorescence excitation almost exclusively from the focal area. A direct comparison of OPE and TPE is shown in figure 3.2. The out-of-focus molecules are excited very ineffectively, that is why a two-photon microscope can be used without a pinhole in the detection path.

The high powers used in two-photon microscopy can damage living tissues. Thus, the power should be well controlled. However, not only the power, but also the laser pulse duration and the wavelength need to be taken care of. It has been established that, for many fluorophores, three-photon damage is a major process that decreases the two-photon induced fluorescence signal [99, 100]. It has also been shown that utilization of picosecond
laser sources is preferable for two-photon imaging as it reduces the peak power of the individual pulses and, therefore, minimizes the effects of photodamage [99, 100]. Some authors even suggest continuous wave sources to be used for two-photon excitation of fluorophores [101]. If possible, it is preferable for live cell imaging to use laser light of $\approx 900 \text{ nm}$ [9]. A minimum of water absorption is around 800 nm; however, two-photon excitation in this region is not recommended due to possible three-photon damage of DNA [100, 102].

In most cases, two-photon microscopy has been implemented using a confocal microscope arrangement. Exotic applications of two-photon excitation in TIRF and even in wide-field microscopy can also be found [103, 104]. Two-photon microscopy is only one of the nonlinear optical microscopy techniques being implemented nowadays. Methods of coherent imaging like coherent anti-Stokes Raman scattering (CARS) [105] and second-harmonic generation (SHG) microscopy do not always require labeling and can be used, e.g., for monitoring the dynamics of microtubules [106] or other highly ordered cellular structures.

3.3 Fluorescent Probes

3.3.1 Probes for One-Photon Microscopy

Fluorescence microscopy requires markers, which are able to highlight specific subcellular structures. Chemically synthesized luminescent dyes offer the largest range of excitation and emission wavelengths. They can be used for staining cellular structures (cell membranes, actin filaments, etc.) or as markers for cell viability and local environment ($\text{Ca}^{2+}$, pH). Synthetic dyes contain conjugated $\pi$-electron systems. Examples of two common fluorescent dyes is shown in figure 3.3. The brightness (product of extinction coefficient
Chapter 3. Techniques of Confocal Microscopy

Figure 3.3: Chemical structures of the fluorescent dyes tetramethylrhodamine (TMR) and Cy3.

and fluorescence quantum yield) is important, but not the only parameter that has to be considered in biological applications. In vivo studies often require red fluorophores, as red emission better penetrates living tissues. Large Stokes shifts (> 100 nm) can be useful for better separation from the background emission. The relatively small size of chemical labels offers the possibility to measure conformational changes of proteins and oligonucleotides by FRET between the chemically attached labels. In dynamic studies on individual molecules it is important for a dye label to show minimal emission fluctuations. This "flickering" results either from the dynamics of intersystem crossing or conformational rearrangements within the dye molecule. Isomerization of labels like Cy3 ([107] figure 3.3) may also result in a short non-exponential fluorescence decay in TCSPC experiments, which limits the use of this dye in fluorescence lifetime-based applications. Luminescent labels based on rare earth elements offer very long excited state lifetimes [108] (and, of course, narrow emission spectra). These labels can be well discriminated against fluorescent background by time-resolved experiments.

Semiconductor nanocrystals (quantum dots, QDs) are bigger in size (2-10 nm in diameter [109]) as compared to synthetic dyes, but extremely photostable [110]. They offer narrow emission bands and a high degree of spectral tunability of their emission (depending on size [109]). Fluorescent proteins can be advantageous for in vivo applications due to their high labeling specificity (by generating fusion proteins) and the possibility to generate these labels directly within the cell. Moreover, photochromic properties of FPs such as EosFP cannot easily be achieved with synthetic labels.

3.3.2 Probes for Two-Photon Microscopy

Brightness of fluorescent labels under TPE and OPE can be totally different. The relation between the chemical structure and nonlinear properties is sometimes difficult to predict. Early studies have shown that electron donor (D) and acceptor (A) groups connected by a π-electron bridge system (D-π-A) have different two-photon excitation cross-sections depending on the bridging part of the molecule [111]. Later, Albota et al. [112] have shown that either a D-A-D or a A-D-A motif is preferable to achieve two-photon cross-sections.
as high as several thousand GM units. Branched structures often exhibit high two-photon excitabilities. *Ab initio* calculations performed by Macak *et al.* [113] suggest that the vibronic contribution to TPE plays an important role in the case of branched dyes. In an experimental study of octopolar molecules, Cho and coworkers [97] have found that, unlike linear quadrupolar molecules having an inversion center, branched octopolar molecules show similar TPE and OPE (at twice the wavelength). This is remarkable, because often fluorophores can be excited in a broad range of wavelengths by TPE. Recently, a smaragdyrin dye with $\delta = 15,525$ GM has been published [114]. Its fluorescence quantum yield was, however, not reported.

Quantum dots, besides featuring high photostability and reasonably high luminescence quantum yield ($\approx 30\%$), offer TPE cross-sections up to 50,000 GM (for CdSe nanocrystals [115]). Owing to these high values, use of QDs can be recommended for nonlinear microscopy. Fluorescent proteins usually show TPE cross-section values on the order of tens of GM units [116], which is still comparable or even higher than those of most synthetic dyes.

### 3.4 Fluorescence Fluctuation Spectroscopy

#### 3.4.1 Correlation Function

In the simplest case, fluctuations of the fluorescence intensity, $\delta I(t) = I(t) - \langle I(t) \rangle$ within the confocal volume arise from fluctuations of the fluorophore concentration and can be viewed in terms of the normalized ACF, $G(\tau) = \langle \delta I(t) \delta I(t + \tau) \rangle / \langle I(t) \rangle^2$. In this case, the ACF provides information on the diffusion coefficient and concentration of the fluorescing molecules. Additionally, FCS data can be analyzed by assuming freely diffusing molecules undergoing a unimolecular reaction between two states with different emissivity. Assuming a 3D gaussian shape of the confocal volume with the ratio $\varpi = r/z$ of the effective radial $r$ and axial $z$ dimensions (defined as a distance over which the intensity decays by a factor of $1/e^2$), the ACF can be written as follows [117, 118, 119]:

$$G(\tau) = 2^{3/2} N \frac{1}{(1 + \tau/\tau_D) \cdot \sqrt{1 + \varpi^2(\tau/\tau_D)}} \left[ 1 + F \frac{\exp(-\Lambda \tau)}{1 - F} \right], \quad (3.2)$$

where $N$ is the time-averaged number of particles inside the confocal volume, $\tau_D$ is the translational diffusion time, $F$ is the dark fraction, and $\Lambda$ is the flicker rate (*vide infra*). The former (diffusional) part of equation 3.2 describes the fluctuations of emission intensity caused by the passing of fluorescent molecules through the confocal volume. The latter (reaction) part describes intensity changes caused by a unimolecular reaction, $A \xrightleftharpoons[k^-]{k^+] B$, where $k^+$ and $k^-$ are the forward and backward reaction rates between the emitting state $A$ and the non-emitting state $B$. An equilibrium coefficient $K = k^+/k^-$ can be expressed in terms of a "dark fraction" $F$:

$$K = \frac{F}{1 - F}.$$
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Figure 3.4: Set of ACFs, presenting light-induced dynamics of eqFP611. The excitation rate ranged between 0.2-3.7 MHz. The figure is adopted from [119].

where $F$ is a relative concentration of molecules in the dark state $[B]$:

$$F = \frac{[B]}{[B] + [A]}.$$

The apparent flicker rate, $\Lambda$, is the sum of the forward and backward reaction rates:

$$\Lambda = k^+ + k^-.$$

A common dynamic process, the transition to the triplet state, can be considered as an example of a unimolecular reaction. Light-driven dynamic processes in fluorescent proteins can also be identified by FCS. In figure 3.4 a set of ACFs for eqFP611 is shown for different powers. The separation between faster reaction and slower diffusion parts is better visible at higher illumination intensities.

3.4.2 Photon Counting Histogram and Cumulant Analysis

FCS analysis can provide information about molecular dynamics and sample concentration, but at the same time discards information on the brightness of the individual molecules. If species with different molecular brightness are present in the observation volume, their contributions to the correlation function will be weighted by the square of their brightness. Besides calculating the ACF, it is also possible to generate a histogram of the collected photons from the measured data, which shows the occurrence of a certain count rate (within a certain bin time $T$). This histogram can be analyzed in terms of molecular brightness, $\eta = fT$, ($f$- number of detected photons per unit time, count rate) and concentration of several species by the photon counting histogram (PCH) [120] and FIDA [121] methods. An alternative approach has been demonstrated by Joachim Müller [122], who introduced
3.4. Fluorescence Fluctuation Spectroscopy

Fluorescence cumulant analysis (FCA). This method is based on the calculation of factorial cumulants from the moments of the histogram of the collected photons and allows one to calculate the brightness and concentration for up to two species of different brightness. The \( r \)-th cumulant \( \kappa_r \) can be related to the molecular brightness \( \eta_i \) and the number of particles \( N_i \) of the \( i \)-th species in the volume:

\[
\kappa_r = \frac{\gamma_r}{\sum_{i=1}^{s} \eta_i^r N_i},
\]

where \( \gamma_r \) is a volume parameter, which is defined via a point spread function (PSF) as follows:

\[
\gamma_r = \frac{\int_V (PSF(r))^r \, dr^3}{\int_V PSF(r) \, dr^3}.
\]

Hence, simultaneous estimation of the molecular brightness and the number of fluorescent particles for one species (\( s = 1 \)) requires calculation of at least two first cumulants:

\[
\kappa_1 = \eta N, \tag{3.3}
\]

\[
\kappa_2 = \gamma_2 \eta^2 N, \tag{3.4}
\]

This analysis (as well as PCH and FIDA) is sensitive to the binning time \( T \), which should be much shorter than the diffusion time \( \tau_D \). However, it is possible to correct for the influence of longer binning times \( T \). The first cumulant (equation 3.3) does not require correction as it is simply the average detected intensity and, therefore, does not depend on binning. The correction of the second cumulant (equation 3.4) can be performed by assuming a 3D gaussian shape for the observed volume [122]:

\[
\tilde{\kappa}_2 = \gamma_2 f^2 NB_2(T),
\]

where \( B(T) \) is the correction factor:

\[
B_2(T) = \int_{-T}^{T} (T - |\tau|)G_D(\tau) \, d\tau = \frac{4\tau_D^2}{\omega^2} \left( 1 - \frac{s^2 + x\omega^2}{\omega} - \frac{\omega^2(1 + x)}{s} \log \left( \frac{(1 - s)(s + \sqrt{1 + x\omega^2})}{\omega^2\sqrt{1 + x}} \right) \right).
\]

Here \( s = \sqrt{1 - \omega^2} \), \( G_D(\tau) \) is the diffusion part of the equation 3.2.
Part II

Experiments
Chapter 4

Experimental Setup

4.1 Bulk Spectroscopy

4.1.1 Absorption and Fluorescence Spectroscopy

Absorption spectroscopy was carried out on two absorption spectrometers: Cary 1E and Cary 14 (Varian, Palo Alto, CA), the latter equipped with a CTI-22 closed-cycle cryostat (Helix Technology Corp., Santa Clara, CA) and a DRC-93CA temperature controller (Lake Shore Cryotronics Inc., Westerville, OH). Fluorescence spectra were measured on a SPEX Fluorolog II fluorimeter (Spex Industries, Edison, NJ) equipped with a 450 W xenon lamp and a CTI-22 closed-cycle cryostat and a DRC-93C temperature controller (Lake Shore Cryotronics Inc.). The spectral resolution was adjusted to 2 nm by the 1.25-mm slits in the emission and excitation path of our fluorimeter. For the estimation of fluorescence quantum yields of the green proteins (green form of EosFP variants), we used a reference solution of rhodamine 6G (Lambda Physik, Göttingen, Germany) in ethanol, while for the red proteins (eqFP611, red form of EosFP), we used Cresyl Violet (Radiant Dyes Chemie, Wermelskirchen, Germany) in methanol. By relating the absorbance $A$ at the excitation wavelength and the fluorescence spectrum area $F(\lambda)$ to that of the reference, the quantum yield of fluorescence $\Phi_F$ could be obtained:

$$\Phi_F = \frac{n^2}{n_{\text{ref}}^2} \frac{\int F(\lambda) \, d\lambda}{\int F_{\text{ref}}(\lambda) \, d\lambda} \left( \frac{1 - 10^{-A_{\text{ref}}}}{1 - 10^{-A}} \right) \cdot \Phi_{F,\text{ref}},$$

(4.1)

where $n$ is the solvent refractive index and $\lambda$ is the wavelength. Samples of different concentrations were used in fluorescence and absorbance experiments at low temperatures. Therefore, estimation of the quantum yield in these experiments requires renormalization of either fluorescence or absorbance spectra according to the known quantum yield. To this end, we measured the quantum yield of eqFP611 in the cryosolvent (vide infra) at $T = 300$ K and absorbance of the cryogenic sample at this temperature to calculate the number of emitted photons. This number of photons was used to normalize the fluorescence spectrum at lower temperatures. Peak positions of absorption, emission and excitation spectra were located within less than 1 nm precision by a gaussian fit to the data. The obtained values in nanometers were rounded to an integer.
Chapter 4. Experimental Setup

4.2 Microscopy

4.2.1 Excitation Light Sources

For our experiments, various excitation sources were used. Coherent (lasers) and incoherent (lamps) light sources can be used to excite fluorescence. For most of the experiments, both kinds of sources can be used. The former, however, offer higher stability, monochromaticity and feature better beam properties, which are required for applications of fluctuation spectroscopy and high-resolution imaging. They can be divided into two main groups: continuous wave (cw) and pulsed sources. Coherent properties of the sources were not important in our experiments.

Continuous Wave Sources

For the single-molecule experiments on eqFP611 we used an Ar$^+/Kr^+$-ion laser (modified model 164, SpectraPhysics, Mountain View, CA), operating at 514 nm. The single-molecule imaging of the tandem complexes was performed with a 532-nm frequency doubled Nd:YVO$_4$ laser (DPGL-2050F, Photop Sueetch Inc., Shanghai, China).

For the imaging of the green form of EosFP, we used 488 nm light from our Ar$^+/Kr^+$-ion laser. The red form was excited in most cases by the same laser except for the FRET experiments of the section 6.5.2, where separate excitation of the red form was needed. In this case we used 532-nm light from our frequency doubled Nd:YVO$_4$ laser (DPGL-2050F). Additionally, a 404-nm laser built from a GaN laser diode (DL-5146351, Sanyo, Japan) was used for EosFP photoconversion in our protein-protein interaction studies (section 6.5.2).

Pulsed Sources

Pulsed sources were required for fluorescence lifetime determination, lifetime imaging and two-photon studies. In the latter case, we used an Ar$^+$-laser-pumped Ti:sapphire laser (Innova Sabre/Mira 900, Coherent, Santa Clara, CA) tuned to 800-1050 nm. Only in the case of two-photon excitation of eqFP611 and red form of EosFP we used the fundamental (1064 nm) of a picosecond Nd:YVO$_4$ laser (GE-100-VAN-IR/SHG, Time-Bandwidth Products AG, Zurich, Switzerland). Pulse repetition rate of the Ti:sapphire and Nd:YVO$_4$ lasers were 76 and 40 MHz, respectively.

Operating the Ti:sapphire laser at different wavelength ranges requires different mirror sets to be installed: short-, mid- and long-wavelength (700-820 nm, 780-920 nm and 900-1000 nm, respectively). The laser operating wavelength was measured by a spectrometer (PulseCheck, APE, Berlin, Germany). The same spectrometer was used to determine the pulse bandwidth, as an autocorrelator (Wavescan, APE) gave less reliable bandwidth estimations for the subpicosecond pulses.

The emission (800-920 nm) from the Ti:sapphire laser was passed through the frequency doubler (TP1B, Chatsworth, CA), and then used as an excitation source in the lifetime experiments with the green form of EosFP. The same arrangement was also used for the single-molecule EosFP photoconversion experiments with the laser tuned to 800 nm. The
4.2. Microscopy

Figure 4.1: The light from various excitation sources is combined by beamsplitters BS2, BS3 and the dichroic mirror DM1 and then fed into the input coupler of a single-mode fiber. After the output collimator, the excitation sources could be combined with one of the infrared sources.

lifetime experiments with the red form of EosFP required much longer wavelengths, therefore the picosecond Nd:YVO$_4$ (GE-100-VAN-IR/SHG) laser was used. We also employed this laser in our estimations of the molecular brightness (section 5.5.1) due to its high stability. For studies of eqFP611 spectral changes upon high-power irradiation we used frequency-doubled 532-nm Q-switched Nd:YAG laser (Surelight II-10, Continuum Inc., Santa Clara, CA)

4.2.2 Excitation Arrangement

The excitation arrangement of our confocal microscope is shown in figure 4.1. The Ar$^+$/Kr$^+$ laser beam is reflected by the M1 and M2 alignment mirrors. It is then combined on the 50/50 beamsplitter BS2 with the 532-nm light from the frequency doubled cw Nd:YVO$_4$ (DPGL-2050F) laser. Emission from both lasers is directed by the mirror M3 to the cold mirror DM1 (Edmund Optics, Barrington, NJ), where it is combined with the 404-nm light from the GaN diode laser. The mirrors M6 and M7 on the GaN path where introduced for alignment purposes. For the same reason, mirrors M4 and M5 were introduced, guiding the frequency-doubled Ti:sapphire beam. This laser can be optionally combined with the Ar$^+$/Kr$^+$ laser by the 50/50 beam-splitter BS3 (Edmund Optics). The combined illumination was fed into a single-mode quartz fiber (QSMJ-3AF35-488-3.5/125-3AS-10, OZ Optics) by a coupler lens (HPUC-23-400/700-S-6AC-12, OZ Optics). This coupling intro-
Chapter 4. Experimental Setup

Figure 4.2: The light from various excitation sources (blue) enters the microscope through the back illumination port and is focused on the sample the by a microscope objective. The fluorescence light is transmitted through the dichroic mirror DM1, which reflects the excitation light. The emission light is then focused on the pinhole by the lens L1. The second lens L2, focuses the emission on the detectors (APD1, APD2 or PMT).

duced simultaneous spatial filtering for all of the excitation sources entering the waveguide. Since the infrared lasers could not be fed into the fiber, they were combined with the emission from the output coupler on the DM2 dichroic (640 DCSPXR, Chroma) mirror. The laser power was regulated by the neutral density (ND) filter wheels. The spatial filter consisting of two lenses (f = 100 mm and f = 50 mm) and a 30 µm pinhole was also used as a simple collimator (without the pinhole) in the measurements of spectral dependencies and in the experiments with the Nd:YVO₄ laser. The former was performed due to a slight wavelength-dependent beam shift, which disturbed the spatial filter alignment. In the latter case, the pinhole was not inserted because of the challenging alignment and low output power of the laser. The non-filtered illumination, however, should not introduce errors in the relative intensity measurements especially in the two-photon case (due to the natural suppression of background fluorescence).

4.2.3 Microscope Description

The confocal system used in this work (figure 4.2) is based on a Zeiss Axiovert 35 inverted microscope (Carl Zeiss AG, Göttingen, Germany) and was first developed by Andreas Schenk and Michael Brehm. A fast photomultiplier tube (PMT), was implemented for time-resolved fluorescence experiments.

The excitation light entering from mirror M1 is fed into the microscope by the dichroic mirror DM1 and further reflected by a 95% reflecting mirror M2 (AHF, Tübingen, Germany) into an objective lens, which focuses the light beam onto the sample. The M2 mirror transmits 5% of the excitation light reflected from the sample, which is directed onto a CCD camera (not shown) used for focus adjustment and alignment purposes. The fluorescence light is transmitted through the dichroic mirror DM1, reflecting the excita-
4.2. Microscopy

The emission light is then focused on the pinhole by the achromatic lens (L1, \( f = 150 \text{ mm} \)). The second lens (L2, \( f = 200 \text{ mm} \)), focuses the passed emission onto the detectors (APD1, APD2 or PMT). The fluorescence light can either be divided in two spectral channels by the dichroic mirror DM2 or detected by a single fast PMT. The optional shutter S2, which is placed in front of APD1, protects the detector from the reflected laser light, when multi-wavelength excitation is used (APD1 was used in the green channel and could not, otherwise be protected against 532-nm light).

Excitation power was determined directly after the M1 mirror and corrected for the excitation losses in all cases except for the two-photon experiments, where the power was measured after the DM1 mirror in order to exclude the influence of the spectral characteristics of DM1 on the measured power.

4.2.4 Sample Positioning

The microscope is equipped with a two-dimensional piezoelectric scanning stage with capacitive positioning sensors and a scan size \( 100 \times 100 \mu\text{m}^2 \) (P-731.20, Physik Instrumente, Karlsruhe, Germany). The stage is connected either to an analog (E509.C3, Physik Instrumente) or a digital controller (E710.3CD, Physik Instrumente). The digital controller was only used for lifetime imaging purposes in a combination with a TCSPC card (TimeHarpp200, PicoQuant, Berlin, Germany).

4.2.5 Filter Arrangement Used in Applications

**Experiments on eqFP611 and its Genetic Variants**

In our experiments on eqFP611, instead of the DM2 assembly with the filters F2 and F3, a single-channel detection (APD2) was used. Here filter F1 was utilized for emission spectral restriction. We used either a HQ 665/170 or a HQ 610/75 (AHF) filter in this position. The former provides a better collection efficiency for eqFP611, but the latter reduces the background contribution from the water Raman band (\( \approx 3500 \text{ cm}^{-1} \)). The latter was only used in the measurements of molecular brightness (section 5.5.1). As dichroic mirror DM1, we used 575 DCXR (AHF). In single-molecule experiments we placed a 80-\( \mu\text{m} \) pinhole (PH) in the emission path, whereas a 50 \( \mu\text{m} \) pinhole was utilized in our estimations of molecular brightness.

**Experiments on EosFP and its Genetic Variants**

In the single-molecule experiments on EosFP, the DM2 assembly consisted of HQ 535/70 (F2, AHF), HQ 610/75 (F3, AHF) filters and a 560 DCXR (DM2, AHF) dichroic mirror. No filter was used at the F1 site. At the DM1 place, a Q495LP (AHF) mirror was installed. A 50-\( \mu\text{m} \) pinhole (PH) was utilized in the single-molecule experiments. For FRET determination in our in vivo experiments (section 6.5.2), the DM1 mirror was replaced by a dual band z488/532RCP dichroic mirror (AHF), which allows the simultaneous excitation of the green and red forms of EosFP. In our two-photon experiments the 50-\( \mu\text{m} \) pinhole was replaced by a 2 mm hole mask. An infrared-blocking BG color glass filter
Figure 4.3: Wavelength dependence of the transmission of objective lenses for two-photon microscopy (the data are received from Carl Zeiss AG, Germany).

(Schott, Mainz, Germany) was introduced at the F1 site, and DM1 position was occupied by a FF495-Di02 (Semrock, Rochester, NY) dichroic mirror because of the high reflection efficiency of the latter in the infrared region.

4.2.6 Objective Lenses

In our experiments, we used three types of objective lenses. For most of the experiments, a water immersion objective Olympus UPlanApo 60x/1,20W (Olympus, Hamburg, Germany) was employed. The only exceptions were the experiments involving two-photon excitation, as these experiments require high transmissivity in the infrared range. For this purpose, two other objectives were selected: Zeiss C-APOCHROMAT 63x/1,2 W and Zeiss Plan-Neofluar 63x/1,25 Oil (Carl Zeiss AG). The former was used in experiments on multiple samples at a single wavelength, where a water immersion objective is advantageous due to the independence of the focal volume on its vertical displacement. The latter objective has transmission characteristics (figure 4.3), which are better suitable for wavelength-dependent measurements. However, for oil immersion objectives the focal volume parameters change as a function of vertical displacement due to the refractive index mismatch between oil (glass) and water. Hence, control over the vertical displacement complicates relative measurements on multiple samples.
4.2.7 Detectors

Most of our experiments were performed using two avalanche photodiodes (APD, SPCM-AQR-14, Perkin-Elmer, Canada). These detectors have high detection efficiency (≈ 60% at 550 nm, up to 70% at 700 nm), which is crucial for the experiments on immobilized single molecules. The performance of these detectors in time-resolved fluorescence experiments is poor as compared to PMTs. The APDs used in our work had a single photon timing resolution of 300 ps, which is worse than the 180 ps value for our fast PMT (PMC-100-4, Becker & Hickl GmbH, Berlin, Germany). We used the latter for estimations of fluorescence lifetimes and for lifetime imaging. PMTs usually have a lower detection efficiency, especially in the red range (above 550 nm in our case), which is a limiting factor for their use in applications.

4.2.8 Computer Hardware and Software

In experiments on individual eqFP611 and EosFP molecules, a home built photon counting card TIPHCO was used. This card, developed by Helmut Lindenthal, allowed two-channel data recording with up to 12.5 ns time resolution. Later, a commercial counter card PCI6229 (National Instruments Corp., Austin, TX) was used in the two-photon conversion experiments and dimerization studies of androgen receptors. Fluorescence autocorrelation functions from dye and FP solutions were measured using a hardware correlator (ALV, 500E Fast).

The home-written data acquisition and analysis software was developed by different people using mainly Visual C++, PV-Wave and Mathlab. Programs for the TIPHCO card including recording of single-molecule traces, confocal images and PCH was developed by Michael Brehm and Elza Kuzmenkina. The program was further evolved by Andrei Kobitski and Jochen Fuchs for image and trace recording using the PCI6229 card. Analysis of the confocal scanning images was performed using programs developed by Elza Kuzmenkina and Andrei Kobitski. The trace screening and autocorrelation programs were developed by Elza Kuzmenkina. Binning on a logarithmic scale of the single-molecule ACFs and their weighted averaging was performed using a home-written C++ program. Weighted fit of all ACFs was performed using a PV-Wave program written by Andreas Schenk. A cumulant analysis PV-Wave program was developed by Hartwig Lehle and Carlheinz Röcker.

4.2.9 Measurements of Fluorescence Lifetime

Fluorescence lifetime experiments were performed using a TimeHarp200 TCSPC card. The trigger signal from the excitation sources was synchronized with the inverted signal from the PMT by adjustment of the cable lengths. Inversion of the signal was required by the TimeHarp200 specifications. To determine the instrumental response function (IRF), we measured a TCSPC histogram for rose bengal (Sigma-Aldrich, München, Germany), a dye with a fluorescence lifetime of 91 ps, dissolved in water. By properly adjusting the parameters of the discriminator (implemented in the TCSPC card), not only stable synchronization but also minimal width of the IRF could be achieved. Data acquisition and analysis was performed with commercial software (MicroTime 200, PicoQuant).
Chapter 4. Experimental Setup

4.3 Sample Preparation and Data Analysis

The stock solutions of fluorescent proteins were received from the group of Dr. Jörg Wiedenmann. The cellular material for our in vivo applications were obtained from the group of Dr. Franz Oswald.

4.3.1 Estimation of Bulk Spectral Properties

Room-temperature spectroscopy was performed in a quartz cuvette (Hellma, Müllheim, Germany). Spectral properties of the different eqFP611 variants were measured in PBS except for the molecular brightness of the individual proteins, which was estimated in pH 7.0 sodium phosphate buffer. In our pH titration experiments on EosFP, a 300 mM sodium phosphate/citric acid buffer was used in the range of pH 3.5-8.5. For pH 9.0 a sodium bicarbonate buffer was used. We tested whether the chemical composition of a buffer (sodium phosphate/citric, sodium phosphate, sodium bicarbonate, tris) at a given pH influences the titration results and found the results to be independent of it.

The extinction coefficient for almost all proteins was determined by relating the absorbance of the aromatic amino acids at 280 nm to the absorbance maximum of the FP chromophore [123]. This method provides an error of ±5%. However, the variation of the extinction coefficients of FPs taken from literature let us assume that purity of the samples and protein folding efficiency may significantly influence the obtained values. Thus, in order to avoid the influence of contaminations, the extinction coefficient of eqFP611 variants was estimated based on the method of alkaline denaturation [37]. To this end, the same amount of protein was diluted in the same amount of PBS buffer and in 1 M NaOH solution. The absorption cross-section of the hydrolyzed chromophore \( \varepsilon_{\text{ref}} = 40,000 \text{ cm}^{-1} \) is known from literature [37]. Relation of the spectra in PBS and 1 M of NaOH yields the extinction coefficient. The latter method is less sensitive to protein purity and provides more precise (higher) values for the FP chromophores. Unfortunately, error of \( \varepsilon_{\text{ref}} \) was not reported, which complicates comparison between the different methods. However, this method offers high reproducibility and independence of preparation artifacts. Otherwise, \( \varepsilon_{\text{ref}} \) can be estimated within 5% error by this method, provided that the reference value of 40,000 determined accurately.

As reference samples for two-photon spectroscopy fluorescein (Sigma-Aldrich) in pH 11 buffer and rhodamine 6G in methanol were used.

4.3.2 Sample Preparation for Low-Temperature Experiments

Use of a cryosolvent is necessary to preserve proteins at low temperatures. Cryosolvent should retain transparency throughout the temperature range of an experiment. The most common cryoprotectors are glycerol and various sugars (e.g., sucrose, trehalose). For our experiments on eqFP611, a mixture 2:1 (by volume) of glycerol (Merck KGaA, Darmstadt, Germany) and 300 mM potassium phosphate pH 8.5 buffer was used. In the case of the cryogenic experiments on EosFP, the mixing ratio was 3:1. This mixture was sealed in a polymethyl methacrylate (PMMA) \( 10 \times 10 \times 2.5 \text{ mm}^3 \) cuvette (figure 4.4 (B)) by a methylmetacrylate glue (Acrifix 192, Röhm GmbH & Co.KG, Darmstadt, Germany).
4.3. Sample Preparation and Data Analysis

Figure 4.4: (A) Sample holder for cryogenic experiments is shown together with the inserted sample. Screw and two copper-beryllium disc springs served to improve temperature contact. (B) Sample for low-temperature experiments.

The concentration of the samples for the absorption measurements was on the order of 100 nM, whereas for the fluorescence experiments, the concentration was reduced down to \( \approx 10 \) nM due to inner filter effects. The cuvette was inserted into a copper sample holder, which contained an indium layer for better thermal coupling (figure 4.4 (A)). For the same purpose a special gold-containing grease (Apiezon Products Ltd., London, UK) was applied between the sample and the holder (design: Kriegel/Forster). In his diploma thesis, Forster had determined that at the lowest achievable temperature the deviation between the temperature of the sample and the cold finger does not exceed 6 K, whereas at temperatures above 50 K the difference is below 2 K [124].

Trehalose (Sigma-Aldrich) was another cryoprotector used in our experiments. We mixed the eqFP611 solution with trehalose powder until a saturated solution was obtained. The mixture was centrifuged at 12,000 g for 10 min to clean the sample from sugar precipitate. A droplet of the clean solution was pipetted on a CaF\(_2\) window and hardened at 65°C until it was still possible to squeeze the sample between two CaF\(_2\) windows. The spectral characteristics of CaF\(_2\) were not important in our experiments. However, in our case, high thermal conductivity of CaF\(_2\) of 9.71 Wm\(^{-1}\)K\(^{-1}\) may be beneficial for better thermal coupling of trehalose glass to the sample holder. The samples looked transparent and contained no cracks. As the sample holder did not allow the 90° excitation-emission geometry to be used, we operated our fluorimeter in the back-scattering geometry. Spectra taken with both geometries were shown to be identical.
4.3.3 Sample Preparation for Single-Molecule Experiments

Sample Cell Description and Surface Treatment

The sample cell for single-molecule experiments was made of 24 × 32 mm$^2$ and 20 × 20 mm$^2$ glass coverslips (Menzel-Glaser, Braunschweig, Germany) held together by two stripes of double-sided tape of ≈ 200 µm (3M, France). The space between the two stripes forms a channel (figure 4.5), which could be filled with the sample solution. The glass coverslips were flamed prior to use to remove possible contaminations. In the measurements of TPE spectra, the cell was sealed with paraffin (Merck) to avoid evaporation of the sample.

For our single-molecule experiments on EosFP, the channel surface of the sandwich cell was functionalized with biotinylated BSA (bovine serum albumin, Sigma-Aldrich). To functionalize the surface, the channel was exposed to 1 mg/ml solution (in pH 7.0 buffer) of biotinylated BSA. After 10 minutes, a homogenous layer of physisorbed BSA is formed. We subsequently replaced the BSA solution with a 15 µg/ml solution of streptavidin (Invitrogen, Eugene, OR) and incubated for another 10 minutes. Streptavidin binds specifically to the biotin labels on BSA with high affinity. After rinsing the cell with the buffer solution, the surfaces were ready to use.

Surface biofunctionalization is an important factor in studies of individual biomolecules. Interaction of biomolecules with the surface can influence their function. This interaction can result in nonspecific adsorption of the biomolecule to the surface. It has been shown that a polyethylene glycol-coated (PEG) surface offers lower nonspecific adsorption as compared to BSA [125, 126]. It was also shown that eqFP611 has a smaller fraction of unfolded protein on a PEG-coated surface as compared to a physisorbed BSA layer [125]. We used a PEG self-assembled monolayer (SAM) to immobilize eqFP611 molecules. The surface preparation consisted of several steps. After the glass slides were cleaned in an oxygen plasma, they were reacted with an aminosilane solution (Vectabond, Vector Laboratories, Burlingame, CA) according to the manufacture’s protocol. This aminosilanization procedure was performed by Sigrid Niederhausen in a clean room. In the next step, the exposed amino groups reacted with the ester groups of methoxy-PEG-succinimidyl pro-
4.3. Sample Preparation and Data Analysis

Pionate (mPEG-SPA, molecular mass = 5000 Da, Nektar Therapeutics, Huntsville, AL) and biotin-PEG-succinimidyl ester (biotin-PEG-NHS, molecular mass = 3400 Da, Nektar Therapeutics). The latter was added in a minor amount (1% by weight) to mPEG-SPA and served as an anchor for protein immobilization. The incubation with the PEG mixture was performed with a 100 mg/ml solution in sodium carbonate buffer at pH 8.2 for 2 hours. After the SAM was formed, the surface was rinsed with deionized water.

Protein Biotinylation

We used two different procedures for attaching biotin labels to our proteins in order to immobilize them by biotin-streptavidin linkage. In our single-molecule experiments on EosFP, the proteins were biotinylated (biotin-XX, sulfosuccinimidyl ester (SSE), Invitrogen) at their accessible primary amines (lysines, N-terminus) by the standard protocol of SSE-amine coupling [127]. A 3:1 molar excess of biotin-XX, featuring a 14-atom long spacer between the biotin and SSE, was taken for the protein labeling. The reaction was completed after 3 hours at room temperature and the protein was purified using a gel filtration cartridge (Edge BioSystems, Gaithersburg, MD).

eqFP611 was biotinylated at one of the two exposed cysteines known from the x-ray structure [128]. Cysteine labeling offers higher specificity than the common method of amine conjugation, because proteins usually contain a much higher amount of lysine than of cysteine residues. The exposed cysteines were reduced with TCEP, then biotin-maleimide (Sigma-Aldrich) was taken in 6 times molar excess as compared to the protein amount. The reaction was performed over night in 100 mM phosphate/150 mM NaCl pH 7.2 buffer. The protein was then purified through a gel filtration cartridge (Edge BioSystems).

4.3.4 Volume Calibration Procedure

In order to gain quantitative results from FCS and FCA measurements, the parameters of the confocal volume have to be determined using a reference sample with known diffusion coefficient. We analyzed the intensity ACF measured on our standard reference, rhodamine 6G (Lambda Physik) in water, where the dye molecules have a diffusion coefficient of \( D = 280 \mu m^2/s \) [129]. The obtained ACF was approximated with the following expression [117]:

\[
G(\tau) = \frac{2^{-3/2}}{N} \frac{1}{1 + \tau/\tau_D} \frac{1}{\sqrt{1 + \left(r_0/z_0^2\right)^2(\tau/\tau_D)}}. \tag{4.2}
\]

Here, \( N \) is the time-averaged number of molecules in the confocal volume, \( \tau_D \) represents the diffusional correlation time of a molecule through the focus, and \( r_0 \) and \( z_0 \) are the radial and axial dimensions of the observation volume, over which the intensity decays by a factor of \( 1/e^2 \). Using equation 4.2 implies that the triplet state of a dye molecule is not significantly occupied. This condition can be met by using lower excitation power. The presence of triplet state dynamics requires an additional term in the equation.
4.3.5 Calculation of the Excitation Rate

The excitation rate is a parameter that determines how many excitations of a single molecule occur in a second. This parameter helps to exclude the excitation wavelength dependence from the analysis and is defined as following:

\[
k_{\text{ex}} \equiv \frac{\lambda \ln(10) \cdot 10^3}{h c N_A} \varepsilon(\lambda) P,
\]

where \(\varepsilon(\lambda)\) is the extinction coefficient in \(\text{M}^{-1} \text{cm}^{-1}\) and \(P\) is the power density expressed in \(\text{W}/\text{cm}^2\). To estimate the power density \(P\), we divided the excitation power by the illuminated area, which can be found from the volume calibration procedure (section 4.3.4), \(A = 4\pi D \tau_D\), where \(D\) is the coefficient of translational diffusion.

4.3.6 Weighted Averaging of Single-Molecule ACFs

Weights for every data point \(G_i\) of the averaged ACF were calculated on the basis of the standard deviations \(\sigma_{ki}\) for the individual ACFs:

\[
G_i = \frac{\sum_k x_{ki}^2}{\sum_k 1/\sigma_{ki}^2}.
\]

Averaging was performed in several iterations. Two ACFs were averaged in each step. After the standard error of the mean for individual ACFs was determined in a first step, the error of the weighted mean \(\sigma_i\) for individual data points was used in all subsequent iterations:

\[
\sigma_i^2 = \frac{1}{\sum_k 1/\sigma_{ki}^2}.
\]

4.3.7 Power Density Correction in Two-Photon Experiments

For proper determination of two-photon excitation and conversion properties of EosFP at different wavelengths, changes of the power density caused by expansion of the illuminated volume at longer wavelengths and variation in pulse width has to be corrected. For the shorter-wavelength data set (800-920 nm) it was technically possible to keep the pulse duration \(\Delta \tau\) constant throughout the range. For the longer wavelengths, the pulse duration had to be varied in order to achieve stable mode-locked operation of the laser at different wavelengths. Variation in the excitation power density \(P_{\text{max}}\) due to changes in pulse width can be taken into account using the relations:

\[
\Delta \nu \Delta \tau \sim 1, \quad \Delta \tau \sim \frac{\lambda^2}{\Delta \lambda}, \quad P_{\text{max}} = \frac{P_{\text{avg}}}{f \Delta \tau},
\]

\[
P_{\text{max}} = \frac{\lambda^2_{\text{ref}} \Delta \lambda}{\lambda^2 \Delta \lambda_{\text{ref}}} \cdot P_{\text{ref}} \cdot P_{\text{max}}.
\]

Here, \(\Delta \nu\) and \(\Delta \tau\) are the spectral (in frequency space) and temporal widths, which are connected by the uncertainty relation; \(f\) is the pulse repetition rate; and \(P_{\text{avg}}\) is average excitation power. Taking into account the wavelength-dependent expansion of the
4.3. Sample Preparation and Data Analysis

illuminated volume \( V \sim \lambda^3 \), we have to modify the equation 4.4 to:

\[
P_{\text{max}} = \frac{\lambda_{\text{ref}}^5 \Delta \lambda}{\lambda^5 \Delta \lambda_{\text{ref}}} \cdot P'_{\text{max}}.
\]  

(4.5)

For correction, reference values were set to the ones measured at 920 nm.

4.3.8 Estimation of Bleaching Yields on the Single Molecule Level

In order to discriminate between the photobleaching event and a transient dark state of a single molecule, we recorded single-molecule time traces until 3 seconds after breakdown of the emission. This 3-second cut-off has been estimated empirically and reduced the probability of data misinterpretation. The number of photons from an individual time trace is the sum of the number of photons emitted by a FP molecule and the background contribution. We measured the average number of background photons per unit time and subtracted this value from the number of acquired photons according to the trace duration.

The histograms of collected photons were fitted with an exponential, \( \exp(-N/N_0) \), where \( N_0 \) represents the average number of emitted photons, before photobleaching occurred. This number was then divided by detection efficiency of our confocal system and the quantum yield of fluorescence to obtain the number of absorbed photons.

4.3.9 Calculation of the FRET Index and Channel Cross-Talk Correction

For all two-channel experiments on EosFP, we first determined the fluorescence intensity of the non-converted green form in both spectral channels. The ratio of the intensities in the red and the green channel defines the cross-talk \( \alpha \).

To calculate our FRET index, we introduce a ratio \( \varsigma' = \frac{I_{\text{FRET}}^{488}}{I_{532}^{488}} \), where \( I_{\text{FRET}}^{488} \) is the intensity detected in the red spectral channel (R) that is caused exclusively by FRET from the green form to the red form upon 488-nm excitation. \( I_{532}^{488} \) is the fluorescence intensity of the red form under 532-nm excitation. Calculation of the ratio \( \varsigma' \) requires an estimation of intensity contributions from the different sources. The intensity in the red channel sums up from \( I_{\text{FRET}}^{488} \), the cross-talk emission of the green form \( \alpha I_{\text{direct}}^{G_{\text{direct}}} \) (\( \alpha \) is the cross-talk ratio) and emission due to the direct excitation of the red form by the 488-nm laser \( I_{488}^{488} \):

\[
I_{488}^{R} = I_{\text{FRET}}^{488} + \alpha I_{488}^{G_{\text{direct}}} + I_{488}^{\text{direct}}.
\]

The correction factor \( C = P_{532}^G / P_{488}^G \) is necessary for comparison between the measurements performed at different excitation powers \( P \). Using \( C \) we can define the FRET index as follows:

\[
\varsigma \equiv \varsigma' C = \frac{I_{488}^{R} - \alpha I_{488}^{G_{\text{direct}}} - I_{488}^{\text{direct}}}{I_{532}^{R}} \cdot C.
\]

We can replace \( I_{488}^{\text{direct}} \) by the expression \( I_{488}^{\text{direct}} = SI_{532}^{R} \), where \( S \) is an excitation scaling factor:

\[
S = \frac{P_{488}^{\text{red}}}{P_{532}^{\text{red}}}.\]
The values $\varepsilon^{\text{red}}$ are the extinction coefficients of the red form at different wavelengths. Thus, the FRET index $\varsigma$ can be expressed as follows:

$$
\varsigma = \left( \frac{I_{R}^{488} - \alpha I_{\text{direct}}^{G488}}{I_{532}^{R}} - S \right) \cdot C. \tag{4.6}
$$
Chapter 5

Red Fluorescent Protein eqFP611

5.1 Introduction: General Description of eqFP611

5.1.1 Spectroscopic Properties

The sea anemone Entacmaea quadricolor (see figure 1.1 B) was found to encode a red fluorescent protein, eqFP611 [44]. This protein, discovered and cloned at the Department of Zoology, University of Ulm by Jörg Wiedenmann and coworkers, has the maximum of absorption and excitation at 559 nm. The large Stokes shift of 52 nm made the protein the most red-shifted naturally occurring RFP, with \( \lambda_{\text{em}}^{\text{max}} = 611 \) nm (see figure 5.1).

The surprisingly large Stokes shift and relatively broad and unstructured emission spectrum can be observed down to temperatures of several kelvins (see section 5.3). These spectra are invariant within the pH range 4-10. The brightness of this FP is governed by the fluorescence quantum yield \( \Phi_F \) of 0.45 and the extinction coefficient of 82,000 M\(^{-1}\)cm\(^{-1}\) at 559 nm [44, 130]. A single-exponential fluorescence decay with a time constant of \( \tau = 2.5 \) ns has also been reported [44].

5.1.2 Structural Properties

Similar to dsRed, the quaternary structure of eqFP611 is tetrameric [128] (figure 5.2). Wiedenmann and coworkers showed, however, that in contrast to dsRed, the eqFP611 tetrameric complex is able to dissociate on a pseudo-native SDS-gel and in single-molecule measurements on surfaces [119].

Although the chromophores of eqFP611 and dsRed are chemically similar, the conformational states of both are different. The chromophore of eqFP611 is built from Met63-Tyr64-Gly65 and thus differs in one amino acid from the one of dsRed (see section 1.2). eqFP611 is the only fluorescent protein to date that has its chromophore in trans configuration (see figure 1.3 (C)). As in most RFPs, the eqFP611 chromophore formation passes through an immature green-emitting stage. During the process of maturation, an additional bond between the \( \text{C}_\alpha \) and the nitrogen of Met63 (Gln66 in dsRed) is formed (see figure 1.3 (B)). Unlike dsRed, the eqFP611 structure shows a \( \pi \)-stacking interaction between His197 and the chromophore.
5.2 Experimental Part: Disruption of the Tetramer

The red fluorescent protein eqFP611, having a number of advantages, still leaves much to be improved. Some of those issues to be addressed are the folding problem at physiological temperatures (37°C) and formation of tetramers already at nanomolar concentrations.

To derive a monomeric eqFP611, Wiedenmann and coworkers modified the A/B and A/C interfaces (see figure 5.2). The choice of mutation sites was based on monomerization studies of dsRed [46]. Disruption of the A/C interface, however, produced only non-fluorescent variants. Surprisingly, we found no influence of the dimerising substitutions T122R and V124T at the A/B interface on the spectral properties of eqFP611 (see figure 5.3).

5.3 Cryogenic Experiments

Although application of low temperature spectroscopy to fluorescent proteins may appear unphysiological, it contains large amount of valuable dynamic information such as conformational energy barriers and the corresponding transition rates [131, 132]. Moreover, at low temperatures, low-energy vibronic modes can be suppressed and therefore, the temperature-associated broadening of the spectra can be reduced (see section 2.2).

To describe the broadening mechanisms of the absorption and to resolve the vibronic structure of the broad emission spectrum, we performed low-temperature experiments on eqFP611. The low-temperature absorbance spectrum ($T = 14$ K) shows the main band centered at $17,980 \pm 1$ cm$^{-1}$, (figure 5.4), while the emission is centered at $16501 \pm 1$ cm$^{-1}$.
5.3. Cryogenic Experiments

Figure 5.2: (A) Quaternary structure of eqFP611 in ribbon presentation. Here A, B, C and D denote individual subunits of the tetramer. The tetramer is stabilized by amino acid interactions at the A/B and the A/C interfaces. (B) Stereoview (cross-eyed) of the eqFP611 chromophore (highlighted in red) and its surrounding. (C) The A/B and (D) the A/C interfaces of the tetramer. Structure data: PDB entry 1UIS.

The temperature-dependent changes of the excitation and emission spectra are plotted in figure 5.5. With increasing temperature, the absorption and emission spectra decrease in intensity and shift to lower frequencies. The expected mirror-like excitation-emission symmetry at low temperatures is not observed here. In our experiments, we did not observe a zero-phonon line (ZPL, purely electronic transition), which stays frequently hidden under the phonon wing in a polar environment [133]. Strong phonon coupling is also one of the reasons why a ZPL may not be observed.

To describe the temperature dependence of the spectral shape, we fitted the absorption and fluorescence maxima with a gaussian function. According to the harmonic model, the spectral width $\Delta\tilde{\nu}$ and peak positions described by equations 2.11 and 2.12, respectively. In figure 5.6 $\Delta\tilde{\nu}$ and the shift of the absorption maximum are plotted versus temperature. Within the harmonic approximation, we can describe temperature dependencies of the $\Delta\tilde{\nu}$...
Figure 5.3: Spectral properties of eqFP611 dimeric variants. Emission is excited at 560 nm, while excitation spectrum is recorded for the emission at 610 nm.

Figure 5.4: Absorption spectra of eqFP611 in glycerol/buffer are shown in the temperature range 14-300 K in 20 K steps (except for the step from $T = 14$ K to $T = 20$ K). The upper black line corresponds to $T = 14$ K and the lower one to $T = 300$ K.
5.3. Cryogenic Experiments

Figure 5.5: Excitation ($\lambda_{\text{ex}} = 610$ nm, solid lines) and emission ($\lambda_{\text{em}} = 540$ nm, dotted lines) spectra of eqFP611 in glycerol/buffer at different temperatures.

Figure 5.6: (A) Temperature dependence of FWHM of absorption (open triangles) and fluorescence (open squares) spectra in glycerol/water, closed triangles show the temperature dependence of FWHM for the protein in trehalose matrix; (B) peak position of the absorbance for eqFP611 (open triangles) in glycerol/water, closed triangles show the temperature dependence of FWHM for the protein in trehalose matrix. The solid lines show the fits to the data below the glass transition temperature of the glycerol/water mixture. This temperature is depicted as the vertical dotted line. The dashed line is a linear fit to the data above the glass transition temperature.
for the emission and fluorescence spectra, and the shift of the absorbance maximum $X_C(T)$ with the same effective phonon mode of $100 \pm 8 \text{ cm}^{-1}$ within the experimental error. The coupling factors $S$ for emission and absorption are $18 \pm 1$ and $2.1 \pm 0.1$ respectively. The quadratic coupling constant $R = 0.1 \pm 0.005$ describes the effect of the peak shift. The behaviors of $\Delta \nu$ and $X_C(T)$ dramatically change above the glass transition temperature ($T_g \approx 180 \text{ K}$ [134, 135]) of the cryogenic solvent and are no longer described by the harmonic approximation (mixture of glycerol and pH 8.5 buffer, see section 4.3.2).

We analyzed the temperature dependence of the centroid (first moment, center of gravity) of the absorption spectrum. This dependence is shown in figure 5.7. It can be approximated with the following equation [82]:

$$M_1 = D + F \coth \left( \frac{hc\langle \nu \rangle}{2kT} \right),$$

(5.1)

where $D$ takes into account all temperature independent contributions, $F$ is an amplitude factor. By the moment analysis we can verify that by limiting the spectral region (only the spectral peak can be fitted with a gaussian) we obtain relevant values for the mean phonon frequency. In the moment analysis, an analyzable part of the spectrum is larger. This analysis yielded the same effective phonon mode of $100 \pm 10 \text{ cm}^{-1}$, which confirms our previous findings.

Obviously, the cryosolvent is an important factor influencing the spectra. At room temperature the quantum yield of eqFP611 in our standard cryosolvent (glycerol/buffer, 70:30 by mass) drops 1.4 times as compared to an aqueous environment, which is opposite to the expected increase due to higher solvent viscosity. This may suggest that glycerol has specific interactions with the protein. The maxima of absorption and emission shift from 559 nm and 611 nm to 561 nm and 609 nm, respectively, in the glycerol/buffer mixture.
5.3. Cryogenic Experiments

Alternatively, we performed additional measurements in trehalose glass, a sugar used by nature to protect living tissues from extreme temperatures. It replaces water molecules that form hydrogen bonds to the surface of a protein [136]. We measured the fluorescence excitation and emission spectra of eqFP611 embedded in trehalose glass at a temperature of 12.5 K. Fluorescence spectra for the protein in both matrices almost match, with the exception of the small shift of the maximum emission wavelength from 16,501 ± 1 cm\(^{-1}\) in glycerol to 16,447 ± 1 cm\(^{-1}\) in trehalose (from 607 to 610 nm). Low-temperature excitation spectrum (\(\lambda_{em} = 610\) nm) of eqFP611 shows an increased band at 19,230 ± 1 cm\(^{-1}\) (or 1270 cm\(^{-1}\)) from the maximum of the absorption band, figure 5.8. We believe that this band can be partially attributed to the green state of eqFP611, as illumination within this band produces a "green" and "red" emission spectrum (not shown). However, we can not exclude that this band is a superposition of the green state protein and vibrational modes in this range. The latter is suggested by Raman studies of dsRed at room temperature, where the strongest vibrations are observed at 1337 and 1391 cm\(^{-1}\) [137] and by low-temperature studies of Bonsma et al. [67, 54]. We found that the intensity of the green band was dependent on a particular preparation, which is illustrated in figure 5.8. The large fraction of the green species in the sample can also be attributed to a preparation artefact. During the preparation, the protein is heated up to 70°C. Trehalose is believed to prevent unfolding of the protein, but possibly it cannot prevent hydrolysis of the chromophore extension (see introductory figure 1.3), which may occur at elevated temperatures. We believe that the product of this hydrolysis contributes to the excitation spectrum, while the excitation band of the red form decreases in intensity.

---

**Figure 5.8:** Excitation (solid lines) and emission (dotted lines) spectra of eqFP611 in trehalose (black) and glycerol (grey) cryoprotection mixtures at \(T = 12.5\) K. A and B depict two different preparations of the sample.
Figure 5.9: Temperature dependence of the fluorescence quantum yield of eqFP611.

If we compare the temperature dependence of ∆ν in both matrices (figure 5.6 (A)), we immediately see that the excitation spectrum for the protein in trehalose is broader than the absorption spectrum for the protein embedded in the glycerol/buffer matrix at the lowest temperature (we believe that possible difference between the excitation and emission spectra has negligible impact on our analysis). On the other hand, at higher temperatures the difference becomes smaller and, finally, the spectral width in glycerol exceeds the one in trehalose at 300 K. The excitation maximum of eqFP611 in trehalose illustrates a very weak dependence on temperature (figure 5.6 (B)).

The quantum yield of eqFP611 (see figure 5.9) changes rapidly above the glass transition temperature of the cryogenic solvent, showing the growing role of non-radiative processes. When ramping up the temperature, passing over the glass transition point changes the cryosolvent from a solid to a viscous liquid. In this phase, the behavior of the fluorescence quantum yield shows irregularities, which may arise from a transition process near Tg. Peculiar dependencies of the spectral moments in this temperature region were frequently observed. Another temperature region, where the data are more scattered, is T ≈ 10 K. We suspect that around this temperature some kind of a light-activated processes may occur.

5.4 FCS on Individual Molecules

In solution experiments, the diffusional decay of the autocorrelation function limits dynamic studies to timescales faster or at least on the order of the diffusional correlation time τD. To extend our investigations beyond this limit, we recorded the fluorescence emission from individual eqFP611 molecules immobilized on a PEG-coated surface and performed autocorrelation analysis of the recorded traces.

Figure 5.10 (A) shows a confocal scan image of immobilized eqFP611 molecules. Fluorescence fluctuations of individual molecules were recorded with a binning time of 625 ns.
5.4. FCS on Individual Molecules

Figure 5.10: Single-molecule fluorescence time traces of eqFP611 and the histogram of the collected photons: (A) Confocal scanning microscopy image (128 × 128 pixels, field of view 18 × 18 µm², 5-ms integration time per pixel, excitation rate 0.14 MHz at 514 nm) of individual eqFP611 molecules attached to a PEG-coated surface. (B) Histogram of the total number of photons collected from 160 individual time traces before photobleaching. The solid line represents an exponential fit. (C) Typical fluorescence time trajectories of individual eqFP611 molecules (traces recorded with 625 ns and plotted with 1-ms resolution). The figure is adopted from [119].

Three typical examples of fluorescence time traces are shown in Figure 5.10 (C). The molecules emit over several hundred milliseconds before photodestruction occurs. There are, however, extended periods during which they are completely nonemitting followed by fluorescence revival. For representation purposes, the data are plotted with a resolution of 1 ms.

In order to characterize the photodynamics of eqFP611, ACFs were calculated from individual time trajectories. To avoid the influence of bleaching on the correlation function, traces shorter than 500 ms were excluded from the autocorrelation analysis. An example of a single-molecule ACF is shown in figure 5.11 (A) (open circles). All ACFs show, besides the fast flickering on the 100 µs timescale, a second, slower dynamic process. The separation between the slower and the faster dynamics shows up as a step in the ACF at ≈ 300 µs. Because of the limited signal-to-noise ratio, we performed weighted averaging of 76 single-
Figure 5.11: (A) Autocorrelation curves of individual eqFP611 molecules dried on a bare glass surface (filled squares) and immobilized on a PEG-coated surface (open circles). The solid line represents a double-exponential fit to the data. The dotted line shows the zero level. (B) Average of 76 correlation functions of individual molecules immobilized on a PEG-covered surface. The solid line shows a double exponential fit to the data. Histograms of the dark fraction (C) and the flicker rate for the faster flickering process (D) are plotted for 76 molecules and fitted with Gaussian distributions. The figure is adopted from [119].

molecule ACFs to unambiguously characterize the slower process (Figure 5.11 (B)). The average ACF clearly exhibits a two-step decay, showing an additional flickering process with smaller amplitude at longer timescales. It can be very well described with a sum of two exponentials:

\[ G(\tau) = A_1 \exp(-\Lambda_1 \tau) + A_2 \exp(-\Lambda_2 \tau), \]

with the amplitude \( A_1 = 0.18 \pm 0.01 \), flicker rate \( \Lambda_1 = 3500 \pm 100 \) Hz, \( A_2 = 0.080 \pm 0.001 \) and \( \Lambda_2 = 105 \pm 5 \) Hz. After the identification of the slow process, we fitted all 76 individual ACFs, keeping the slow flicker rate \( \Lambda_2 \) fixed at 105 Hz. Figure 5.11 shows histograms for the dark fraction \( F = A_1/(1 + A_1) \) in panel (C) and for the rate coefficient \( \Lambda_1 \) of the fast flicker process in panel (D), as determined from the analysis of the individual traces. For the distribution of the dark fraction, we obtained a mean, \( \langle F \rangle = 0.17 \), and the observed distribution can be fitted by a Gaussian peaking at 0.166 ± 0.005 with a standard deviation of 0.062 ± 0.005. The distribution of the flicker rate yields \( \langle \Lambda \rangle = 3400 \) Hz, the maximum at 3160 ± 60 Hz and has a standard deviation of 1170 ± 60 Hz. The mean parameters
are consistent with those obtained by the solution FCS data, when using an average excitation rate of 0.14 MHz [119]. Because of the low fluorescence intensity observed from the individual molecules, the role of background (weakly fluorescent contaminations in particular) cannot be neglected. We performed control experiments, which showed only negligible effects of photobleaching of the fluorescent background on our single-molecule ACFs.

As can be seen from figure 5.11 (A), for the molecules dried on a bare glass surface almost no flickering can be observed. This observation points to strongly suppressed dynamics within the dried protein.

5.5 Photostability of Individual eqFP611 Molecules

Characterization of the photobleaching yield can either be done at the bulk or at the single-molecule level. In the former case, fluorescence time decays can be compared against a known reference dye. Another option is to estimate the incident power, dye concentration, extinction coefficient and quantum yield. Then, based on these data, fluorescence decays can be analyzed in terms of the photobleaching efficiency. These approaches are relatively easy to realize, however, in the case of multi-exponential decays, the data can easily be misinterpreted. Single-molecule bleaching experiments can potentially discriminate between various protein populations and resolve heterogeneity, which stays commonly unresolved in bulk experiments.

Figure 5.10 (B) shows a histogram of the observed numbers of photons from 160 eqFP611 molecules. The exponential fit on the figure yields an average number of detected photons of 1234 ± 88. Estimating the overall detection efficiency of the system as ≈ 5%, this number corresponds to 24,200 emitted photons. Taking the fluorescence quantum yield Φ_F = 0.45 (at room temperature) into account [44], a total of ≈ 54,000 excitations occur on average before photobleaching occurs, corresponding to a yield of photobleaching, Φ_B = (1.9±0.3)×10^{-5}. This number represents the probability of a single photon to cause photobleaching. We also estimated the photobleaching yield from 48 eqFP611 molecules dried on a glass coverslip, Φ_B = (1.3 ± 0.4) · 10^{-6}.

5.5.1 Molecular Brightness of eqFP611 Tandems

The brightness of a fluorescent protein can be characterized by the product of its molar extinction coefficient ε and fluorescence quantum yield Φ_F. Michael Wolff and co-workers constructed functional eqFP611 tandems using a 12-amino acid spacer. In the ideal case, the observed brightness of the the merged (or tandem) dimeric FP should be equal to the brightness of the original (non-fused) dimer. Unfortunately, calculation of brightness based on a product of ε and Φ_F has a serious drawback. The fusion of two identical proteins can mutually influence their folding efficiency. Therefore, a mixture of tandems with different numbers of properly formed fluorophores may be present.

Absorption spectroscopy allows one to estimate the fraction of improperly folded protein in solution. The characteristic absorption of the aromatic amino acids at 280 nm yields the overall protein concentration. It can be compared with the characteristic absorbance
Chapter 5. Red Fluorescent Protein eqFP611

Figure 5.12: (A) Confocal scanning microscopy image (128 × 128 pixels, field of view 18 × 18 µm², 5-ms integration time per pixel, excitation: 3 µW at 532 nm) of individual eqFP630 (teqFP611 N143S/T122R/S171F/V184D) tandems.

of the chromophore, which gives the concentration of the intact protein. For this method, however, a high concentration of purified protein is necessary. Large amounts of protein can only be obtained if the FPs are bacterially expressed. The folding problems of bacterially expressed FPs can, however, be irrelevant if the protein is developed for expression in mammalian organisms. Owing to the low production yield of FPs in mammalian cells, the application of absorption spectroscopy for the brightness quantification is not promising. We tried alternative approaches.

We observed bacterially expressed tandem constructs of a red-shifted variant of eqFP611 with improved folding properties on a single-molecule level. This variant contains four substitutions: N143S, T122R, S171F and V184D. The last two mutations caused folding improvement at elevated temperatures, whereas N143S induces red shift (section 5.6.1). The tandem constructs were immobilized on a BSA-coated surface. Most of the individual tandem complexes exhibited two-step photobleaching (see figure 5.12). However, this method cannot give a good estimation for the fraction of improperly folded proteins because two-step bleaching is often difficult to resolve.

Alternatively, the molecular brightness of the protein extracted from mammalian cells can be estimated directly by the methods of fluctuation spectroscopy, for which low concentrations of the sample are advantageous. Therefore, the methods of photon counting histogram and particularly the fluorescence cumulant analysis (see section 3.4.2) are more adequate for the analysis of dilute fluorescent protein (lysate) solutions. For the expression of the tandem variants of eqFP611 we used human embryonic kidney (HEK293) cells.

In order to estimate molecular brightness, histograms of the collected photons (see section 3.4.2) for each variant were acquired within 10 minutes at three different excitation powers: 20, 10 and 5 µW, with λex = 532 nm using our confocal microscope setup. Measurements at different powers are necessary to avoid the effects of saturation (FPs...
have lower saturation threshold as compared to most synthetic dyes due to their prominent light-induced dynamics). We found these effects to be negligible at powers below 10 \( \mu \text{W} \); therefore, the comparison of the molecular brightness between the different FPs was performed at this excitation power. In order to convert these numbers into power density, we determined the parameters of the confocal volume by measuring FCS functions from our reference R6G solution (see section 4.3.4 for details). This analysis yielded a beam waist of \( r = 0.35 \mu \text{m} \) and an axial dimension of \( z = 2.5 \mu \text{m} \). The sample concentration could be obtained as a result of the FCA method. However, preliminary estimations of the sample concentrations had to be performed. These were estimated from the intensity ACFs that were measured using a hardware correlator.

Examples of the collected histograms are shown in figure 5.13. The cumulant analysis of these histograms yields simultaneously the molecular brightness and the concentration of the sample. The results of the analysis are summarized later in section 5.7. The brightness is quantified here by the number of detected photons per one second per a molecule (\( P_{ex} = 10 \mu \text{W} \)). These values are corrected for the detection and excitation efficiency of the system and the different excitation efficiencies of the FPs at 532 nm. The detection efficiency was determined by multiplication of the normalized emission spectra by the spectral transmission window of our system. The correction for the different excitation efficiencies at 532 nm was obtained from the normalized excitation spectra of the different FPs. Reproducibility of the data was verified for different concentrations. These measurements allowed us to estimate the error of the method to be 15%.

The obtained molecular brightness of the dimeric eqFP611 variants and the correspon-
Chapter 5. Red Fluorescent Protein eqFP611

...ting tandem constructs is approximately the same, indicating that eukaryotic expression of tandem and dimeric proteins results in equal folding efficiency of both. The obvious advantage in brightness of the tandem constructs compared to hcRed1 and mRFP1 can be seen from table 5.1. In our experiments, however, we found a very high expression level for mRFP1 in HEK293 cells. The coloring of cellular lysates containing mRFP1 were visible to the naked eye that is rarely observed for other FPs. Comparison of dimeric and tetrameric FPs shows that their molecular brightness does not exactly track the oligomerization state. We can only suggest that a fraction of dissociated tetramers might be present in the solution, which decreases the overall molecular brightness.

5.6 Photochromic Properties of eqFP611

5.6.1 Red-Shifted Variants Show KFP-like Photochromic Properties

Substitution of Asn143 with serine in eqFP611 unexpectedly created a fluorescent protein with an emission maximum at 630 nm. This variant with a longer emission wavelength was denoted as eqFP630 (figure 5.14). The N143S mutation, however, does not abolish the original 611 nm-emitting species. Changes of the environment, such as addition of glycerol or variation of temperature and pH cause exchange between these two states. The pH-dependent absorption spectra of eqFP630 are shown in figure 5.15. The most obvious changes are the absorbance variations of the immature form, which becomes almost completely protonated upon decreasing the pH from 8.0 down to 5.0. Below this pH, the major changes in the spectrum arise from the red form. The protonation of the red form causes appearance of the band peaking at \( \approx 450 \) nm \[138\], while the band at \( \approx 390 \) nm can be assigned to the protonated green species, which can either be the immature form or the hydrolyzed form of the red chromophore.

We believe that, at low and high pH, the hydrogen bonds stabilizing the chromophore can be destabilized (residues Ser143 and Ser158 can stabilize the chromophore in the cis and the trans state by hydrogen bonds). This allows easier isomerization of the chromophore. Thus, the two spectroscopic forms represent different conformational states of the chromophore (vide infra).

Similar structures of the eqFP630 and KFP1 chromophore pockets (see section 5.8.1) make us believe that the trans and cis states of the eqFP630 chromophore are counterparts of the off- and on-states of KFP in structural terms. To verify this idea, we performed continuous illumination at 450 nm (conversion into the A state), followed by illumination at 560 nm (conversion into the B state). We indeed observed these KFP-like reversible light-induced transitions (figure 5.16 (A)). Moreover, the spectral difference between the forms A and B shows resemblance to the corresponding KFP difference spectra (see figure 5.16 (B) and discussion figure 5.20 for comparison).

These light-induced conformational changes can also be visualized as either a fluorescence rise or decay when the protein is excited at 450 nm or 560 nm, respectively (see figure 5.17). The kinetic data were acquired at 600 nm emission wavelength and, therefore, several contributions to these kinetics must be taken into consideration. Illumination at 450 nm shifts the emission spectrum from 630 nm towards 611 nm, and also improves the
5.6. Photochromic Properties of eqFP611

**Figure 5.14**: Comparison of absorption (solid line) and emission (dotted line) spectra of eqFP630 (black) against eqFP611 (grey).

**Figure 5.15**: Absorption spectra of eqFP630 at pH 8.0-5.0 (A) and pH 4.5-3.0 (B). The pH interval between the individual spectra $\Delta pH = 0.5$. 
Figure 5.16: (A) Illumination of eqFP630 at $\approx 450$ nm induces changes in the absorption spectrum. (B) The calculated difference between the initial spectrum and the spectrum after illumination at 450 nm (blue line). The opposite effect can be induced by illumination at 560 nm (green line). In order to achieve these small changes, prolonged illumination for at least one hour under 450 nm (or 560 nm) light from a 450 W xenon lamp passed though a SPEX 1680 Spectramate f/4 high performance double monochromator at $\approx 14$-nm spectral bandwidth.
5.6. Photochromic Properties of eqFP611

Figure 5.17: Illumination of eqFP630 at ≈ 450 nm produces a fluorescence increase at 600 nm (upper curve); illumination at 560 nm produces the opposite effect (lower curve).

quantum yield of fluorescence (0.45 for eqFP611 vs 0.3 for eqFP630), whereas illumination at 560 nm does the opposite.

5.6.2 High-Power Photoconversion

The photoconversion experiments carried out on some common FPs (dsRed, hcRed, YFP) under very intense illumination have inspired us to treat eqFP611 in a similar way. Using 532 nm illumination from a Q-switched laser (peak power density 700 kW/cm²), we observed light-induced changes in the absorbance spectrum at room temperature (figure 5.18).

As a result of intense illumination, the major absorption band of eqFP611 broadens and shifts to the red while reducing in amplitude. The red shift may be related to a minor fraction of a new red species. We believe that this effect is related to the formation of an eqFP630-like chromophore isomer. Simultaneously with the red shift, an absorption band at ≈ 390 nm appears. This band is normally assigned to the protonated green chromophore, which appears as the main photoproduct. This band can be assigned the hydrolyzed chromophore of eqFP611 (see introductory figure 1.3 (B), [37]), which means that hydrolysis of the Cα = N bond is one of the mechanisms of photobleaching of eqFP611.

A similar effect was found by Bonsma et al.[67], and by Cotlet et al. in bulk and single-molecule experiments [66]. Bonsma et al. have attributed this effect to a conformational change.
5.7 Spectral Properties of eqFP611 Variants

The properties of the different eqFP611 variants are summarized in this section. The mutations T122R and V124T yield dimeric variants of eqFP611; S171F, V184D, I57V and F102I help protein folding at 37°C; N143S allows a red-shifted (cis) conformation of the protein chromophore.

In our experiments, $\lambda_{max}$ values were defined with subnanometer precision (materials section 4.1). The error of the extinction coefficient cannot be calculated precisely as the error of the reference value is not known. Otherwise, the error of these measurements does not exceed 5%, as derived from the instrumental precision.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Oligomerization</th>
<th>$\lambda_{em/ex}^{\text{max}}$, nm</th>
<th>$\varepsilon$, cm$^{-1}$M$^{-1}$</th>
<th>$\Phi_F$</th>
<th>MB, kHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>eqFP611</td>
<td>tetramer</td>
<td>559/611</td>
<td>82.000</td>
<td>0.45 ± 0.02</td>
<td>-</td>
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<tr>
<td>eqFP611 T122R</td>
<td>dimer</td>
<td>559/611</td>
<td>84.000</td>
<td>0.41 ± 0.03</td>
<td>-</td>
</tr>
<tr>
<td>eqFP611 V124T</td>
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<td>559/611</td>
<td>74.000</td>
<td>0.43 ± 0.03</td>
<td>-</td>
</tr>
<tr>
<td>eqFP611 N143S (eqFP630)</td>
<td>tetramer</td>
<td>583/630</td>
<td>50.000</td>
<td>0.35 ± 0.02</td>
<td>-</td>
</tr>
<tr>
<td>eqFP611 N143S/S171F/V184D (eqFP630 K18)</td>
<td>tetramer</td>
<td>560-582/≈620*</td>
<td>- *</td>
<td>0.35 ± 0.02</td>
<td>45 ± 7</td>
</tr>
<tr>
<td>eqFP611 N143S/S171F/V184D/T122R (deqFP630 K18)</td>
<td>dimer</td>
<td>560-582/≈620*</td>
<td>- *</td>
<td>0.35 ± 0.02</td>
<td>32 ± 5</td>
</tr>
<tr>
<td>teqFP611 N143S/S171F/V184D/T122R (teqFP630 K18)</td>
<td>tandem dimer</td>
<td>560-582/≈620*</td>
<td>- *</td>
<td>0.35 ± 0.02</td>
<td>30 ± 5</td>
</tr>
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<td>112.000</td>
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<td>45 ± 7</td>
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<td>89.000</td>
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<td>43 ± 7</td>
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<td>teqFP611 I57V/F102I/T122R (Starttandem)</td>
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<td>2x103.000</td>
<td>0.46 ± 0.03</td>
<td>32 ± 5</td>
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<td>89.000</td>
<td>0.45 ± 0.03</td>
<td>38 ± 8</td>
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<td>0.47 ± 0.03</td>
<td>45 ± 7</td>
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<td>557/607</td>
<td>90.000</td>
<td>0.44 ± 0.03</td>
<td>-</td>
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<td>mRFP1</td>
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<td>584/607**</td>
<td>50.000**</td>
<td>0.25**</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>hcRed1</td>
<td>dimer</td>
<td>588/618**</td>
<td>20.000**</td>
<td>0.015**</td>
<td>17 ± 3</td>
</tr>
<tr>
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<td>tetramer</td>
<td>561/587**</td>
<td>43.800**</td>
<td>0.55**</td>
<td>48 ± 7</td>
</tr>
</tbody>
</table>

*The spectral data for all N143S mutants are ill-defined because of the two conformational states of the protein. Therefore, the values of the extinction coefficient will lie somewhere in between the values of the eqFP630 and eqFP611 dimeric variants.

**Values taken from literature[46, 139, 140].
Chapter 5. Red Fluorescent Protein eqFP611

5.8 Discussion and Conclusions

5.8.1 eqFP611 versus KFP: Structural Comparison

A surprise comes when the chromophore surroundings of eqFP611 and KFP are put in direct comparison (figure 5.19). They are very much alike. However, there are also a few differences. The chromophore of eqFP611 and the one of KFP in the off-state are in trans conformation. In the on-state, the chromophore of KFP is in the cis conformation. Unlike eqFP611, the chromophore of KFP stays non-planar in both off- and on-conformations. The structural comparison is in agreement with the spectroscopic analysis. The difference spectra between the quenched and activated state of eqFP630 resemble the spectrum of KFP found in literature [40] (figure 5.20).

After many cycles of activation and deactivation of KFP, an increasing fraction of permanently activated species can be observed [58, 11]. This effect can be seen better at higher powers and was for a long time without an explanation. A stabilizing hydrogen bond is formed between the chromophore and a water molecule (see figure 5.19). We believe that the water molecule can escape from the protein after many cycles of activation. This may create a permanently fluorescent KFP.

5.8.2 Precursor Species in eqFP630 Photoisomerization

The difference spectrum shown in figure 5.16 (B) gives a hint as to how the process of isomerization occurs. Just as it was assumed for KFP, the pKa of the chromophore is higher in the cis state than in the trans state [40]. The difference in the preferred protonation states for the two conformations results in the increased absorption at 450 nm. The absorption of the red protonated chromophore around this wavelength can also be seen from figure 5.15. Therefore, the protonated red form is a precursor species, which is necessary for isomerization. As seen from our titration experiments, by changing the pH, it is possible to achieve redistribution of the two forms even without illumination.

5.8.3 Maturation of the eqFP611 Chromophore

A plausible mechanism of dsRed maturation was proposed in 2001 (see section 1.2). In 2005, Tubbs et al. proposed an alternative mechanism, where maturation is catalyzed by cation-π interaction between Lys70 and the phenol ring of the chromophore [41]. In the case of dsRed, replacement of this amino acid by methionine produced a green fluorescent protein that was unable to mature to the red-emitting stage.

Replacement of the corresponding Lys67 in eqFP611 does not prevent maturation of the protein. Moreover, substitution of Met160, which is opposed to His197 with respect to the phenol ring of the chromophore, by lysine produced a green fluorescent protein from eqFP637. On the other hand, this residue is occupied by lysine in dsRed and does not affect maturation. All these facts together sum up to a picture where the maturation mechanism is similar to the one shown in section 1.2. The role of the helper residue, which keeps Glu212 in its anionic form, however, is now played by the His197 residue instead of Lys70 for dsRed. We believe that in the recently generated M160K mutant of eqFP637,
Figure 5.19: Stereo views of the eqFP611 (top) and KFP (bottom) chromophore pockets (cross-eyed viewing). The structure data: PDB entries 1UIS, 2A53 and 2A56.
Based on these observations we suggest that maturation from the green-emitting to the red-emitting chromophore can be induced in EosFP, whose chromophore environment is almost identical to the one of eqFP630 (PDB codes 1UIS vs. 1ZUX), by introducing the two mutations H62M and N65S.

### 5.8.4 Protonation State of Mature and Immature Chromophores

The pKa value of the green immature form is higher than for the mature form of eqFP630, as can be easily concluded from figure 5.15. A lower pKa of red compared to green chromophores is a general property [141]. The difference between the pKa values of the two chromophores can be translated into a change in free energy \( \Delta \Delta G \),

\[
\Delta G = \text{pKa} \cdot RT \ln(10),
\]

\[
\Delta \Delta G = \Delta \text{pKa} \cdot RT \ln(10).
\]

On the other hand, the difference in free energy can be obtained from the difference in the resonance energy, which is a measure of the additional chemical stability caused by electron density delocalization (see eg. [142]). Electron delocalization can be viewed in terms of Lewis (or resonance) structures. This picture can be especially useful, when comparing different types of red chromophores. As illustrated in figure 5.21, the red chromophore of eqFP611 has three resonance structures in the deprotonated state, whereas green chromophores (see eg. figure 1.4) generally have only two. The increased number of resonance structures indicates a higher degree of electron delocalization and, therefore, a higher stability of the red chromophore. The higher chemical stability of the eqFP611 red
5.8. Discussion and Conclusions

Figure 5.21: Resonant structures of the eqFP611 chromophore.

chromophore does not contradict the fact that hydrolysis is observed, since this process involves acylimine, which is absent in GFP.

5.8.5 Effects at Cryogenic Temperatures

Different temperature dependencies of spectral shapes of \( N \)-acetyl-L-tryptophanamide (NATA) embedded in glycerol and trehalose/sucrose (TS) matrices have been observed by Wright et al. [133]. They have shown that the absorption spectrum of NATA in glycerol/water environment is narrower than in TS at different hydration levels. The fluorescence spectrum of NATA in glycerol/water shifts towards higher wavenumbers as the temperature decreases, while it was less pronounced in the sugar glass, especially in drier preparations. The fluorescence spectrum turns out to be more blue-shifted in glycerol/water environment than in trehalose, the effect is again smaller in drier TS. The same authors studied tryptophan fluorescence in proteins as a function of temperature and found similar effects of the matrices. The fact that these effects are consistent with our data for eqFP611 dissolved in similar environments suggests the same origin of these phenomena. It is in general difficult to disentangle the dynamic behavior of the protein from the dynamics of the surrounding glass matrix, as protein conformational and vibrational dynamics is strongly coupled to (slaved by) a solvent [143]. Fenimore et al. suggest, that the process of slaving arises mostly from dipole interactions. On the other hand, Vanderkooi and coworkers have shown that the hydrogen bonds between the backbone amide groups and water strengthen with lowering the temperature, which results in slaving [144].

The mean frequency of 100 cm\(^{-1}\) observed in our experiments lies within the typical range of acoustic phonons (\( \approx 10 \text{ to } 100 \text{ cm}^{-1} \)) and compares well with the low-frequency (soft) modes of heme proteins (\( \langle \nu \rangle = 120 \text{ to } 180 \text{ cm}^{-1} \)) [145]. On the other hand the
\( \langle \nu \rangle \approx 100 \text{ cm}^{-1} \) effective phonon mode may arise (at least partially) from the bath of solvent low-frequency modes, which are observed as boson peak by Raman, neutron and x-ray scattering techniques. These modes couple well to the soft modes of the protein. Studies of \( s \)-tetrazine and its dimethyl derivatives in polar and non-polar media have shown that phonon-induced contributions to \( \Delta \tilde{\nu} \) are comparable for polar and non-polar solvents [92]. For glycerol, this contribution can be described by a phonon mode of 150 cm\(^{-1}\) at 120 K, while for water contributions from 50 cm\(^{-1}\) and 180 cm\(^{-1}\) (\( T = 8 \text{ to } 10 \text{ K} \)) can be found for aluminium phthalocyanate. The latter suggests that for a mixture of glycerol and water, the value of \( \approx 100 \text{ cm}^{-1} \) can be expected.

The coupling parameter \( S = 2.1 \) extracted from the temperature dependence of absorption \( \Delta \tilde{\nu} \) of eqFP611 is higher than the one obtained for dsRed (\( S = 1.25 \)) from hole-burning experiments [69] as well as the effective phonon frequency (\( \langle \nu_{\text{eqFP611}} \rangle = 100 \text{ cm}^{-1} \) against \( \langle \nu_{\text{dsRed}} \rangle = 50 \text{ cm}^{-1} \)). Unfortunately, we can not properly compare these numbers as the sample description is not given in the paper. The effective phonon frequency varies between 0.3-0.7 for low frequency modes of heme proteins [145] and equals 0.76 for the photosystem II reaction center of green plants [146]. The change in the temperature behavior of the absorption \( \Delta \tilde{\nu} \) can be attributed to an additional \( \alpha \)-relaxation process [86].

The origin of the excitation-emission asymmetry and particular of the broad and unstructured emission of eqFP611 at low temperatures remains unclear. Wright and coworkers [133] argued that the broad and unstructured emission of NATA at low temperatures is caused be relaxation of water around the chromophore in the excited state. The dipole moment of a molecule in the excited state is often higher than the dipole moment of the ground state [133, 85]. A higher dipole moment suggests a stronger coupling to the polar environment. In the case of eqFP611 we were able to describe the broadening of the emission spectrum within the harmonic approximation with the same effective phonon mode of 100 cm\(^{-1}\), but an almost 10 times higher coupling parameter than for the absorption spectra. To understand, whether the analysis is correct and the change in the dipole moment is responsible for the broad and unstructured emission of eqFP611 at low temperatures, or the increased chromophore mobility induces broadening, additional experiments should be performed. Among the RFPs bearing a chromophore similar to eqFP611, only the emission spectrum of dsRed was resolved at \( T = 1.6 \text{ K} \) [67], where it showed an additional high-frequency vibronic feature at 1250 cm\(^{-1}\) (from the maximum of emission). To verify the influence of the chromophore phenolate mobility, an alternative study of the protein in the immature state could be performed.

However, some speculations can already be made. Studies of the split variant of EGFP have shown that by destabilizing the structure, the maximum emission wavelength shifts to 524 nm from initial 507 nm position [147]. The vibronic structure is less resolved in this case. This reminds us of eqFP611, the structure of which is also not very stable as can be suggested from inefficient folding at elevated temperatures. In this case, however, we find different broadening for the mature (red) and immature (green) protein. The room-temperature spectra of immature eqFP611 show the vibronic sideband in the emission spectrum at room temperature (data not shown). In contrast to immature, for mature
5.8. Discussion and Conclusions

eqFP611, no vibronic structure of the emission spectrum can be resolved even at low temperature (figure 5.5). This difference can either be interpreted in terms of change of the vibronic structure caused by acylimine formation or by increased dipole moment of the red chromophore as compared to the green one. In the latter case, larger dipole moment may induce higher coupling to the surrounding solvent thereby producing broad red-shifted emission.

Studies of Wang et al. of mRFP1 derivatives show that the width of the spectrum increases with the red shift of emission, which is not surprising as the red shift arises mostly from the enlarged Stokes shift (up to 59 nm) [49]. Moreover, red-shift of the emission spectra did not always correlate with the shift of the excitation spectra for these proteins (e.g., excitation maximum of mRaspberry is red-shifted as compared to mPlum, however the latter shows further red-shifted emission). In our case, independence of the low-temperature emission spectrum of eqFP611 from the host matrices may indicate that an internal process, probably a vibronic mode, might be responsible for the broad spectrum at low temperatures. In the study of Wang et al., the red shift was caused by the mutations in close proximity or including the acylimine of the chromophore. Vibrational relaxation dynamics of this group in the excited state might be responsible for the broad unstructured emission spectrum of eqFP611 even at low temperatures.

The behavior of the spectral width of emission below and above $T_g$ temperature (figure 5.6 (A)) is not typical of fluorescent dyes. In fact, the $\Delta \tilde{\nu}$ of emission is expected to grow faster above the transition because of an additional relaxation process ($\alpha$-relaxation) observed in molecular glasses [86]. In our case, the slower increase of emission spectral width with temperature may be explained by a change in the solvation character (transition from the discrete-state to the continuous model [85] above $T_g$) or by some other general mechanisms of motional narrowing. Time-resolved spectroscopic studies should shed light on the origin of the unstructured emission band and its odd temperature behavior.

5.8.6 Photodynamics of Fluorescent Proteins

In our experiments, eqFP611 molecules dried on a bare glass surface show one order of magnitude lower bleaching yield as compared to that of the PEG-immobilized protein. We try to explain this keeping in mind that our the single-molecule method can overestimate the yield of photobleaching due to the limited ability to distinguish between a long-living non-emitting state and irreversible photobleaching (for proteins like PA-GFP dark state has lifetime of days [56]). Kinetics of the dark state may result in non-exponential photobleaching traces in bulk experiments, which is observed for many FPs. If we consider strongly suppressed dynamics of eqFP611 molecules on a bare glass surface, we can suggest that in the case of the dried eqFP611 the probability for the protein to enter a reversible non-radiative state maybe lower as compared to the protein in solution. Therefore, one may assume, that in the case of the dried protein on a glass surface we might observe a "pure" photobleaching, meaning the photochemical destruction of the chromophore, while in the case of eqFP611 in buffer, the apparent photobleaching rate is strongly influenced by dark states of the fluorescent protein.
CHAPTER 5. RED FLUORESCENT PROTEIN EQFP611
EosFP has been discovered in *Lobophyllia hemprichii* (figure 1.1 D) by Dr. Jörg Wiedenmann and co-workers. The distinctive feature of this green fluorescent protein is that it can be permanently converted to a red fluorescent protein by illumination with blue/UV light. In this chapter we will dwell on this process in more detail. We will also discuss the structural basis of the conversion process and applications.

### 6.1 Introduction: Overview of Structural Properties

#### 6.1.1 Quaternary Structure

wtEosFP is comprised of four identical subunits, similar to other anthozoan FPs (see figure 6.1). The A/B interface is mainly stabilized by hydrophobic interactions of Ile100, Ile102, Val123 and by π-stacking interaction of His121-His121 [148]. The A/C interface is stabilized by several hydrogen bonds (Thr158-Thr158 and Thr143-Thr143 interactions) and additionally by salt bridges Arg170-Asp156, Glu96-Arg149. Three hydrophobic residues (Tyr147, Tyr189, Phe191) reside in the interface.

It is remarkable that only two mutations are sufficient to break up both A/B and A/C interfaces. The A/B interactions can be effectively disrupted by introducing threonine instead of valine at the position 123. This substitution produced d1EosFP – a dimeric variant of EosFP. On the other hand, a single mutation, T158H, is able to take apart A and C subunits, revealing another dimeric variant, d2EosFP. The combination of these two mutations yields monomeric mEosFP. These alterations of the interfaces had almost no effect on the spectral properties of the protein (see table 6.1).

#### 6.1.2 Molecular Basis of Photoconversion

It was shown that a mechanism of photoconversion involves cleavage of the peptide backbone $N_\alpha - C_\alpha$ of His62 as shown in figure 6.2. As concluded from spectroscopic studies, photoconversion requires the chromophore to be in the protonated state (see section 6.2).
Figure 6.1: (A) Quaternary structure of wtEosFP. Here A, B, C and D denote individual subunits of the tetramer, the chromophore is highlighted in red. (B) Stereoview (cross-eyed) of the EosFP chromophore (shown green, except for His62, being a part of the red chromophore only) and its surrounding. The green dotted lines show hydrogen bonds, the arrow points to His62 Nα − Cα bond, which is cleaved during photoconversion. Thereby, the chromophore is extended to the imidazole ring of the His62. (C) The A/B and (D) the A/C interfaces of the wtEosFP tetramer; the chromophore is highlighted in green. The structure data: PDB entry 1ZUX.
6.2. Experimental Part: Spectroscopic Properties

The mechanism proposed by Nienhaus et al. [148] suggests that a $\beta$-elimination reaction is responsible for formation of the red chromophore. In order to explain why excitation of the protonated chromophore is necessary for the photoconversion reaction, the authors suggested a light-induced proton transfer from the phenolate oxygen to the nitrogen of the His62 (see figure 6.2). The positive charge of the imidazole ring pulls the electron density away from the C$_{\beta}$ of His62, making the C$_{\beta}$ more acidic. It is, therefore, easier for the Glu212 to abstract a proton from the His62 backbone, which after all triggers the $\beta$-elimination reaction. By termination of the backbone on this site, a double bond extending the $\pi$-system from the green chromophore to the imidazole ring of the His62 is formed. The chromophore conformation and its environment are not significantly perturbed during this process [148]. The substitution of the histidine by any other amino acid extinguishes the conversion property of the protein [32].

The fact that the yield of photoconversion decreases strongly at high pH, low temperatures and in D$_2$O environment, makes proton transfer a plausible component of the conversion process. The substitution of Glu212 by glutamine causes complete loss of the conversion [148, 149]. This underscores the ultimate role of this amino acid as a proton acceptor.

6.2.1 Determination of Conversion Rates

The wild-type EosFP absorbance as well as the excitation spectrum shows a maximum at 506 nm, which is shifted 10 nm away from the maximum of emission, peaking at 516 nm (see figure 6.3 (A)). Upon illumination of the protonated species ($\lambda_{max} \approx 395$ nm, see figure 6.3 (C)) does not excite fluorescence, but instead leads to a photoreaction, formation of a new type of red chromophore. Upon illumination within the conversion band (see action spectrum in figure 6.3) the green form irreversibly transforms into the red form. The maximum of absorption of the red form is at 571 nm; the emission peaks at 581 nm (figure 6.3 (B)).

Both forms show pH-dependent absorption and emission spectra. With decreasing pH, the intensity of absorption of the anionic chromophore decreases. At the same time, the intensity of the absorption band associated with the protonated chromophore increases in the blue part of the spectrum. The pH titration of absorbance and emission of both forms yields the same pKa value of 5.7 ± 0.1 (see figure 6.4) within the experimental error. This property of having the same pKa for a green and a red chromophore is not typical (see sections 5.8.4 and 6.8.1).

The EosFP P141A variant shows increased absorption at $\approx 390$ nm as compared to the wild type (figure 6.5), which is a sign for a higher pKa. Wiedenmann and co-workers have shown that the increase is associated with higher conversion yield for this protein [?].
Figure 6.2: Photoinduced formation of the red EosFP chromophore. The initial deprotonated chromophore is presented here in the quinonoidal resonant form, see section 1.1 for details.
6.2. Experimental Part: Spectroscopic Properties

Figure 6.3: Absorption (solid lines), emission (dotted lines) and excitation (dashed lines) spectra of green (A, C) and red (B, D) forms of EosFP are shown for pH 7.0 and pH 5.5 for comparison. Emission (excitation) spectra of the green and red form were recorded at $\lambda_{\text{ex}}^{\text{green}} = 490$ nm ($\lambda_{\text{em}}^{\text{green}} = 520$) and $\lambda_{\text{ex}}^{\text{red}} = 560$ nm ($\lambda_{\text{ex}}^{\text{red}} = 590$), respectively. The blue dots represent the action spectrum for conversion scaled to the absorbance.

portional to the absorption, we performed additional measurements at various pH values.

The experimental arrangement for photoconversion, which was based on a fluorimeter and an additional external lamp, did not allow us to achieve reproducible measurements and to observe both forms at the same time. Therefore, we performed experiments on a dense layer of EosFP immobilized on a BSA-coated surface (see section 4.3 for details). We used our confocal setup to simultaneously excite and convert the immobilized protein with 488 nm and 400 nm while recording time traces in the green and the red channels (see section 4.3) at different pH. An example time trajectory at pH 7.0 is shown in figure 6.6. The set of kinetic data at different pH was analyzed and represented in figure 6.4 in terms of a pH-dependent conversion yield. In order to describe the conversion kinetics correctly, we subtracted the intensity signal in the green channel multiplied by the cross-talk ratio from the signal in the red channel. After this step, a bi-exponential fit was applied to the data. A sum of rising and falling exponentials determines, respectively, conversion and bleaching rates of the sample.
Figure 6.4: Normalized pH dependencies of photoconversion yield, absorbance and emission. Green species: absorbance at 506 nm (filled green circles), absorbance at 390 nm (blue diamonds), fluorescence at 506 nm (open green circles), green-to-red conversion yield (blue stars); red species: absorbance at 572 nm (filled red squares), absorbance at 581 nm (open red squares). Within the experimental error, all data follow the Henderson-Hasselbalch relation with pK = 5.7 ± 0.1 (solid lines).

Figure 6.5: Comparison of EosFP P141A (black) and wtEosFP (grey) absorption spectra.
6.2. Experimental Part: Spectroscopic Properties

Figure 6.6: Kinetic record of EosFP conversion. The falling (green) and rising (red) signals are anticorrelated and represent the depletion of the green state and population of the red state with time. The black line represents a bi-exponential fit to the data.

6.2.2 EosFP at Low and High pH

The green form of the chromophore is chemically stable in the range of pH 3-11, whereas the red form is only stable in range of pH 4.5-11. The instability of the red form may arise from the peptide bond break, which accompanies photoconversion (see section 6.1.2 for details). In general, the red form is much less stable at acidic than at basic conditions. Thanks to this property, EosFP can serve as an intracellular pH indicator.

With lowering pH, the fluorescence intensity decreases significantly. Below pH 4.0 the spectral fine structure is lost (figure 6.7). This irreversible broadening of the emission spectrum is observed only for the red form. The broadening of the spectra may be a sign of an increased chromophore mobility within of the chromophore pocket due to destabilization of the chromophore pocket at low pH. When compared to the green form, the red form is destabilized by the backbone cleavage, which occurs during the photoconversion process. Another feature of the emission spectrum, which is best visible at pH 4.5, is an additional peak at $\approx 545$ nm. This "yellow" form should result from a shortened $\pi$-conjugated system of the chromophore.

Unlike dsRed, the red chromophore of EosFP is not hydrolyzed at high pH, whereas the dsRed-like chromophore of eqFP630 follows a typical hydrolysis pathway (see introductory figure 1.3 (B)). The high-pH absorption spectra of the green and the red forms of EosFP are presented in figure 6.8. Therefore, by observing the alkaline denaturation, one can distinguish between the different types of red chromophores in almost any particular case.
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Figure 6.7: A set of emission spectra of the red form of EosFP is shown for pH 6.5 - 3.5. All spectra are normalized to unit area to compensate for changes in absolute intensity of fluorescence. With decreasing pH, the red species decays faster than the green one (the non-converted green species is always present in a sample and is visible here as the small band at 516 nm at the highest pH).

Figure 6.8: Absorbance spectra of the green and the red forms of EosFP in 1M NaOH solution. The spectrum of eqFP630 is shown for comparison. Unlike the red form of EosFP, hydrolysis of the red chromophore of eqFP630 removes the double-bond extension.
6.2. Experimental Part: Spectroscopic Properties

6.2.3 Fluorescence Lifetime of Green and Red EosFP

The results of lifetime analysis of different EosFP variants are summarized in table 6.1. The TCSPC histograms show single-exponential decays. The green form was excited with 404-nm pulses from a frequency-doubled Ti:Sapphire laser, whereas for excitation of the red form, a 532-nm frequency-doubled mode-locked neodymium-doped yttrium vanadate laser was used. We found similar fluorescence lifetimes among the different variants of EosFP. The photoconverted form in general feature extended fluorescence lifetimes. Similarity in fluorescence lifetime and fluorescence quantum yield indicates that monomerization does not influence the structure of the chromophore pocket significantly.

6.2.4 Excitation and Emission Spectra of wtEosFP at Low Temperatures

Figure 6.10 shows fluorescence spectra at 10 K. Upon cooling, the excitation spectra of the green and the red forms shift to the blue by $197 \pm 1$ cm$^{-1}$ and $123 \pm 1$ cm$^{-1}$ (506 → 501 nm and 571 → 567 nm), respectively. The corresponding emission spectra shift by $267 \pm 1$ cm$^{-1}$ and $240 \pm 1$ cm$^{-1}$ (516 → 509 nm and 581 → 573 nm). All spectra narrow markedly towards lower temperatures, revealing prominent vibronic sidebands with average phonon frequencies $\approx (1400 \pm 100)$ cm$^{-1}$ for both the red and the green species. A further peak in the red excitation spectrum at 502 nm probably contains higher-order vibrational excitation. The strong vibronic coupling of this chromophore could be caused by the extension of the conjugated $\pi$-electron system to the imidazole ring of His62 instead of the typical conjugation to the carbonyl oxygen of the backbone seen in other RFPs.
Figure 6.10: Excitation (solid line) and emission (dotted line) spectra of wtEosFP at room (A) and low temperature (10 K). In panel (B), the emission of the red form is excited via FRET by the green form. Green excitation (emission) spectra were recorded with emission (excitation) set to 520 (490) nm; red excitation (emission) spectra were recorded with emission (excitation) set to 590 (560) nm.
6.2.5 Spectroscopic Properties of a Non-Convertible EosFP Variant

Substitution of His62 by any other amino acid causes complete loss of photoconversion ability. We studied one of this mutants, namely, the H62M variant. The maximum of absorption and excitation resides at 504 nm, which is 2 nm blue-shifted from the wild type. The emission spectrum peaks at 515 nm (see figure 6.11). Titration of this protein at different pH yields the same pKa of the chromophore, 5.7 ± 0.1, as for wtEosFP within the experimental error (figure 6.12).

6.3 Nonlinear Properties of wtEosFP

6.3.1 Two-Photon Excitation of the Green Form of wtEosFP

We explored the applicability of EosFP as a marker for two-photon microscopy. The one-photon action spectrum of wtEosFP suggests that the wavelength region around 800 nm is preferable for two-photon conversion. At the same time, we could analyze the two-photon excitability at the same wavelength. When exciting at 808 nm, wtEosFP as well as the reference sample (rhodamine 6G in methanol) show a quadratic dependence of emission intensity on excitation power (see figure 6.13), which proves the two-photon character of the excitation process.

Analysis of the slopes yields a two-photon excitation cross-section of $\delta_{808} = 0.6 \pm 0.3$ GM for wtEosFP (see eq. 3.1). We also analyzed mEosFP by the same method and obtained a value of $\delta_{808}^{mEosFP} = 0.2 \pm 0.1$ GM. The lower value might be explained by
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Figure 6.12: Absorption of EosFP H62M in the pH range 3.0-9.0. The dotted line shows the Henderson-Hasselbalch fit to the data. Absorption data of wtEosFP are shown for comparison.

Figure 6.13: Dependence of the two-photon excited fluorescence on the excitation power for wtEosFP in buffer and R6G in water and methanol ($\lambda_{ex} = 808$ nm).
a higher bleaching rate of mEosFP as compared to wtEosFP. All of the dependencies depicted in figure 6.13 show that, at higher powers, the fluorescence intensity saturates. This effect is most likely associated with the effect of three-photon bleaching known for other dyes [99, 100].

To excite EosFP more effectively, longer wavelengths are preferable. We have performed determination of the two-photon excitation cross-section \( \delta(\lambda) \) in the wavelength range 800-1050 nm. In these experiments linearly polarized excitation was used. The acquired data sets in the ranges 800-920 nm, 900-1010 nm and 1000-1050 nm were normalized to the value of the two-photon cross-section measured at 920 nm. At this wavelength, our reference solution of fluorescein (100 nM) at pH 11 has a two-photon cross-section of 28±7 GM [150], which yields \( \delta_{920}^{wtEosFP} = 2.0 \pm 0.5 \) GM for wtEosFP (1 \( \mu \)M), when directly compared. The obtained spectrum is shown in figure 6.14. Since saturation effects are more pronounced for higher excitabilities, we limited the excitation power by setting a maximum threshold on the detected fluorescence intensity. For every point, a minimum set of four points at different powers was taken to verify the two-photon character of excitation (see figure 6.15). The slopes taken from the linear fit of these dependencies at different wavelengths were used to obtain individual excitabilities (eq. 3.1). The overlapping points from different data sets are averaged. The larger relative error of the longer-wavelength data sets is caused by a narrower spectral width \( \Delta \lambda \) as compared to the shorter wavelengths, which could probably indicate the presence of water vapor being not completely removed from the laser cavity. The corrections of the power density, variation in the focal waist due to variation in laser pulse width and the diffraction-limited volume must be considered. We measured the length of the laser pulses by using either an autocorrelator or a spectrometer. The latter gave more reproducible and plausible results. The power correction procedure can be found in the methods section 4.3.7.

The difference between the shapes of one- and two-photon excitation spectra is demonstrated in figure 6.14. The comparison of the one-photon and two-photon spectral shapes shows different vibronic structure. One can also notice that the maximum of the TPE spectrum is blue shifted and resides at 1000 ± 5 nm, while direct comparison to OPE predicts 1012 nm to be the excitation maximum (see figure 6.14). At the maximum, wtEosFP shows a reasonably high two-photon excitability of 19 ± 5 GM, which compares well with the values found for other GFPs [116].

We also compared the two-photon excitability of rhodamine 6G diluted in water against the one dissolved in methanol. The measured value of \( \delta_{808}^{R6G_{water}} = 11 \pm 3 \) GM shows that water environment provides lower values for two-photon excitation cross-section of R6G, as compared to methanol (40 GM [151]).

### 6.3.2 Two-Photon Conversion of wtEosFP

Given that the TPE spectrum of wtEosFP resembles the OPE spectrum at twice the wavelength, one should expect two-photon conversion to take place after irradiation at around 800 nm.

Two-photon conversion requires careful control over the IR power. Too weak illumination causes no conversion, while illumination at high powers causes strong bleaching. To
Figure 6.14: Solid line: two-photon excitation spectrum of the green form of wtEosFP; dotted line: one-photon excitation spectrum of wtEosFP, with wavelengths doubled for comparison.

Figure 6.15: wtEosFP emission versus squared excitation intensity at two different wavelengths: 920 nm and 1000 nm. The power corrections are applied (see 4.3.7).
6.3. Nonlinear Properties of wtEosFP

Figure 6.16: Lines drawn by Ti:Sapphire laser on a wtEosFP-coated surface represent production of the red form as a result of two-photon conversion (upper panels). The corresponding vertically averaged profiles are shown in the lower panels. (A) Two-photon conversion of the green form of wtEosFP at average powers ranging from 1 mW (leftmost) to 7.5 mW (rightmost) at 800 nm. (B) Two-photon conversion of wtEosFP in the wavelength range 800-880 nm done in 10 nm steps (from left to right).

Figure 6.17: (A) Two-photon conversion spectrum of the green form of wtEosFP, (B) production of the red form versus power at $\lambda_{ex} =$800 nm.
set the low and the high power limits, we converted the immobilized protein at powers ranging from 1.0-7.5 mW (see figure 6.16 (A)). We noticed that, at powers above 30 mW, the converted red form can not be observed because of strong bleaching. In a set of lines shown in the figure, each line is drawn with the focused beam of the Ti:Sapphire laser. The length of each line is $\approx 30 \text{ } \mu \text{m}$, the scanning velocity was 2.3 $\mu \text{m/s}$. For the analysis, the averaged line profiles were fitted with a sum of gaussian functions having the same width and a fixed relative position. The gaussian amplitudes are plotted in figure 6.17 (B) as a function of the squared power. An exponential fit $A(P) = A_0(1 - \exp(-P^2/P_0^2))$ yields $P_0 = 3.7 \text{ mW}$.

We performed two-photon conversion of wtEosFP immobilized on a BSA coated surface at different conversion wavelengths ranging from 800 - 880 nm in 10 nm steps. In a set of lines shown in figure 6.16 (B), each line is drawn with the focused beam of the Ti:Sapphire laser (scan speed 4.7 $\mu \text{m/s}$) and corresponds to an individual excitation wavelength. Special care was taken to keep the laser pulse duration $\Delta \tau$ constant throughout the wavelength range. Additionally, due to the wavelength-dependent expansion of the focal waist, we compensated for loss in power density by increasing the excitation intensity. In our experiments with the immobilized (2D) samples, we corrected only for radial expansion of the illuminated volume $S \sim \lambda^2$ because of the plain geometry of the sample. These correction resulted in an increase in average power power from 7 mW at 800 nm to 8.5 mW at 880 nm. After correction for cross-talk (17%) and integration of the individual lines, we obtained the two-photon conversion spectrum (see figure 6.17 (A)).

### 6.3.3 Two-Photon Excitabilities of the Red Form of wtEosFP and eqFP611

According to the TPE spectrum of wtEosFP, the protein is practically non-excitable at wavelengths longer than 1050 nm. Therefore, we can selectively excite the red form with a common picosecond neodymium laser emitting at 1064 nm.

We compared the two-photon excitation cross-sections of two fluorescent proteins, the red form of wtEosFP and eqFP611. As a reference, we used a rhodamine 6G solution (87 $\mu \text{M}$ in methanol). The two-photon excitation cross-section of our reference sample according to Bradley et al. is 3.6 GM when excited at 1064 nm. The measured dependencies of the emission intensity on the excitation power confirm the two-photon character of the excitation process (see figure 6.18). The analysis of the slopes yields the values for TPE cross-sections after correction for change in concentration, detection losses and fluorescence quantum yield. The concentrations of eqFP611 and the red form of wtEosFP were estimated from absorption measurements as 32 $\mu \text{M}$ and 83 $\mu \text{M}$, respectively. The quantum yields for rhodamine 6G, wtEosFP red and eqFP611 are 0.88, 0.55 and 0.45, respectively. Detection losses for the individual fluorophores were determined based on filter spectral properties. Finally, the values extracted from the slopes for eqFP611 and the red form of EosFP are $4.2 \pm 0.3$ and $5.0 \pm 0.4$ GM, respectively.

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6.4. Experiments on Individual EosFP Molecules

6.4.1 Single-Molecule Photoconversion

To explore the switching dynamics of individual EosFP molecules and, therefore, to avoid ensemble averaging, we immobilized the biotinylated protein on a BSA-coated surface via streptavidin linkage.

Figure 6.19 (A) shows wtEosFP molecules excited at 488 nm only as green spots, which have gaussian intensity distributions, with standard deviation $\sigma = 0.16 \mu m$, as derived from the analysis of 21 molecules. In panel (B), the 488-nm excitation is superimposed with the 404 nm conversion light. As seen from comparing both, illumination with 488 nm alone does not cause conversion, whereas the green spots turn red with additional 404 nm conversion light. Although irreversibility of the photoconversion is known from bulk experiments, scanning over the red spots makes some of them suddenly turn green again. By taking a closer look at intensity fluctuations from individual spots under both 488 and 404-nm illumination (D), an alternating green and red signal sequence can be detected.

Another observation, four-step bleaching of an individual spot (figure 6.19 (C)), suggests that the tetrameric structure of wtEosFP is preserved even on the single-molecule level. The fact that this red-to-green switching behavior is never observed for the monomeric mEosFP variant (figure 6.19 (E)) suggests FRET to be the origin of this effect. The consecutive conversion of a single subunit within the tetramer creates a FRET acceptor. Since the stability of the acceptor is usually lower under intense illumination, it bleaches
Figure 6.19: Single-molecule conversion of EosFP on a BSA-coated surface: (A) Confocal scan images were taken with 488-nm excitation only and (B) additional 400-nm irradiation. (C) Fluorescence emission traces from an individual EosFP tetramer (488-nm excitation) showing sequential bleaching of all four subunits. (D) An individual EosFP tetramer (488-nm excitation plus 400-nm irradiation) showing green-to-red switching and sequential bleaching under 488-nm and 400-nm combined irradiation. (E) mEosFP (488-nm excitation plus 400-nm irradiation) showing single-step green-to-red conversion and bleaching. The picture is adopted from [10].

first, which results in appearance of the green emission again, which is then followed by another cycle of conversion and bleaching.

6.4.2 Photostability of Individual EosFP Molecules

We acquired statistics on the number of emitted photons from individual EosFP molecules immobilized on a BSA-coated surface. To this end, we recorded sets of individual emission time traces for each of the EosFP variants: d1EosFP, d2EosFP and mEosFP. The number of recorded traces was 188, 277 and 545, respectively. Single step photobleaching of the individual d1EosFP and d2EosFP molecules immobilized on a BSA-coated surface suggests that they dissociate into monomers at the single-molecule level (figure 6.20). We determined the average number of emitted photons from individual EosFP molecules under 488-nm excitation before photodestruction occurred. d1EosFP, d2EosFP and mEosFP emit on average 31,000 ± 2,000, 42,000 ± 3,000 and 33,000 ± 1,000 photons (see photon histograms in figure 6.21). The calculated yields of photobleaching $\Phi^\text{green}_B$ are summarized in section 6.7. One should notice, however, that it is in general very difficult to distinguish between long-living dark states and irreversible photodestruction of the fluorophore.
6.4. Experiments on Individual EosFP Molecules

Figure 6.20: Intensity trace from an immobilized d1EosFP molecule. On-off blinking of the molecule proves its monomeric state.

Figure 6.21: Histograms of the detected photons from individual molecules of (A) d1EosFP, (B) d2EosFP and (C) mEosFP.
Chapter 6. Photoconvertible Fluorescent Protein EosFP

Figure 6.22: Fluorescence decay kinetics from a dense layer of immobilized wtEosFP (solid line) and d2EosFP (dotted line) upon 488-nm excitation.

Since determination of the bleaching yield on the single-molecule level is problematic for a tetrameric protein, we recorded the bleaching decay of wtEosFP and d2EosFP, both densely immobilized on a BSA-coated surface, upon 488-nm excitation. This allowed us to study bulk properties and perform multiple experiments in a short amount of time. The decay kinetics is presented in figure 6.22. We fitted each decay with two exponentials. Both exponential components for wtEosFP were slower by a factor of 2.3 from the corresponding components of d2EosFP. By dividing the bleaching yield of d2EosFP, found in our single molecule experiments, by 2.3 we obtain $\Phi_{\text{green}} = (1.0 \pm 0.1) \cdot 10^{-5}$ for the wild type protein. The bleaching yield is, therefore, lower for the protein in the tetrameric state. As a consequence, one should notice that $\Phi_{\text{green}}$ for FPs in the dimeric state may differ from the obtained values, as in our single-molecule experiments dimers were dissociated into monomers.

6.5 Applications of EosFP

As many other FPs, EosFP can be used as an intracellular fluorescence marker. Moreover, thanks to its photactivation properties, this FP can be used in local optical marking. It is possible to optically highlight subcellular structures and follow their dynamic evolution. Possible applications include studies of embryo development, intracellular trafficking and protein-protein interaction.

6.5.1 Application of EosFP for Optical Labeling

To test EosFP as a fusion marker, we performed in vivo optical labeling of EosFP constructs. In figure 6.23 (A), active transport of recombination signal-binding protein (RBP2N–d2EosFP) is visualized by local conversion of a part of a nucleus of a HEK293 cell. This
6.5. Applications of EosFP

Figure 6.23: Local photoconversion of EosFP fusions in cells: (A) Tracking of movement of the fusion protein RBP2N-d2EosFP in the nucleus by localized photoconversion, observed after 0.5-s irradiating with 400-nm light (1 μW) in our confocal microscope. (B) HeLa cells expressing dimeric d2EosFP (Upper) and monomeric mEosFP (Lower) fused to MLS. (C) HEK293 cell expressing cytokeratin 18-d2EosFP. (Upper) One-photon excitation at 488 nm. (Lower) Two-photon excitation by 808-nm Ti:sapphire laser pulses. The picture is adopted from [10].

protein is a splice variant of the DNA-binding transcriptional repressor RBP-Jκ, it binds specifically the nucleotide sequence 5’ GTGGGAAA 3’ [152]. After local illumination of the EosFP fusion construct with 400 nm light, the illuminated region appears as a red spot. This optically labeled fraction of RBP2N−d2EosFP can be monitored for several minutes after photoactivation until it distributes over the nucleus.

Tracking of single mitochondria within the mitochondrial network, observation of their fusion and fission events can easily be visualized by converting the mitochondrial targeting sequence from subunit VIII of cytochrome c oxidase (mitochondrial localization signal, MLS) fused to d2EosFP (figure 6.23 (B) Upper) or mEosFP (Lower), which yields selective staining of mitochondria. Mitochondrial dynamics is better presented in figure 6.24, where the two converted areas (shown in circles) of the mitochondrial network undergo fusion and fission transformations. When mitochondrial fusion occurs, the converted EosFP flows from the labelled mitochondrion to the other one, thereby highlighting the fusion event by a sudden color change. This experiment shows an advantage of EosFP as a photoconvertible protein, since such studies can be challenging without using a photoactivable probe.

6.5.2 EosFP to Probe Protein-Protein Interactions via FRET

Fluorescence resonance energy transfer between the green and the red form of EosFP can be utilized to probe protein-protein interactions [149]. In order to test the protein applicability, several constructs were generated by Michael Wolff. HEK293 cells were
Figure 6.24: Tracking of movement of individual mitochondria labeled with a MLS-d2EosFP fusion construct in a HeLa cell by localized photoconversion (converted areas are shown in circles), observed after 0.5-sec irradiating with 400-nm light (6 µW) in our confocal microscope. Frequent fusion and fission of mitochondria are observed in the image series. It shows the HeLa cell viewed in the green channel (Upper), the red channel (Middle) and the overlay of the two (Lower). The picture is adopted from [153].
transfected with d2EosFP dimer, the two subunits of which were fused together into one tandem protein (tEosFP). A corresponding nucleotide sequence for a tandem-linking peptide GHGTGSTGSGSS was additionally subcloned into the cDNA.

As a reference construct, where no FRET can occur, we used the same tandem construct, where either the first (t1EosFP) or the second (t2EosFP) chromophore was knocked out by a Tyr63Gly substitution.

The following procedure was used in order to verify FRET between the tandem subunits. First, the cells expressing the EosFP-tandem construct were imaged under 488-nm excitation from an Ar$^+$ ion laser. Most of the emission coming from the non-converted green protein was detected in the green channel. Emission of the red form was detected only in the red channel. A part of the emission from the green form is also detected in the red channel due to channel cross-talk (see section 4.3.9). We observed the emission of the non-converted construct in both spectral channels (see 4.2.3) to estimate the amount of the cross-talk for future correction. The cross-talk was determined to be $\approx 17\%$. Next, the individual cells were partially converted by scanning with a 404-nm diode laser. The cells were once again imaged under 488-nm excitation and then under 532-nm light to directly excite the red form of EosFP.

Unfortunately, it is not trivial to estimate FRET efficiencies from the intensities in green and red channels. However, an alternative approach of FRET indices [154] can help distinguishing cells with different FRET levels without calculating the FRET efficiency.

We define our FRET index $\varsigma$ as a ratio of the intensity in the red channel due to FRET $I_{\text{FRET}}^{488}$ and direct excitation of the red form $I_{532}^{R}$:

$$\varsigma = \frac{I_{\text{FRET}}^{488}}{I_{532}^{R}} \cdot C,$$

where $C$ is a correction factor necessary for comparison of measurements performed at different excitation powers. The direct relation of $\varsigma$ to the measured values is derived in the methods section 4.3.9 and presented in equation 4.6. This index should be zero if no FRET occurs and exceed zero if FRET is present. Of course, due to systematic errors, the index never equals zero, but the index value is expected to grow as either FRET efficiency or the fraction of FRET pairs increases. This implies that, in order to obtain comparable values, the same fraction of FRET pairs is needed and, therefore, the conversion power should be constant.

This analysis shows that $\varsigma$ has 5-10 times higher values for the tandems with both intact chromophores as compared to tandems without one of the chromophores. The deviations between the different sets (days) of measurement are caused by the different UV powers used.

We applied this method to studies of testosterone-induced dimerization of the androgen receptors (AR), whose nuclear translocation can be observed upon hormone stimulation within one hour. To this end, the fusion constructs of the androgen receptor and t1EosFP, t2EosFP were prepared. We were able to observe the translocation of the AR at temperatures around 37°C (figure 6.25). Unfortunately, we could not detect any changes of our FRET indices upon hormone stimulation. We also bleached red-converted form by
Figure 6.25: Confocal scanning image of a HEK 293 cell expressing AR-t1EosFP construct before (A) and after (B) hormone stimulation. (C) shows a lifetime-colored image of a HEK 293 cell after the stimulation. Red color corresponds to longer fluorescence lifetimes, while green is attributed to moderate (≈ 3 ns) and blue to shorter lifetimes.

532-nm irradiation, but no enhancement of the donor emission was observed. Although the FRET index method is technically very simple, it has two major drawbacks. First, the method operates with too many parameters, which altogether introduce a large error. Second, this method does not give values of the FRET efficiency and the fraction of the molecules exhibiting energy transfer.

FRET estimation based on analysis of the fluorescence lifetime is free of these flaws. Only one (donor) spectral channel is observed in this case. The efficiency of fluorescence resonance energy transfer $E$ can be calculated from the exponential decay of the donor fluorescence: $E = 1 - \tau'/\tau$, where $\tau'$ and $\tau$ are the fluorescence lifetimes in the presence and absence of an acceptor. The single-exponential character of the fluorescence decay of the marker is crucial, making the commonly used CFP/YFP FRET pair not applicable for this method (the same is true for the Cy3/Cy5 dye pair). The decays of the both spectral forms of EosFP are monoexponential, making this protein beneficial for fluorescence lifetime-based applications. The lifetime-colored image is shown in figure 6.25 (C). Blue clusters observed in the cell could either be aggregates of the fluorescent proteins or autofluorescent lipofuscins, which most frequently stain for agglomerates of intracellular junk [155]. In spite of the potential advantages we could not detect any changes in FRET efficiency even when a fusion construct AR-mEosFP(A69T) was used. The mEosFP A69T variant is a recently derived monomeric thermostable variant of EosFP. Two alternative ways for ARs to form a dimer were discussed in literature. These are head-to-tail or head-to-head orientations. The former would explain why no change in FRET can be found upon dimerization as in this case the distance between donor and acceptor is greater. Haelens et al. [156] predicted the
6.5. Applications of EosFP

androgen receptors to dimerize in head-to-tail configuration. Crystallographic studies of Shaffer and coworkers [157] found, however, that DNA-binding domains form a homodimer in head-to-head configuration.

6.5.3 Estimation of EosFP Concentration in HEK293 Cells

The protein oligomerization state is always dependent on the protein concentration. Therefore, the oligomerization state of a protein should be related to expression levels. We determined the expression levels of EosFP P141A in stably transfected HEK293 cells either by measuring absorption of cellular lysates or by measurements of fluorescence intensity from individual cells using a confocal microscope.

In the former method $8 \times 10^6$ cells were lysed and centrifuged; the lysate was measured on an absorption spectrometer. The cellular dimensions were estimated on a light microscope for the cells in suspension. In this state, the cell shape could be well approximated by sphere of 8.25 $\mu$m diameter. After subtraction of a scattering-induced baseline (an inverse polynomial), the measured absorption spectrum in figure 6.26 was analyzed in terms of the EosFP concentration. The absorption spectrum contains three major bands. The bands around 415 and 550 nm correspond to porphyrin absorption, while the peak at 506 nm is related to EosFP. Absorption yielded an intracellular concentration of 6 $\mu$M.

In the fluorescence-based method, we related intensity of an EosFP solution with known concentration (1 $\mu$M) to the intensity within individual HEK 293 cells. The method gave an estimation of 1.5 $\mu$M for the intracellular concentration. Therefore, the typical concentration of fluorescent proteins in stably transfected cells can be expected to be on the order of 1-10 $\mu$M.

Figure 6.26: Absorption spectrum of a lysate from HEK293 cells stably transfected with EosFP P141A.
Apart from EosFP and Kaede, which all have very similar spectroscopic properties and chromophore structures, there are some of the members of the family, which have their own distinctive features. The fluorescent protein lhOFP, isolated from Lobophyllia hemprichii, is a green FP with absorption and fluorescence maxima peaked at 506 nm and 517 nm respectively (see figure 6.27). Upon irradiation at around 400 nm, the protein solution in PBS turns orange. The orange form of the protein features unexpectedly broad absorption and excitation spectra peaked at 541 nm, while the emission spectrum is relatively narrow and has a maximum at 575 nm. The orange color of the photoprodut gave the protein its name—orange fluorescent protein (lhOFP). Although the spectral properties of the orange form of lhOFP and the red form of EosFP look different at neutral pH, the comparison of absorption spectra of the alkali denatured proteins suggests that the chromophores of the photoconverted proteins have an identical chemical structure. To define the origin of the orange form, an additional analysis is required.

The protein cmFP506 found deep in the sea in Cerianthus membranaceus is able of "reversible photobleaching". It has the maxima of excitation and emission at 493 nm and 506 nm respectively (figure 6.28 (A)). Upon excitation at the absorption maximum,
6.6. Other Photoactivable Fluorescent Proteins

Figure 6.28: (A) Excitation (solid) and emission (dotted) spectra of cmFP506; (B) fluorescence decay under 488-nm excitation followed by a minute in dark (axis brake). During this time this FP gains back its fluorescence activity, which is illustrated as another fluorescence decay on the same plot.

The protein enters a non-radiative state, from which it can thermally relax back to the emitting state in the absence of illumination. Such quenching-activation kinetics is very similar to Dronpa, which suggests a Dronpa-like protonation mechanism to be the origin of the off-state.
6.7 Spectral Properties of EosFP Variants

In this section we summarized properties of EosFP variants, which were studied in this work. The studied variants are the tetrameric wild type EosFP, dimeric variants d1EosFP (V123T) and d2EosFP (T158H), the monomeric mEosFP (V123T/T158H), and tetrameric EosFP P141A, which features higher photoconversion cross-section.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\lambda_{\text{em/ex}}^\text{max}$, nm</th>
<th>$\varepsilon$, cm$^{-1}$M$^{-1}$</th>
<th>$\Phi_F$</th>
<th>$\tau$, ns</th>
<th>$\Phi^B \times 10^{-5}$</th>
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</thead>
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<tr>
<td>wtEosFP green</td>
<td>506/516</td>
<td>72,000</td>
<td>0.7 ± 0.02</td>
<td>2.9 ± 0.1</td>
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<td>74,000</td>
<td>0.68 ± 0.03</td>
<td>3.1 ± 0.1</td>
<td>3.2 ± 0.2</td>
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<tr>
<td>d2EosFP green</td>
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<td>84,000</td>
<td>0.66 ± 0.03</td>
<td>3.1 ± 0.1</td>
<td>2.4 ± 0.2</td>
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<tr>
<td>mEosFP green</td>
<td>505/516</td>
<td>76,200</td>
<td>0.64 ± 0.03</td>
<td>2.6 ± 0.1</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>EosFP P141A green</td>
<td>506/516</td>
<td>53,400</td>
<td>0.72 ± 0.03</td>
<td>2.9 ± 0.1</td>
<td>-</td>
</tr>
<tr>
<td>wtEosFP red</td>
<td>571/581</td>
<td>41,000</td>
<td>0.55 ± 0.03</td>
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<td>-</td>
</tr>
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<td>d1EosFP red</td>
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<td>40,000</td>
<td>0.62 ± 0.03</td>
<td>3.6 ± 0.1</td>
<td>-</td>
</tr>
<tr>
<td>d2EosFP red</td>
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<td>0.60 ± 0.03</td>
<td>3.1 ± 0.1</td>
<td>-</td>
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<tr>
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<td>0.62 ± 0.03</td>
<td>3.9 ± 0.2</td>
<td>-</td>
</tr>
<tr>
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<td>571/581</td>
<td>-</td>
<td>0.60 ± 0.03</td>
<td>3.5 ± 0.1</td>
<td>-</td>
</tr>
</tbody>
</table>

6.8 Discussion

6.8.1 Protonation State of the EosFP Chromophore

From our titration experiments, the pKa values of the green and red EosFP chromophores are the same within the experimental error. In order to understand this untypical phenomenon we first compare the chemical structures of eqFP611 and the red form of wtEosFP. The comparison shows, that in the case of eqFP611 three resonant structures of equal stability are present, whereas in the case of the red EosFP chromophore, one of the resonant structures is replaced by a less stable one. The lower stability of this structure is based on lower electronegativity of the nitrogen atom in the imidazole ring of His62. As already discussed in section 5.8.4, lower electron delocalization results in higher pKa. Therefore, the pKa of the eqFP611 chromophore is expected to be lower than the pKa value of the red EosFP chromophore.

On the other hand, higher electron delocalization within the red chromophore of EosFP still suggests lower pKa for the red form as compared to the green one. However, if we take into consideration the chromophore environment, we see that a negative charge of Glu212, residing near the His62, makes it improbable for the negative charge to appear on the imidazole ring, thereby destabilizing the corresponding Lewis structure of the red chromophore. Moreover, proximity of the positively charged residues Arg66, Arg91 and His194 to the rest of the chromophore (see figure 6.1) makes the contribution of the last resonant form practically non-observable.
6.8. Discussion

The above discussion can also explain the unexpectedly short maximum emission wavelength of the red form of EosFP. The influence of Glu212 on the emission spectrum of the red form can not be easily verified by substitutions as this amino acid is essential for the photoconversion process [149]. Nevertheless, the negative charge of Glu212 probably contributes to the reduced electron delocalization as compared to dsRed or KFP. The red form of EosFP potentially has a longer conjugated \( \pi \)-electron system, but the negative charge of Glu212 probably reduces the electron delocalization, thereby shifting the emission and absorption spectra to shorter wavelengths.

6.8.2 Vibronic Structure of EosFP Two-Photon Excitation Spectrum

The blue shift of two-photon excitation is typical of many fluorophores [151] and most of the measured TPE spectra of fluorescent proteins confirm this phenomenon [116]. It is known that the TPE spectrum of tyrosine is 2000 cm\(^{-1}\) blue-shifted as compared to the OPE spectrum, and the TPE spectrum of phenylalanine is more structured than the corresponding OPE spectrum [158]. Generally, two-photon spectra often appear blue-shifted because of the more pronounced vibronic coupling as compared to OPE case [85]. As compared to other FPs, the observed shift of 120 cm\(^{-1}\) for EosFP is 6 times smaller than the one for EGFP and two times smaller than the value for ECFP [116]. Given that the parity selection rules are in general different for OPE and TPE, exact coincidence of the spectra can not be expected. In order to explain different shifts for different proteins, Blab et al. suggested the presence of an additional, low-symmetry vibrational mode, which couples well to the electronic transition. In this work, TPE spectra of different FPs showed different spectral shifts, suggesting that depending on the FP chromophore, a different vibronic modes can couple to the TPE process. Differences from the OPE vibronic structure of EosFP in the two-photon case indicates the plausibility of this explanation. However, in order to make these conclusions more solid one needs to resolve the symmetry properties of the states by measuring e.g. the polarization ratio \( \Omega \) of the TPE spectra under circular and linear polarized excitations [158]. \( \Omega \) can be directly related to the trace squared of the transition tensor.
Outlook

The discovery of the first GFP has opened a new dimension in studies of vital processes. Since red-shifted fluorescence is beneficial for in vivo studies, lots of efforts are being made to create a near-infrared FP. Currently, fluorescent proteins with emission maximum up to 663 nm are found [45]. Unfortunately, all far-red FPs exhibit very low brightness, which makes their implementation problematic for biological studies. Stabilization of the FP structure and planarization of the chromophore may increase brightness. However, in order to shift the emission above 800 nm new types of FP chromophores are required. These chromophores would need a longer \( \pi \)-electron conjugation length, which results in larger size and, therefore, an expanded chromophore cavity. Thus, the development of new IR biosynthetic fluorescent markers is very challenging.

A dramatic increase of resolution in fluorescence microscopy can be potentially achieved with photochromic FPs [11, 159]. Although first experiments have demonstrated a rather mediocre gain in resolution [11], the potential of this technique is still unexplored. Control over photodynamic processes, that occur in fluorescent proteins, requires their deeper understanding. The structure of eqFP611 offers a good basis for design of photochromic FPs with superior properties.

Two-photon conversion of EosFP offers precise localization of the photoconverted region in three dimensions, thereby increasing labeling specificity even further as compared to the one-photon case. The reduced stability of EosFP at low pH, however, limits the benefits of the marker. Additional covalent bonds (e.g. sulfur bridges) at specific points may be helpful to stabilize the protein structure, thereby expanding the number of potential applications.
Outlook
Bibliography


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Bibliography
Summary

Development of novel fluorescent marker proteins was for a long time driven by the evolutionary approach of random mutagenesis. The relation of many spectroscopic phenomena to structure remains poorly understood, which prevents researchers from rational development of fluorescent proteins (FPs).

In the presented thesis, spectroscopic properties of fluorescent proteins eqFP611, EosFP and their derivatives are investigated. The former is a red fluorescent protein, having excitation and emission maxima at 559 nm and 611 nm, respectively, which makes it the most red-shifted naturally occurring FP. The latter protein, EosFP, is a green FP, which undergoes irreversible photoconversion from a green to a red-emitting form upon UV illumination. EosFP is a next step in labeling techniques as it offers a more precise definition of the region of interest compared to standard FPs. By using a focused UV beam, it is possible to locally label a smaller fraction of FP-tagged proteins and follow its migration pathway.

EosFP and eqFP611 show a tetrameric quaternary structure, which is stabilized by interactions at the A/B and A/C interfaces. Modifications of the protein interfaces, performed by the group of Dr. Wiedenmann, have shown that disruption of the A/B interface retains the spectroscopic properties, while disruption of the A/C interface causes folding problems at physiological temperatures. A set of beneficial substitutions, which promote folding was discovered by the method of random mutagenesis. We performed spectroscopic investigations of these proteins on bulk and single-molecule level.

The fluorescence quantum yield of eqFP611 has been determined as $0.45 \pm 0.02$ with minor deviations for the dimeric variants of the protein. We estimated the molecular brightness of several eqFP611 variants extracted from mammalian cells using the fluorescence cumulant analysis and found that the dimeric and pseudo-monomeric tandem variants (obtained by fusion of the two subunits) are comparable in brightness.

We found that the fluorescence spectrum of eqFP611 stays surprisingly broad, even at the lowest attainable temperature of 10 K. Analysis of the temperature-dependent absorption spectra enabled us to determine the linear coupling factor for the effective low-frequency mode of 100 cm$^{-1}$ to be $2.1 \pm 0.1$. In the same time, the analysis of spectral broadening of fluorescence yields a coupling factor of $18 \pm 1$ for the same effective phonon mode. We explored the temperature dependence of the fluorescence quantum yield in the temperature range 10-300 K and found that below the glass transition temperature $T_g \approx 180$ K the quantum yield remains constant, whereas above this temperature, the quantum
yield drops dramatically, indicating an additional non-radiative relaxation process.

Studies of photoinduced dynamics of individual eqFP611 molecules immobilized on a PEG-coated surface were performed. We characterized the flickering of individual molecules in terms of the so-called dark fraction and the flicker rate. An additional light induced process was found to occur on a milliseconds timescale. Additionally, we estimated the bleaching yield of these molecules ($\Phi_B = 1.9 \cdot 10^{-5}$) and compared it with the molecules dried on a bare glass surface. In the latter case we observed brighter molecules with $\approx 10$ times lower bleaching yield.

A unique structural feature of this FP is a fluorescent trans conformational state of the chromophore. Illumination of the protein with a pulsed laser at 532 nm caused reduction and red shift of the main absorption band and appearance of a band at 390 nm, which can be attributed to the hydrolyzed red chromophore. The light-induced red shift can be explained by a formation of new red-shifted species. We found that introducing a single mutation N143S promotes trans-cis isomerization of the chromophore and creates two spectroscopically distinct species emitting at 611 nm and 630 nm. The transition from the latter state into the former can be induced by changes in the environment of the protein. We were able to reversibly switch the isomeric state of the chromophore by illuminating the protein at either 450 nm or 560 nm.

EosFP changes its emission color from green ($\lambda_{\text{max}} = 516$ nm) to red ($\lambda_{\text{max}} = 581$ nm) upon near UV illumination. We determined the quantum yield of the green and the red form to be 0.7 and 0.55 respectively. The maximum excitation and emission wavelengths as well as the quantum yields of fluorescence for dimeric (d1EosFP and d2EosFP) and monomeric (mEosFP) variants of EosFP appeared similar to the wild type protein. No major changes were observed in fluorescence lifetime between the different mutants. Cryogenic studies of EosFP show narrow emission and excitation spectra, in contrast to eqFP611. The low-temperature spectra of the green and red form of EosFP display vibronic side-bands with average phonon frequencies of $\approx 1400$ cm$^{-1}$.

In our pH-titration experiments on EosFP, we found that the absorption and emission bands follow the Henderson-Hasselbalch equation with the same $pK_a = 5.7 \pm 0.1$ for the green and red forms of the protein within the experimental error. Moreover, we established a correlation between the conversion rate and the absorbance at 390 nm at different pH. Our fluorescence studies of the red form of EosFP at low pH show an additional feature at 545 nm, which is strongly reduced at pH values higher than 6.0. The absorption spectrum of the red EosFP form at pH 14 deviates from most of the other RFP chromophores at this pH, which indicates differences in the chromophore structures.

In our single-molecule experiments a four-step bleaching was observed for the wild type protein as opposed to the mEosFP variant. This confirms the tetrameric state of the protein even at the single-molecule level. In contrast, the dimeric EosFP variants appeared as monomers in our single-molecule experiments. We demonstrated fluorescence resonance energy transfer to take place between the green and red subunits of the partially converted tetrameric wild type protein. The bleaching yield of the dimeric and monomeric variants was calculated to be $\approx 3 \cdot 10^{-5}$. We determined the two-photon excitation spectrum for
the green form of wtEosFP, which yielded a two-photon excitation cross-section, $\delta = 19 \pm 5$ GM, at $\lambda_{\text{max}} = 1000 \pm 5$ nm. Moreover, the two-photon conversion spectrum of the green form of the wild type protein was determined. The two-photon excitation cross-sections for eqFP611 and the red form of wtEosFP at 1064 nm were estimated as 4.2 and 5.2 GM, respectively.

Finally, we applied EosFP to \textit{in vivo} studies of the nuclear transport of a DNA-binding protein and dynamics of the mitochondrial network. In the latter case, mitochondria could be marked individually within a HeLa cell, which allowed tracking of fusion and fission events.
Zusammenfassung

Die Entwicklung von neuartigen fluoreszierenden Proteinen wurde seit langem durch den evolutionären Ansatz der Zufallsmutagenese geprägt. Der Zusammenhang zwischen den spektroskopischen Eigenschaften und der Struktur ist noch kaum verstanden, was eine zielgerichtete Entwicklung von Fluoreszierenden Proteinen (FPs) verhindert. In dieser Arbeit werden spektroskopische Eigenschaften der fluoreszierenden Proteine eqFP611, EosFP und ihrer Varianten untersucht. EqFP611 ist ein rot fluoreszierendes Protein mit Anregungs- und Emissionsmaxima bei 559 bzw. 611 nm, und damit das am stärksten rotverschobene natürliche FP. Das zweite Protein, EosFP, ist ein grünes FP, das durch UV Beleuchtung irreversibel von einer grün emittierenden in eine rot emittierende Form umgewandelt wird. Dies ermöglicht eine neue Markierungstechnik, die im Vergleich mit üblichen FPs eine genauere Lokalisation erreicht. Mit Hilfe eines fokussierten UV Strahls kann ein kleiner Bereich von FP-fusionierten Proteinen lokal markiert und auf seinem Weg verfolgt werden.


Die Fluoreszenz-Quantenausbeute von eqFP611 wurde als 0.45 ± 0.02 bestimmt mit nur geringen Abweichungen für die dimeren Varianten des Proteins. Wir schätzten die molekulare Helligkeit für mehrere Varianten von eqFP611 die aus Säugetierzellen isoliert wurden mittels der Fluoreszenzkumulantanalyse ab und fanden, dass die Dimeren und die pseudomonomeren Tandem-Varianten (Fusion von zwei Untereinheiten) ähnliche Helligkeiten aufweisen. Spektroskopische Untersuchungen bei tiefen Temperaturen zeigten, dass das Fluoreszenzspektrum von eqFP611 überraschend breit ist, sogar bei der niedrigsten erreichbaren Temperatur von 10 K. Die Analyse der temperaturabhängigen Absorptionspektren ermöglichte uns, den linearen Kopplungsfaktor für die effektive niederfrequente Mode von 100 cm⁻¹ als 2.1 ± 0.1 zu bestimmen. Gleichzeitig ergibt die Analyse der spektralen Bandbreite der Fluoreszenz einen Kopplungsfaktor von 18 ± 1 für diese Mode. Wir ermittelten die Temperaturabhängigkeit der Fluoreszenz-Quantenausbeute im Temperaturbereich von 10-300 K und fanden, dass unter der Glasübergangstemperatur $T_g \approx 180 \text{ K}$
Zusammenfassung

die Quantenausbeute konstant bleibt, während sie oberhalb dieser Temperatur drastisch abfällt, was einen zusätzlichen nicht-strahlenden Relaxationsprozess andeutet.


Die Emissionsfarbe von EosFP ändert sich unter UV-Beleuchtung von grün ($\lambda_{\text{max}} = 516$ nm) nach rot ($\lambda_{\text{max}} = 581$ nm). Wir bestimmten die Quantenausbeute der grünen und der roten Form als 0.7 bzw. 0.55. Die maximale Anregungs- und Emissionswellenlängen sowie die Fluoreszenz-Quantenausbeuten für die dimeren (d1EosFP und d2EosFP) und monomeren (mEosFP) Varianten unterschieden sich kaum von wtEosFP. Auch bei der Fluoreszenzlebensdauer fanden wir keine wesentlichen Unterschiede zwischen den verschiedenen Mutanten. Im Gegensatz zu eqFP611 zeigten Tauatemperaturuntersuchungen an EosFP schmale Emissions- und Anregungsspektren. Die Tauatemperaturspektren der grünen und roten Form von EosFP zeigten vibronische Seitenbanden mit einer mittleren Phononenfrequenz von $\approx 1400$ cm$^{-1}$. In unseren pH-abhängigen Experimenten an EosFP fanden wir, dass die Absorptions- und Emissionsbanden der Henderson-Hasselbalch Gleichung mit dem selben $pK_a = 5.7 \pm 0.1$ für die grüne und rote Form des Proteins innerhalb des experimentellen Fehlers folgen. Außerdem stellten wir eine Korrelation zwischen der Konversionsrate und der Absorption bei 390 nm bei verschiedenen pH fest. Unsere Messungen an der roten Form von EosFP bei niedrigem pH zeigen eine zusätzliche Bande bei 545 nm, die bei pH-Werten über 6.0 stark reduziert ist. Das Absorptionsspektrum der roten Form von EosFP bei pH 14 weicht von meisten anderen RFP Chromophoren ab, was Unterschiede in den Chromophor-Strukturen nahelegt.

In unseren Einzelmolekül-Experimenten wurde für wtEosFP ein Fluoreszenzbleichen in vier diskreten Schritten festgestellt, im Gegensatz zu der monomeren Variante mEosFP. Dies bestätigt, dass die tetramere Struktur des Proteins sogar auf Einzelmolekül-Niveau
erhalten bleibt. Im Gegensatz dazu erschienen die dimeren EosFP Varianten als Monomere in unseren Einzelmolekül-Experimenten. Wir demonstrierten, dass Fluoreszenz Resonanz Energie Transfer zwischen den grünen und roten Untereinheiten des teilweise konvertierten tetrameren wtEosFP stattfindet. Die Bleicheffizienz der dimeren und monomeren Varianten wurde als \( \approx 3 \cdot 10^{-5} \) berechnet. Wir ermittelten das zwei-Photonen Anregungsspektrum für die grüne Form von wtEosFP, sowie einen zwei-Photonen Anregungsquerschnitt von \( \delta = 19 \pm 5 \) GM, bei \( \lambda_{\text{max}} = 1000 \pm 5 \) nm. Außerdem wurde das zwei-Photonen Konversionsspektrum der grünen Form von wtEosFP bestimmt. Die zwei-Photonen Anregungsquerschnitte für eqFP611 und die rote Form von wtEosFP bei 1064 nm wurden als 4.2 bzw. 5.2 GM abgeschätzt.

Schließlich verwendeten wir EosFP für in vivo Studien des Kerntransports eines DNA-bindenden Proteins und Untersuchungen der Dynamik des Mitochondrien-Netzwerks. Im letzteren Fall konnten einzelne Mitochondrien innerhalb einer HeLa Zelle markiert werden, sodass die Fusions- und Spaltungsereignisse im Netzwerk zeitlich verfolgt werden konnten.
Zusammenfassung
Achievements

Publications


Zusammenfassung

Posters

3. 47th Annual Meeting of the Biophysical Society, March 1-5, 2003, San Antonio, TX;
4. Annual Meeting of the American Physical Society, March 3-7, 2003, Austin, TX;
6. 49th Annual Meeting of the Biophysical Society, February 12-16, 2005, Long Beach, CA

Workshops

1. Common Workshop GRK 328 & SFP 569 October 12-14, 2003, Heinrich-Fabri-Institut, Blaubeuren;

Attended Lectures

1. Graduiertenkolleg Student Seminars,
2. Graduiertenkolleg Interdisciplinary Lectures.
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