Optimization of fluorescent proteins for novel quantitative multiparameter microscopy approaches
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Citation for published version (APA):

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Introduction
1.1 Introduction

During the past two decades, cell-biological and biomedical research has greatly benefited from innovations in fluorescence microscopy. Both the increase in the repertoire of fluorescence staining techniques at the (sub)cellular level and the development of a multitude of novel fluorescence microscopy techniques, contributed significantly. Digital imaging microscopy has been one of the key developments. Advances in technology have resulted in improved light sources for excitation, more versatile optical components (i.e. excitation/emission filters, dichroic mirrors), and faster, more sensitive detectors. This has enabled more complex image acquisition schemes, which in turn have allowed significant improvements in both optical and temporal resolution. Just as important is the shift from qualitative to quantitative microscopy.

Before the 1990s, labeling of proteins with fluorescent probes inside cells has been mainly achieved by immunocytochemistry. However, to enable fluorescent antibodies to enter cells need to be fixed and permeabilized and therefore immunolabeling is not suited for the study of proteins in living cells. Direct labeling with organic fluorophores is possible. However, this is laborious because it requires protein-purification, chemical labeling and microinjection into cells. Therefore, there has been great urge for methods allowing non-invasive and site-specific labeling of proteins in living cells or tissue. This was satisfied with the revolutionary discovery of the genetically encoded green fluorescent protein. This protein contains a fluorophore that is synthesized within the protein itself, without the need for additional substrates for generating fluorescence. Today, fluorescent proteins are available in a variety of colors which are generally named visible fluorescent proteins or VFPs. Specific fluorescent labeling of multiple proteins simultaneously with VFPs can now be achieved.

Quantitative fluorescence microscopy of VFP-labeled proteins in living cells has been essential for the understanding that intracellular signaling is not the result of disordered chemical reactions. Instead, signaling appears to be highly regulated, both spatially and temporally. Development of novel quantitative microscopy techniques and optimized fluorescent proteins is crucial to further increase our knowledge of subcellular signaling.

Therefore, the aim of the research described in this thesis is to create a set of fluorescent proteins with optimized properties for application in a wide range of organisms, including mammalian cells, plant cells and bacteria and to develop novel quantitative microscopy methods for studying signal transduction in living cells using these optimized VFPs. In the remainder of this chapter a description is given of the fluorescence microscopy techniques used for the research described in this thesis. Special attention will be given to fluorescence lifetime imaging microscopy (FLIM) and fluorescence resonance energy transfer (FRET) imaging, as these microscopy techniques allow imaging of protein-protein interactions and conformational changes in living cells. Furthermore, an overview is giving of the current status in fluorescent protein research and several applications of fluorescent proteins in cell biology are described.
1.2 Fluorescence microscopy

Fluorescence microscopy is a non-invasive technique, ideally suited for observing the localization of fluorescent molecules inside the living cell. Fluorescence can be detected with high contrast and sensitivity. Furthermore, the use of fluorescent proteins as genetically encoded fluorescent labels and the wide range of organic-dye based staining procedures provide unprecedented biomolecular specificity (Horan et al., 1990; Miyawaki et al., 2003).

1.2.1 Widefield versus confocal microscopy

Widefield fluorescence microscopy is the oldest microscopy technique for detecting fluorescence and still is widely used. In widefield fluorescence microscopy the lens directly forms an image which can be projected onto a camera or through an eye piece. Therefore the whole image is recorded at once. The resolution of widefield microscopy is suboptimal, because the contribution of out of focus fluorescence results in blurring of the image.

In confocal fluorescence microscopy, true 3D-resolution (x,y,z) is achieved by suppression of out of focus fluorescence (Müller, 2006). This is achieved by placing a pinhole in front of the detector so that fluorescence from the in-focus plane passes through the pinhole, whereas fluorescence originating from out of focus planes is blocked. Blocking of out of focus fluorescence results in "optical sectioning" capabilities. With confocal microscopy, a 3D representation of the sample can be reconstructed after acquisition of a series of images taken along the z-axis. Because of the confocal pinhole, only a single point in the object plane can be measured at a time. In order to produce an image, the sample has to be scanned. Therefore, confocal imaging is slower than wide-field imaging, in which the whole image is recorded at once. Several alternative modes of confocal imaging have been developed to improve the speed of imaging. One is spinning-disc confocal microscopy, which uses a fast rotating disc with thousands of pinholes to scan the whole image at once (Tadakuma et al., 2001; Graf et al., 2005). Another approach is slit-scanning (Brakenhoff and Visscher, 1992). In this case, the confocal pinhole is replaced by a confocal slit, to enable line-by-line scanning instead of point-by-point scanning. This approach has recently found renewed interest and has seriously improved temporal resolution of confocal microscopy (LSM 5 Live, Zeiss). Yet both alternative approaches do trade off axial resolution for speed.

1.2.2 Fluorescence lifetime imaging microscopy

Fluorescence lifetime imaging microscopy (FLIM) is a technique to determine the spatial distribution of excited state lifetimes in microscopic samples (Gadella et al., 1993; Clegg and Scheider, 1996; Gadella et al., 1997; Gadella et al., 1999; Lakowicz, 1999; van Munster and Gadella, 2005). In contrast to fluorescence intensity, the fluorescence lifetime is a kinetic parameter, which is independent of probe concentration, excitation light intensity and moderate levels of photobleaching.
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The fluorescence lifetime of a fluorophore represents the average amount of time the fluorophore spends in the excited state after absorption of a photon (Lakowicz, 1999). Deactivation from the excited state generally takes 1 to 10 ns. The excited-state energy can be discharged, by i) radiatively by the release of a (red-shifted) photon, ii) non-radiatively as thermal energy, iii) through triplet state formation (sometimes resulting in phosphorescence) or iv) by fluorescence resonance energy transfer (FRET, see paragraph 1.2.3).

The fluorescence lifetime is linearly proportional to the quantum yield (QY) of the fluorophore (i.e. the probability of releasing excited state energy by emission of a photon, 0<QY<1). The QY of a fluorophore is dependent on the direct molecular environment and factors that influence QY are for example pH, local hydrophobicity, the presence of fluorescence quenchers or the proximity of energy transfer acceptors (Gadella et al., 1993; Lakowicz, 1999).

The fluorescence intensity measured in a microscope is always the product of the excitation light intensity, probe concentration, probe QY and the detection efficiency of the microscope. Probe concentration and probe QY can vary in the sample and cannot be determined independently of each other based solely on the fluorescence intensity. In other words, fluorescence intensity does not discriminate between a high concentration of weakly fluorescent probes and a low concentration of intensely fluorescent probes. In contrast, FLIM enables the generation of separate images of fluorescence intensity and fluorescence lifetime. The lifetime image enables independent estimation of the QY, thereby providing the capability to image both the local fluorophore concentration and the local fluorophore quantum yield reporting on the probe microenvironment.

FLIM can be implemented in many different ways (for a recent review see van Munster and Gadella, 2005), nevertheless fluorescence lifetimes are generally measured using frequency-domain or time-domain approaches. FLIM requires measurements in the ns time

![Figure 1.1 Principle of frequency-domain FLIM.](image)

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domain. This can be achieved by using (fs-ps) pulsed light sources for excitation and time-correlated detection of the fluorescence decay (generally referred to as time-domain FLIM), or by using intensity-modulated (in the 10-100Mhz region) excitation light and detecting the delay (phaseshift) of the fluorescence decay (referred to as frequency-domain).

In frequency-domain FLIM, the intensity of the excitation light is continuously modulated by a sinusoid or other shaped profile. As a result, the emitted fluorescence will be intensity modulated as well (figure 1.1). Due to the fluorescence decay (i.e. fluorescence lifetime), the fluorescence will display a phase-shift ($\Delta \phi$) and decrease in modulation depth ($M$) with respect to the excitation light (Weber, 1981; Gadella et al., 1994).

$$\Delta \phi = \Delta T \cdot \omega; \quad \omega = 2 \pi f$$
and
$$M = \frac{M_F}{M_E} = \frac{B}{A} \quad (1a,b)$$

where $f$ is the frequency of modulation. The observed phase-shift and change in modulation depth can both be used to calculate the fluorescence lifetime, using the following equations:

$$\tau_\phi = \frac{1}{\omega} \tan(\Delta \phi) \quad \text{and} \quad \tau_m = \frac{1}{\omega} \sqrt{\frac{1}{\Delta M^2}} - 1 \quad (2a,b)$$

In case of a mono-exponential lifetime decay, $\tau_\phi$ and $\tau_m$ are equal. If $\tau_\phi < \tau_m$, this is an indication that the fluorescence decay is multi-exponential and has different lifetime components (Weber, 1981; Gadella et al., 1993).

### 1.2.3 Fluorescence Resonance Energy Transfer

Fluorescence resonance energy transfer (FRET) is a phenomenon, by which excited-state energy of a fluorophore (donor) is transferred to another chromophore (acceptor) ( Förster, 1948; Förster, 1951). FRET can occur, if the emission spectrum of the donor has overlap with the absorbance spectrum of the acceptor. This energy transfer occurs nonradiatively; that is, the donor does not actually emit a photon and the acceptor does not absorb a photon (Herman, 1989; Clegg, 1996). FRET causes a decrease in fluorescence of the donor and if the acceptor is a fluorophore, FRET causes an increased or sensitized emission of the acceptor. A fluorescent acceptor does allow ratiometric imaging. Alternatively, FRET can be measured by a decrease in the fluorescence lifetime of the donor.

The FRET efficiency ($E$) is highly dependent on the distance between donor and acceptor and is defined by Försters theory (Förster, 1948):

$$E = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6} \quad (3)$$

in which $r$ is the distance between donor and acceptor and $R_0$ is the Förster radius for donor-acceptor pair. $R_0$ is defined as the distance at which 50% FRET occurs and can be calculated from the following equation (Förster, 1948; Patterson et al., 2000):

$$R_0 = \frac{c \kappa^2 \eta^{-4} \phi_d \epsilon_a J(\lambda)}{c} \quad (4)$$

Where $c$ is 8.786·10^{-11} mol L^{-1} cm nm^{2}, $\kappa^2$ is the orientation factor of the interacting dipoles, $\eta$ the refractive index of the medium separating donor and acceptor chromophore, $\phi_d$ the
quantum yield of the donor, \( \varepsilon_a \) the extinction coefficient of the acceptor with dimensions \( \text{mol}^{-1} \text{ L cm}^{-1} \) and \( J(\lambda) \) the overlap integral. Generally, \( \kappa^2 \) is set to 2/3, which is true if donor and acceptor are rapidly randomly orientated (Stryer, 1978; Clegg, 1996; van der Meer, 2002). Depending on the donor-acceptor pair, \( R_0 \) ranges from 2 to 6 nm (Wu and Brand, 1994; Patterson et al., 2000). Because of the inverse sixth power distance-dependence, \( E \) quickly drops to zero when donor and acceptor are separated beyond \( R_0 \) (figure 1.2) (van Munster and Gadella, 2005). Hence, FRET is only significant at distances \( r < 1.5R_0 \) (i.e. 0 - 9 nm).

FRET is well suited to measure protein-protein interaction between fluorescently labeled proteins, since the close proximity required for FRET generally only occurs upon interaction of the two proteins. Although the resolution of fluorescence microscopy is limited to approximately 200 nm, FRET enables measurements at the nanometer scale. Hereby, FRET microscopy effectively combines nm molecular resolution with \( \mu \text{m} \) subcellular spatial resolution.

### 1.2.4 Imaging FRET

The occurrence of FRET can be imaged by microscopy in several ways. One method is to measure quenching of donor fluorescence and sensitized emission of the acceptor by imaging of donor and acceptor fluorescence intensity (Gordon et al., 1998). Ratiometric FRET imaging of donor and acceptor fluorescence is preferred over imaging only the donor fluorescence, because by measuring intensity at one wavelength, one cannot discriminate the donor concentration from the (reduced) donor-quantum yield. For quantitative filter-FRET applications, many correction factors have to be taken into account (Berney and Danuser, 2003; van Rheenen et al., 2004). An alternative method is to use acceptor bleaching to abolish FRET. This results in donor dequenching, i.e. an increase in donor

![Figure 1.2 FRET efficiency versus distance (R0 = 4.75 nm).](image-url)
fluorescence. The relative increase can be quantitatively related to the FRET efficiency (Bastiaens et al., 1996; Wouters et al., 1998; Wouters and Bastiaens, 1999; Kenworthy, 2001; Gu et al., 2004). However, acceptor bleaching is inherently irreversible and can be prone to many error sources such as acceptor dark states, acceptor photochromicity, sample movement, etc.

An alternative is to use an imaging spectrograph in FRET microscopy (SPIM) (Gadella et al., 1997; Gadella et al., 1999). Here the fluorescence originating from a line in the sample is focused onto the entry slit of a spectrograph and the generated spectrum is imaged onto a CCD detector. In this way, complete emission spectra are obtained for all pixels along a line in the object plane. Decomposing multicomponent fluorescence by spectral unmixing provides much more detail than just using two averaged regions of the spectra of fluorophores, as is the case in filter-FRET imaging. In the past, considerations for using SPIM have been that the spectral information is obtained at the expense of one spatial dimension. This limitation has been overcome by the development of spectral detectors in confocal microscopy (Leica TCS SP6, Zeiss LSM 510 META), enabling 3D (x,y,λ) and 4D (x,y,z,λ) pixel-by-pixel spectral imaging (Zimmermann et al., 2003; Thaler et al., 2005).

As mentioned before, the fluorescence lifetime is dependent on the molecular environment of the fluorophore. In case of fluorescent proteins, the chromophore is buried inside the protein (for details see paragraph 1.3.4), therefore its direct molecular environment is the protein itself. Because of the rather effective shielding of the chromophore from the chemical environment of the protein, the fluorescence lifetime of VFPs is relatively stable (also inside cells). Since FRET takes place through space the protein shell does not obstruct energy transfer to acceptors that are in close proximity. Hence, FRET is almost the only process influencing the quantum yield and fluorescence lifetime of VFPs. This makes VFPs excellent probes for FRET microscopy. As FLIM provides a quantitative spatially resolved estimation of fluorescence lifetimes, FRET-FLIM using VFPs is ideally suited for the study of protein-protein interactions in live cells.

1.3 Fluorescent proteins

1.3.1 Discovery of the fluorescent proteins

The first reported observation of green fluorescent protein (GFP) fluorescence dates back to the early 1960s. A protein exhibiting a greenish glow was observed as a byproduct after purification of the chemiluminescent protein aequorin from the jellyfish Aequorea victoria (Shimomura et al., 1962). In the following 3 decades GFPs were also discovered in several other light emitting marine species, such as the sea pansy Renilla reniformis (Morin and Hastings, 1971).

The first major breakthrough for the application of fluorescent proteins occurred 30 years later, when the gene encoding Aequorea victoria GFP (avGFP) was isolated (Prasher et al., 1992), followed 2 years later by the discovery that GFP could be expressed in organisms other than Aequorea victoria and also became fluorescent (Chalfie et al., 1994; Inouye and Tsuji, 1994). This showed that avGFP is an autofluorescent protein and requires no
additional (jellyfish-specific) substrates or enzymes, in contrast to the well known chemiluminescent proteins aequorin and luciferase (Inouye and Shimomura, 1997; Greer and Szalay, 2002).

The second major breakthrough for the application of fluorescent proteins was the isolation of the red fluorescent protein drFP583 or DsRed from the Anthozoa Discosoma sp, a mushroom-shaped anemone found in the warm waters of the indo-pacific ocean (Matz et al., 1999). The breakthrough was not only the discovery of the first true red fluorescent protein, but equally important was the fact that it was discovered in a non-bioluminescent species and that the gene was cloned immediately.

1.3.2 Evolution of fluorescent proteins

The isolation of fluorescent proteins from non-bioluminescent species has led to the discovery of a whole superfamily of GFP-like proteins (Shagin et al., 2004; Chudakov et al., 2005). Recently, six additional GFP-like proteins were isolated from Aequorea victoria related jellyfish (Xia et al., 2002; Gurskaya et al., 2003; Shagin et al., 2004). Furthermore a large number of GFP-like proteins have been isolated from Anthozoa species, ranging in fluorescence from green to orange-red, as well as non-fluorescent purple-blue chromoproteins (Gurskaya et al., 2001; Labas et al., 2002; Matz et al., 2002). Hydrozoa, like Aequorea victoria, and Anthozoa both belong to the phylum Cnidaria. Recently, fluorescent proteins have been isolated from planktonic Copepods, which belong to the evolutionary distant phylum Arthropodia (Shagin et al., 2004). This wide phylogenetic distribution of GFP-like proteins might implicate that these proteins developed early in evolution and hence that almost every animal taxon can potentially contain GFP homologs (Chudakov et al., 2005).

1.3.3 Biological function of fluorescent proteins

The biological function of fluorescent proteins is not completely clear. In Aequorea victoria and other bioluminescent species containing GFP-like proteins, GFP is bound to aequorin, where it converts the blue light emitted by aequorin into green light in a proces called bioluminescence energy transfer (BRET) (Tsien, 1998). The advantage might be that green light is less absorbed by the surrounding ocean and the emitted fluorescence might be used as a defense strategy against predators or to attract prey.

Although this might be one of the functions of fluorescent proteins, it is certainly not the only function. The association of GFP with aequorin is (most likely) a special case, since most bioluminescent species apparently lack fluorescent proteins and in addition, the majority of species containing GFP-like proteins are not bioluminescent (Chudakov et al., 2005). Furthermore, the existence of highly absorbing but non-fluorescent chromoproteins, together with the fact that many of the organisms containing fluorescent proteins have been found in subtropical climates, suggests a possible role for fluorescent proteins in protection against UV-radiation from the sun (Salih et al., 2000). Another function proposed for GFP-
like proteins in Copepods is that they are involved in visual mate-recognition (Marshall and Oberwinkler, 1999; Mazel et al., 2004; Chudakov et al., 2005).

1.3.4 Protein structure of VFPs

The crystal structure of avGFP was first solved in 1996, independently by two groups (Ormo et al., 1996; Yang et al., 1996a). The structure revealed a cylindrical protein, consisting of 11 β-strands (figure 1.3), which was named 11-stranded β-barrel. A single α-helix runs along the axis inside the β-barrel and was found to contain the chromophore, the source of fluorescence. The chromophore is placed almost exactly in the centre of the protein and therefore is buried deep inside the GFP protein. This protective environment effectively shields the chromophore from its aqueous surroundings.

Remarkably, although there is little sequence homology between the members of the GFP super family (DsRed and avGFP share less than 30% sequence homology), their crystal structures are highly similar (Wall et al., 2000; Yarbrough et al., 2001). The β-barrel structure is a feature common to all members of the GFP super family for which the crystal structure has been solved. However, whereas avGFP is present mainly as a monomer, many other VFPs form obligate di- or tetramers.

1.3.5 Chromophore formation in avGFP and DsRed

The chromophore of avGFP is formed by amino acids Ser65, Tyr66 and Gly67 by a series of autocatalytic reactions (figure 1.4). Although the precise sequence of events is still under debate (Rosenow et al., 2004; Barondeau et al., 2005), it is generally accepted that chromophore formation involves cyclization of the tripeptide by nucleophilic attack of the

**Figure 1.3** Ribbon diagram of the avGFP crystal structure (PDB entry 1EMA). The chromophore is buried in the protein’s interior and shown in sticks representation.
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amide nitrogen of Gly67 on the carbonyl carbon of Ser65 (Prasher et al., 1992; Cody et al., 1993; Cubitt et al., 1995; Reid and Flynn, 1997). This results in ring closure and the release of one H2O molecule. The mature chromophore is formed upon oxidation of the Tyr66 Cα-Cβ bond by molecular oxygen, causing the formation of a p-hydroxybenzylidene-imidazolidinone derivative with a large conjugated π-electron system. Since the chromophore is created by autocatalytic reactions within the protein itself, no co-factors are required for fluorescence, except for a single O2 molecule during chromophore maturation (Heim et al., 1994).

The red fluorescent chromophore of DsRed is formed through a green intermediate formed by the tripeptide Gln66-Tyr67-Gly68, analogous to avGFP. Red fluorescence develops only after additional oxidation of the bond between the α-carbon and the nitrogen of Gln66, which extents the conjugation of the chromophore (see also figure 1.6) (Baird et al., 2000; Gross et al., 2000; Yarbrough et al., 2001). The second oxidation step requires an additional O2 molecule and occurs rather slow and incomplete. This results in an immature green chromophore. Importantly, since DsRed is an obligate and closely packed tetramer, as soon as one chromopore in the tetramer is fully matured (red), then the residual green fluorescence is efficiently converted to red via FRET.

1.4. Variants of avGFP

Use of avGFP is limited by inefficient folding at temperatures above 20°C. Therefore much effort has been put into optimizing protein folding at elevated temperatures to facilitate GFP expression at 37°C for applications in mammalian cells. Furthermore, a range of mutations have been found, which alter the spectral properties of avGFP. This has resulted in a variety of color variants of avGFP with fluorescence ranging from blue to greenish-yellow.
1.4.1 Mutations for improving expression of avGFP

Random mutagenesis has yielded several mutations that do not increase the intrinsic brightness of fluorescence, but rather increase the amount of expressed protein that reaches a fluorescent state. Some of these mutations are present close to the chromophore (Phe64Leu, Val68Leu and Ser72Ala; Delagrave et al., 1995; Cormack et al., 1996), whereas others are located more distant and can be buried inside the protein (Val163Ala; Crameri et al., 1996) or exposed to the outside (Met153Thr, Ser175Gly, Ala206Lys; Heim and Tsien, 1996; Siemering et al., 1996; Zacharias et al., 2002)). Although the exact mechanism of most of these mutations is unknown, mutations close to the chromophore are presumed to directly improve chromophore formation, whereas the more distant mutations Val163Ala and Ser175Gly are likely to prevent protein misfolding at elevated temperatures (Cubitt et al., 1999). Mutation Met153Thr presumably improves solubility, by decreasing the surface hydrophobicity. Mutation Ala206Lys was found to abolish the tendency of GFP to dimerize, because the charged lysine disrupted the hydrophobic interphase required for dimerization (Zacharias et al., 2002). Although the affinity for dimerization is low (kd = 0.11 mM), it can induce artifacts when using GFP fusion proteins to study intermolecular interactions (Zacharias et al., 2002).

Furthermore, the Aequorea victoria codon usage has been changed into an optimized mammalian codon usage, thereby eliminating several rare codons (Yang et al., 1996b; Zolotukhin et al., 1996). Codon optimization has been shown to increase levels of protein expression four-fold at 37°C and GFP variants with such optimized codon usage are generally named enhanced fluorescent proteins or EVFPs (enhanced visible fluorescent proteins) (Yang et al., 1996b; Yang et al., 1998).

1.4.2 Wild type avGFP and variants with neutral chromophore

The chromophore in avGFP can exist in two states with different spectroscopic features. These states depend on the protonation-state of the phenolic hydroxyl group of Tyr66 and are strongly influenced by a hydrogen bond network involving amino acids Ser205 and Glu222 and a H2O molecule buried inside the protein. In wild type avGFP, the chromophore is mainly present in a neutral form in which the hydroxyl group of Tyr66 is protonated, because Glu222 donates a proton by electrostatic repulsion via the hydrogen bond network (figure 1.5A) (Brejc et al., 1997).

In a minor fraction of the protein, the chromophore is in a deprotonated, anionic state, in which the positive charge is relayed back to Glu222. Additional rearrangements, for example formation of a hydrogen bond between Thr203 and the chromophore, cause further stabilization of this conformation (figure 1.5B).

Wild type avGFP has a major excitation peak at 395 nm arising from the protonated chromophore and a minor excitation peak at 475 nm corresponding to the deprotonated anionic chromophore (Tsien, 1998). Excitation at either excitation maximum gives rise to similar but not identical fluorescence with peak emission at approximately 505 nm. The similar fluorescence is the result of excited-state proton transfer upon excitation of the
neutral chromophore (Chattoraj et al., 1996). Because the phenolic hydroxyl group of Tyr66 becomes more acidic in the excited state, it can now donate a proton to Glu222 via the hydrogen bond network (Heim et al., 1994; Brejc et al., 1997).

avGFP variants have been described, in which the minor excitation peak at 475 nm is abolished, due to a point mutation Thr203Ile (Heim et al., 1994; Ehrig et al., 1995). The absence of deprotonated chromophore can be explained by the fact that Ile, in contrast to Thr, is unable to form a hydrogen bond with the phenolic hydroxyl group of Tyr66, which has been shown to be necessary to stabilize the anionic form of the chromophore (Kummer et al., 2000; Jung et al., 2005). An optimized avGFP variant, T-Sapphire, contains several folding mutations in addition to mutation Tyr203Ile (Zapata-Hommer and Griesbeck, 2003). T-Sapphire is very interesting, as it can be detected separately from avGFP variants with stabilized anionic chromophores (discussed next).

### 1.4.3 avGFP variants with anionic phenolate chromophore

For cell biological applications, excitation at 475 nm is more favorable than excitation at 395 nm, because the risk of (near-UV) radiation damage is decreased and the levels of cellular autofluorescence are less. Therefore much effort has been put into improving excitation of avGFP at 475 nm. Random mutagenesis of wild type avGFP has yielded several mutations that efficiently abolish chromophore protonation, resulting in increased excitation around 475 nm at the expense of excitation at 395 nm. Key mutations include changing Ser65 into Thr, Gly, Ala, Leu or Cys (Delagrave et al., 1995; Heim et al., 1995; Cormack et al., 1996). These mutations generally act by disrupting the hydrogen bond network between the chromophore and Glu222 (figure 1.5). Gly, Ala and Leu can not donate hydrogen bonds and Cys is too large to adopt the right conformation (Tsien, 1998), therefore Glu222 can not transfer a proton to the chromophore (Brejc et al., 1997). The
most commonly used mutation to prevent chromophore protonation is Ser65Thr (Heim *et al.*, 1995). Compared to serine, threonine contains an additional methyl group and the increased size causes steric hindrance, preventing hydrogen bonding to Glu222 (Brejc *et al.*, 1997).

In addition to disrupting the hydrogen bond network between the chromophore and Glu222, chromophore protonation can be prevented by direct mutagenesis of Glu222. Replacing Glu222 with Gly or Gln has been shown to effectively abolish excitation at 395 nm (Ehrig *et al.*, 1995; Jung *et al.*, 2005; Sniegowski *et al.*, 2005). However, Glu222Gln seems to reduce the efficiency of chromophore maturation (Sniegowski *et al.*, 2005).

The most commonly used avGFP variant today is enhanced GFP or EGFP (Cormack *et al.*, 1996; Yang *et al.*, 1996b). EGFP has an optimized codon usage for expression in mammalian cells and contains the mutations, Phe46Leu and Ser65Thr (figure 1.6A). Because of the excitation maximum at 488 nm and emission maxima at 507 nm (figure 1.7), EGFP is compatible with the standard fluorescence filter sets used for green fluorescent dyes, *i.e.* fluorescein.

Emerald is another optimized GFP variant with spectra resembling EGFP and is supposed to be superior to EGFP (Tsien, 1998; Shaner *et al.*, 2005). Emerald contains several additional mutations to improve protein folding, however fast bleaching kinetics cause a 50% reduction in fluorescence brightness within seconds after excitation, after which the photostability is comparable to EGFP. The absence of folding mutations in EGFP suggests it might be possible to further improve brightness and protein folding of EGFP.

### 1.4.4 Blue fluorescent variants of avGFP

One of the first color variants of avGFP was a blue fluorescent protein (BFP). BFP has gained interest, because the blue-shifted fluorescence permitted, for the first time, dual-
color imaging together with GFP (Heim and Tsien, 1996; Rizzuto et al., 1996; Yang et al., 1998). Replacing Tyr66 for His in the chromophore (figure 1.6B) results in blue shifted fluorescence with excitation and emission maxima at 380 nm and 450 nm, respectively (figure 1.7) (Heim et al., 1994), however the brightness of this fluorescent protein is poor. Optimization of the codon usage and incorporation of the additional mutation Tyr145Phe have been shown to improve expression and the brightness of enhanced BFP (EBFP; Yang et al., 1998), nevertheless fluorescence remains dim and susceptible to photobleaching (Heim and Tsien, 1996; Patterson et al., 1997).

For many cases, the UV-excitable GFP-variant T-Sapphire might be a better alternative to EBFP for dual-color imaging with EGFP, since it is more fluorescent and permits use of a longer wavelength for excitation.

### 1.4.5 Cyan fluorescent variants of avGFP

Cyan fluorescent proteins (CFPs) have blue-shifted excitation and emission spectra, because of the mutation Tyr66Trp inside the chromophore (figure 1.6C) (Heim et al., 1994). CFP fluorescence (Ex 435 nm / Em 474 nm; figure 1.7) is less blue-shifted than for EBFP and CFP excitation is intermediate to the excitation of the neutral and anionic chromophores of avGFP (Tsien, 1998). The main advantage of CFP is that it can be used for dual-color and FRET applications with yellow fluorescent protein (YFP, paragraph 1.4.6).

The first reported CFP variants were only weakly fluorescent and the extra mutation Asn146Ile was needed to efficiently accommodate the bulkier chromophore (Heim and Tsien, 1996; Tsien, 1998). ECFP is a widely used CFP variant with mammalian optimized codon usage and contains the additional mutations Phe64Leu, Ser65Thr, Met153Thr and Val163Ala. Although not as bright as EGFP, ECFP fluorescence is readily monitored, due to the high photostability and the high intensity output of a pressured mercury lamp at 436 nm.

Recently, novel CFP variants CyPet and Cerulean have been developed (Rizzo et al., 2004; Nguyen and Daugherty, 2005). CyPet contains several novel mutations and was presented as an improved donor for FRET assays. However, CyPet has been found to express relatively poorly at 37°C, which can limit its application (Nguyen and Daugherty, 2005;
Shaner et al., 2005). Cerulean is a brighter fluorescent variant than ECFP, mainly because of the mutation His148Asp. The increased brightness removes many of the drawbacks of using cyan fluorescent proteins (Rizzo et al., 2004). Since Cerulean is well expressed at 37°C it probably is the best general-purpose CFP. However, the photostability of Cerulean is under debate and might limit prolonged excitation (Shaner et al., 2005).

### 1.4.6 Yellow fluorescent variants of avGFP

Although a true red fluorescent protein has never been found among the many color variants of avGFP, it was possible to create red-shifted GFP variants (Ex 514 nm / Em 526 nm; figure 1.7). These fluorescent proteins have been called yellow fluorescent proteins (YFPs) because of the yellowish appearance of their fluorescence (Tsien, 1998). Whereas all other color variants of avGFP were initially found by random mutagenesis strategies, YFP was rationally designed, based on the crystal structure of GFP (Ormo et al., 1996). Ormø and co-workers hypothesized that the phenolic ring of a tyrosine residue at position 203 might result in π-π stacking with the phenolic ring of the chromophore and hence would reduce the excited state energy. This hypothesis was later confirmed by the crystal structure of YFP (figure 1.6D) (Wachter et al., 1998). YFPs have found wide application, because for several years YFP together with CFP was the best combination of fluorescent proteins available for dual-color imaging and for FRET applications.

Use of early YFP variants, for example EYFP, suffered from several disadvantages, since they did not express well at 37°C, were sensitive to photobleaching and to environmental factors, for example pH and Cl− concentration (Llopis et al., 1998; Elsliger et al., 1999; Jayaraman et al., 2000; Griesbeck et al., 2001; McAnaney et al., 2005). The recently developed optimized YFP variant, Venus, has overcome most of the drawbacks of early YFP variants (Nagai et al., 2002). Venus is a fast maturing fluorescent protein and well expressed at 37°C. Furthermore Venus is less sensitive to changes in pH and insensitive to the Cl− concentration. However Venus has been implied to be less photostable than EYFP (Shaner et al., 2005).

### 1.5 Variants of DsRed

DsRed, is a bright red fluorescent protein with excitation and emission maxima at 558 nm and 583 nm, respectively. Despite the bright red fluorescence, application of DsRed has been restricted, because of slow and inefficient maturation and its tetrameric form (Lauf et al., 2001; Mizuno et al., 2001). The poor maturation efficiency has been overcome by random mutagenesis, which resulted in the fast maturing variant DsRedT1 (Bevis and Glick, 2002). However, DsRedT1 remains tetrameric.

#### 1.5.1 Monomeric color variants of DsRed

DsRedT1 has served as the basis for further mutagenesis by directed evolution to finally yield a monomeric red fluorescent protein, mRFP1 (Campbell et al., 2002). An additional feature of mRFP1 is a further red-shift in excitation and emission of approximately 25 nm,
resulting in excitation and emission maxima at 584 nm and 607 nm, respectively. For two years, mRFP1 has been the only monomeric red fluorescent protein available. Recently, a whole series of bright monomeric fluorescent proteins has been developed by further mutagenesis of mRFP1 (Shaner et al., 2004; Wang et al., 2004). These fluorescent proteins range in color from green and orange to red and far-red. A brighter alternative for mRFP1, mCherry, is now available. mCherry shows fast and efficient maturation and high photostability (Shaner et al., 2005). The excitation and emission spectra of mCherry are 3 nm further red-shifted, with maxima at 587 nm and 610 nm, respectively (figure 1.7).

1.5.2 Far-red fluorescent proteins

The development of even further red-shifted fluorescent proteins is of great interest. One reason is the expansion of the color palette of fluorescent proteins, however even more important is the potential of far-red fluorescent protein for use in tissues and whole-body mapping (Weissleder and Ntziachristos, 2003; Chudakov et al., 2005). In biological tissue, autofluorescence is minimal and light penetration is efficient in the region between 650 nm and 1100 nm, as these wavelength are too long to be absorbed by hemoglobin and too short to be absorbed by water.

The most red-shifted native fluorescent protein at present is eqFP611 isolated from the sea anemone Entacmaea quadricolor, which has its emission maximum at 611 nm (Wiedenmann et al., 2002). Although brightly fluorescent, eq611 is tetrameric.

Non-fluorescent chromoproteins constitute a surprising alternative source of far-red fluorescent proteins. These GFP homologs have highly absorbing chromophores, however without the ability to fluoresce. Mutagenesis of chromoproteins has resulted in far-red fluorescent proteins (Gurskaya et al., 2001; Bulina et al., 2002). Currently, the most far-red shifted fluorescent protein is the tetrameric protein AQ143 with excitation and emission maxima at 595 nm and 655 nm, respectively, and fluorescence extending beyond 750 nm (Shkrob et al., 2005). The most far-red monomeric fluorescent protein is mPlum, with excitation and emission maxima at 590 nm and 649 nm, respectively (Wang et al., 2004). Until now, far-red fluorescent proteins remain only weakly fluorescent, due to a low quantum yield (0.04 for AQ143; 0.1 for mPlum). However, because of the high molar extinction coefficient (90.000 M⁻¹·cm⁻¹ for AQ143; 41.000 M⁻¹·cm⁻¹ for mPlum), far-red fluorescence can be readily measured.

1.6. Photoconvertable fluorescent proteins

Among the large number of fluorescent proteins known today several proteins have been identified which contain unique properties, for example the ability to change the color of their fluorescence (Chudakov et al., 2005; Lukyanov et al., 2005). These fluorescent proteins can be specifically "turned on" at will, where and when you want.
1.6.1 Photoactivatable fluorescent protein

Photoactivatable visible fluorescent proteins (PA-VFPs) become fluorescent upon intense illumination with violet light of around 400 nm. At present, three PA-VFPs are known: PAGFP, PS-CFP2 and PAmRFP1. PAGFP was created by inserting mutation Thr203His into avGFP (Patterson and Lippincott-Schwartz, 2002). This mutation prevents chromophore deprotonation. Therefore, before activation PAGFP does not fluoresce when excited with 488 nm light. Upon irradiation with intense violet light, the chromophore is photoconverted into its anionic deprotonated state, thereby enabling excitation at 488 nm and producing a 100-fold increase in fluorescence (figure 1.8). Photoconversion has been shown to result in decarboxylation of Glu222, making photoconversion irreversible (van Thor et al., 2002; Bell et al., 2003).

PAmRFP1, is a variant of the red fluorescent proteins DsRed and mRFP1 (Verkhusha and Sorkin, 2005). Upon irradiation with 380 nm light, PAmRFP1 displays a 70 fold increase in red fluorescence (Ex 578 nm / Em 605 nm). However, use of PAmRFP1 is limited by the dim red fluorescence and because the red fluorescence fades with an apparent half-life of 9 hours at 37°C.

PS-CFP2, actually a photoswitchable fluorescent protein (see paragraph 1.6.2), emits cyan fluorescence (468 nm) when excited with low intensity violet light (Chudakov et al., 2004; Chudakov et al., 2005). Upon illumination with intense violet light, PS-CFP2 fluorescence

![Figure 1.8 Overview of the chromophore structures and rearrangements of photoactivatable fluorescent proteins. Reproduced with permission from Nature Reviews Molecular Cell Biology. Lukyanov et al., 2005. Copyright 2005 Macmillan Magazines Ltd. For color figure see page 139 of this thesis.](image-url)
converts to green (Ex 490 nm / Em 511 nm), resulting in a more than 400-fold increase in green fluorescence and over 2000-fold change in the ratio green/cyan fluorescence. The mechanism of photoconversion is irreversible and thought to be similar to that of PAGFP (Chudakov et al., 2004).

1.6.2 Kaede-like photoswitchable fluorescent proteins

Kaede-like photoswitchable visible fluorescent proteins (PS-VFPs) change the color of fluorescence from green (Ex \(~\sim\) 507 nm / Em \(~\sim\) 517 nm) to red (Ex \(~\sim\) 570 nm / Em \(~\sim\) 580 nm) upon illumination with intense violet light. An advantage of Kaede-like PS-VFPs is that the excitation wavelength required for fluorescence does not cause photoconversion. In Kaede (Ando et al., 2002), the green fluorescent chromophore before photoswitching consists of His65-Tyr66-Gly67 (amino acid numbering according to avGFP). Photoconversion involves cleavage of the protein backbone between the N\(\alpha\) and C\(\alpha\) of His65 and the subsequent formation of a double bond between C\(\alpha\) and C\(\beta\) of His65 (figure 1.8). This extends the conjugated \(\pi\)-electron system of the chromophore to the imidazole ring of His65 and results in red shifted fluorescence.

At present, three Keade-like PS-VFPs have been isolated, all of which are obligate tetramers. Kaede and EosFP (Wiedenmann et al., 2004) are native PS-VFPs, whereas KiKGr (Tsutsui et al., 2005) was rationally engineered. A monomeric variant of EosFP (mEosFP; Wiedenmann et al., 2004) has been created, however expression of mEosFP is limited to temperatures under 30°C.

1.6.3 Reversible photoactivatable fluorescent proteins

Reversible photoactivatable fluorescent proteins (rPA-VFPs) have the ability to be repeatedly turned on and off. Only two rPA-VFPs, KFP1 and Dronpa have been studied in detail (Chudakov et al., 2003; Habuchi et al., 2005). Dronpa is a monomeric green fluorescent protein (Ex 503 nm/Em 518 nm) and is switched on by near UV irradiation and turned off by intense blue light. KFP1 is a tetrameric red fluorescent protein (Ex 572 nm/Em 695 nm) that is switched on by illumination with green light and is switched off again by blue light. Photoactivation of KFP1 can be made irreversible by using high intensity green illumination. The mechanism of reversible photoactivation has been proposed to involve a cis-trans isomerization of the chromophore, with fluorescence associated to the cis-conformation (figure 1.8) (Lukyanov et al., 2005; Quillin et al., 2005). Recently, the crystal structure of KFP1 in its dark state has been solved and the structural information supports this hypothesis (Quillin et al., 2005; Wilmann et al., 2005).

1.7 Application 1: Direct labeling with VFPs

1.7.1 Promoter activity studies

The first and probably most straightforward application of VFPs is the visualization of up and down regulation of promoter activity and gene expression in vivo (Chalfie et al., 1994;
Cirillo et al., 1998; Soboleski et al., 2005). Although enzymatic assays can be more sensitive, the availability of different colored VFPs offers the ability to study the activity of multiple promoters at once. Fast maturing VFPs are recommended for monitoring promoter activity, to minimize the delay between promoter activation and onset of fluorescence. Furthermore, in order to measure promoter deactivation, VFP fluorescence should be transient. Therefore special destabilized VFP variants have been developed, which contain peptide sequences that promote protein degradation (Li et al., 1998).

Living cells can be labeled using fluorescent proteins, either as single cells in culture or in whole organisms. Within organisms, specific labeling of cell types, tissues or organs can be achieved by using a selective promoter (Hara et al., 2003; Gunawardana et al., 2005; Yu et al., 2005). Especially interesting in this regard is Timer (Verkhusha et al., 2001; Mirabella et al., 2004; Kozel et al., 2006), a DsRed analogue that changes from green to red over time. Monitoring the ratio of green versus red fluorescence of Timer gives information on the history of promoter activity. When expressed by a ubiquitous active promoter, Timer can reveal cell lineage during embryo development. Photoconvertable VFPs can be used to specifically highlight single cells, for example single neurons (Tsutsui et al., 2005) and enable tracking of cells without the need for continuous illumination.

VFP expressing fluorescent cancer cells are a powerful tool to study tumor development and metastasis (Weissleder and Ntziachristos, 2003; Tsuji et al., 2006). Whereas a less scientific use of VFPs is the development of fluorescent pets, for example fluorescent fish (Gong et al., 2003).

1.7.2 Cell organelle labeling

For many proteins the appropriate subcellular location is encoded within the protein by stretches of amino acids, termed targeting sequences. Chimeric proteins, consisting of a VFP fused to a targeting sequence, enable fluorescent labeling of virtually any cell organelle (Gerdes and Kaether, 1996; Patterson et al., 2001). Depending on the targeting sequence even sub-organelle structures can be specifically labeled, for example the inner matrix or the inter-membrane space of mitochondria (Llopis et al., 1998; Porcelli et al., 2005). With the recent discovery of photoactivatable and photoswitchable fluorescent proteins, it is now even possible to highlight and track single organelles (Patterson and Lippincott-Schwartz, 2002; Chudakov et al., 2004).

1.7.3 Labeling of proteins

The most commonly used application of VFPs is to fluorescently label proteins. Before the discovery of VFPs, protein tagging could only be done by chemically labeling purified proteins and microinjecting the labeled proteins into cells (Wang et al., 1982a; Wang et al., 1982b). This is a laborious process and experiments were limited to one or a few cells at a time. Today, with the aid of standard recombinant DNA techniques, chimeric genes encoding fusion proteins of fluorescent proteins are easily constructed and expressed in cells. Although the size of VFPs (∼27 kDa) is considerable, many VFP-tagged proteins
remain fully functional. Nevertheless, it is essential to check whether the VFP label interferes with the biological function.

A prerequisite for using VFPs for protein-labeling is that they should be monomeric, because oligomerization can easily induce aggregation. A drawback in this respect is that many VFPs form dimers or tetramers. In some cases aggregation of dimeric fluorescent proteins can be prevented by using a tandem dimer of the fluorescent protein (Shaner et al., 2004; Peyker et al., 2005). The larger size of the dimer will however increase the risk of artifacts due to steric hindrance. Therefore, it is of key importance to generate monomeric variants of oligomeric VFPs by means of mutagenesis.

1.7.4 Visualization of protein dynamics in the living cell

The most important feature of VFPs is that they enable non-invasive labeling. As a result it is no longer necessary to study fixed cells. It is now possible to study proteins live in action and to track proteins for longer periods of time. With the introduction of confocal microscopy, three dimensional time-lapse imaging has become a standard (x,y,z,t; 4D-imaging). Furthermore, it is possible to use VFP color variants for multi-color imaging to study two or three proteins simultaneously (x,y,z,t,λ; 5D-imaging).

In addition to protein localization, VFP-labeled proteins are excellent for studying protein mobility in living cells. Methods like fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) measure diffusion of labeled proteins (Lippincott-Schwartz and Patterson, 2003; van Drogen and Peter, 2004). The recent discovery of photoactivatable and photoswitchable fluorescent proteins enables novel methods to study protein dynamics (Lukyanov et al., 2005). The advantage of photoswitchable fluorescent proteins is that the photoconverted proteins remain fluorescent and usually less light is necessary for photoconversion in comparison with bleaching.

1.7.5 Visualization of protein-protein interactions

In addition to investigation of protein dynamics, visualization of protein-protein interactions is a key aspect for understanding the regulation of protein-function (especially in signal transduction). Because of the limited spatial resolution of conventional light microscopy, it is impossible to distinguish between two differently labeled proteins that actually interact or merely co-localize. To detect protein-protein interactions in living cells, methods are required which are sensitive to changes in the nanometer range.

Detection of FRET between fluorescently labeled proteins has become an extremely powerful tool for studying protein-protein interaction in living cells. Since FRET is restricted to distances < 9 nm, it provides resolution on the nanometer scale (Bastiaens and Squire, 1999; Gadella et al., 1999; Truong and Ikura, 2001; Zhang et al., 2002). FRET has been used to visualize interaction between a variety of proteins, including oligomerization of receptors (Gadella and Jovin, 1995; Overton and Blumer, 2000; Tertoolen et al., 2001; Patel et al., 2002), complex formation between heterotrimeric G-proteins (Ruiz-Velasco
and Ikeda, 2001; Zhou et al., 2003), transcription factor interactions (Day, 1998; Immink et al., 2002; Tonaco et al., 2006) and many others.

Until now, most studies involving VFP-based FRET used CFP and YFP variants, however with the expansion of the spectral palette of VFPs, novel FRET-pairs have become available. Some of the novel FRET-pairs are superior to CFP/YFP, due to improved contrast and higher FRET efficiencies (Karasawa et al., 2004; Shaner et al., 2005).

An alternative method to measure protein-protein interactions with VFPs is bimolecular fluorescence complementation (BiFC) (Hu et al., 2002). For this, two proteins are labeled with non fluorescent VFP fragments. These fragments can form a functional fluorescent protein only if their fusion partners associate. Simultaneous visualization of multiple protein interactions can be achieved by combining fragments of different colored VFPs (Hu and Kerppola, 2003). Limitations of the BiFC approach are slow maturation of the associated VFP molecule, as maturation of the chromophore is initiated only after association of two VFP fragments. Furthermore, the stereospecific requirements for association increase the risk of false negative interactions and BiFC results in irreversible dimerization of the fusion partners, prohibiting the use of BiFC for dynamic interaction studies.

1.8 Application 2: Indirect labeling with VFPs

An emerging application of VFPs is their use as fluorescent biosensors to sample the interior of living cells. VFP-based biosensors are chimeric fusion proteins, consisting of a VFP fused to a protein domain with affinity for a (non-protein) compound of interest. VFP-based biosensors take genetically encoded labeling beyond the labeling of proteins alone. Fluorescent biosensors can be divided into three groups: sensors with variable fluorescence intensity, sensors that change localization and FRET-based sensors.

1.8.1 Fluorescent biosensors with variable fluorescence intensity

One of the first fluorescent biosensors were cellular pH-sensors, exploiting the fact that the fluorescence of many VFPs is decreased at reduced pH (Kneen et al., 1998) (Llopis et al., 1998). VFP variants have been developed with pKa values close to physiological pH. These pH-sensitive VFPs have been used for pH measurements within the cytosol, mitochondria, golgi and endoplasmatic reticulum. More versatile is the avGFP variant pHluorin, a ratiometric pH-sensor (Miesenbock et al., 1998). Acidification causes a reduction in pHluorin excitation at 400 nm, and a concomitant increase in excitation at 470 nm. Fluorescence of the first generation YFP variants was uniquely quenched by halide ions, due to a cavity in the β-barrel structure, close to the chromophore. Such YFPs have been used as reporters of cytosolic Cl− ion concentration (Wachter and Remington, 1999; Wachter et al., 2000). In addition, a special avGFP variant has been engineered that is sensitive to the redox state of the cell (Dooley et al., 2004; Hanson et al., 2004). For this purpose, surface exposed residues in avGFP were substituted for cysteines which can form a disulfide bond, depending on the redox state of the environment. Formation of a disulfide
bond favored the protonated neutral form of the avGFP chromophore, thereby quenching fluorescence.

Certain fluorescent biosensors contain circularly permutated (cpVFPs) (Baird et al., 1999). The N- and C-terminus of cpVFPs have been connected by a flexible linker and novel termini have been created inside the β-barrel structure of the protein. Since the novel termini are located close to the chromophore, cpVFP fluorescence is very sensitive to conformational changes at the new termini. A fusion of cpYFP to calmodulin and the M13 peptide has been used to create a Ca^{2+}-sensors (Baird et al., 1999; Nagai et al., 2001). Binding of Ca^{2+} to calmodulin causes a conformational change and protonation of the chromophore in cpYFP, which results in a decrease in fluorescence.

1.8.2 Fluorescent biosensors that change localization

Many proteins contain binding sites for recruitment and regulation by (non-protein) second messengers. Well known examples of high affinity binding domains are the C1 and C2 domains of protein kinase C (PKC), which mediate PKC activation by diacylglycerol (DAG) and Ca^{2+} (Hurley et al., 1997; Hurley and Misra, 2000). Another example is the affinity of pleckstrin homology (PH) domains for phosphoinositides (Lemmon et al., 1996; Lemmon and Ferguson, 2001). These regulatory domains consist of conserved motifs, which keep their affinity when fused to a VFP. Lipid synthesis will result in binding of such fusion protein or biosensor to the membrane where the lipid is produced, whereas breakdown will cause release of the sensor. This redistribution (or translocation) can be used to indirectly follow lipid-signaling in time and space. This approach has been used to develop fluorescent biosensors for diacylglycerol (Oancea and Meyer, 1998; Oancea et al., 1998) and phosphoinositides, for example PtdIns3P (Burd and Emr, 1998; Gillooly et al., 2000), PtdIns(4,5)P_{2} (Stauffer et al., 1998; Varnai and Balla, 1998; van der Wal et al., 2001) and PtdIns(3,4,5)P_{3} (Gray et al., 1999; Varnai et al., 1999). The C2-domain of PKCγ is an aspecific lipid binding domain, however the requirement of Ca^{2+} for binding and translocation to the plasmamembrane, provides a highly sensitive biosensor for Ca^{2+}-release (Oancea and Meyer, 1998).

1.8.3 FRET-based biosensors that change conformation

FRET-based biosensors consist of a pair of VFPs capable of fluorescence resonance energy transfer fused to a domain that can undergo a conformational change. The structural rearrangements change the distance and/or the spatial orientation of the fluorophores and hence influence the FRET efficiency.

Conformational changes are the key to a myriad of signaling events, including ligand binding and protein phosphorylation. Numerous FRET-based biosensors have been developed to visualize these events and include biosensors for second messengers, for example Ca^{2+} (Miyawaki et al., 1997; Nagai et al., 2004), cAMP (Nagai et al., 2000; Ponsioen et al., 2004), cGMP (Honda et al., 2001) and Ins(1,4,5)P_{3} (Tanimura et al., 2004; Remus et al., 2006). FRET-based biosensors for specific protein modifications include
biosensors for kinase and phosphatase activity (Violin et al., 2003; Niethammer et al., 2004; Schleifenbaum et al., 2004; Green and Alberola-Ila, 2005). FRET-based sensors for protease activity have been developed by direct fusion of donor and acceptor VFP, using a linker encoding a protease cleavage site (Mitra et al., 1996; Nagai and Miyawaki, 2004; Zhang, 2004). Protease activity will result in irreversible loss of FRET.

1.9 Outline of this thesis

Fluorescent proteins and fluorescence microscopy provide the unique possibilities to study target proteins in their natural environment. This provides more realistic experimental results, especially with respect to protein localization and kinetics. In the complex environment of cells, many parameters contribute to the performance of the protein of interest. Therefore, it is desirable to monitor simultaneously as many of the relevant cellular processes as possible (multiparameter imaging). The large variety of fluorescent proteins and of advanced fluorescence microscopy techniques have extreme potential for multi-parameter monitoring of cellular events. However, to exploit their full potential it is of paramount importance to optimize fluorescent proteins for use in cell biological applications. Furthermore, the expansion of the array of available fluorescence microscopy techniques will generate novel methods to study the spatial and temporal regulation of the living cell. Of special importance is the ability to generate quantitative data from live cell microscopy. In this thesis I focus on the generation of novel optimized fluorescent proteins and the generation of new quantitative microscopy techniques for extracting qualitative information from cells using these optimized fluorescent proteins.

In chapters 2 and 3 the development and characterization of improved folding variants of avGFP variants EYFP, ECFP, EGFP and EBFP is described. Chapter 2 reports the results for the yellow and cyan fluorescent proteins and chapter 3 reports the results obtained for the green and blue fluorescent proteins. Multiple mutants of each color variant are obtained by site-directed mutagenesis. Purified fluorescent protein is used for characterization of the spectral properties, as well as protein folding and chromophore formation. Furthermore, expression in E. coli bacteria and mammalian cells is evaluated.

In chapter 4 a novel procedure is presented to image FRET between fluorescent proteins by means of gradual acceptor-photobleaching. The theoretical framework for the technique is discussed in detail, as well as the benefits and potential pitfalls of this new procedure. Performance of the novel procedure is studied in vitro, using purified protein, and in vivo in mammalian cells.

In chapter 5 a method is presented for quantifying the fractional composition of binary mixtures of fluorophores, based on single-frequency FLIM data. The method of lifetime unmixing can be used regardless of the complexity of fluorescence decay of the fluorescent probes involved. The feasibility of lifetime unmixing for discriminating spectrally identical fluorescent proteins is explored. This is done in vitro, using purified proteins, and in vivo in mammalian cells. The theoretical framework for the technique is discussed in detail, as well as its potential for quantitative multiparameter imaging.
Chapter 6 summarizes and discusses the conclusions that can be drawn from the work described in this thesis. Furthermore, an outlook for the future for the application of fluorescent proteins and fluorescence microscopy is given.

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