1 Introduction and outline of this work

1.2 Outline

In the work presented here, confocal microscopy was combined with high resolution time– and frequency–resolved spectroscopy in order to investigate autofluorescent biomolecules. Section 2 gives a short introduction to the basic concepts of fluorescence microscopy and spectroscopy. Since parts of the work presented here were carried out in living plants, the basics of photosynthesis and the characteristics of plant cells are discussed as well. Furthermore, autofluorescent proteins, representing an important class of fluorescent labels, are introduced. Chapter 3 presents the instrumentation employed for the spectro–microscopic investigations in this work. In addition to that, the molecular biological methods used to manipulate and generate autofluorescent proteins are shortly presented.

Autofluorescent proteins have been engineered such that they act as local sensors to their nanoenvironment. Section 4 presents a precise photophysical characterization of a redox–sensitive green fluorescent protein variant called roGFP2. Redox–sensitivity in roGFP2 is achieved by the introduction of two adjacent cysteine residues into the protein structure that reversibly form a disulfide bond. By means of ensemble and single–molecule spectroscopy, the underlying mechanism of how roGFP2 achieves redox–sensitivity is probed.

When fragments of different AFPs are brought into close contact they may form autofluorescent proteins with unprecedented physical and chemical properties. The analysis of these so–called Bimolecular Fluorescence Complementation (BiFC) complexes in vitro was largely impeded up to now because of their fragments' low solubility. In section 5, a strategy to overcome intrinsic problems of these investigations is presented, and novel AFPs are thereby generated and thoroughly analyzed. Combinations of AFPs are identified that provide favorable photophysical and biochemical properties and single mutations in the amino acid sequence of AFPs are discussed in a broad context.

In section 6, a spectro–microscopic readout technique is used to investigate protein–protein interactions in living plant cells. To this end, different AFPs with the capability of intermolecular energy migration are employed. The fluorescence lifetime measurements are used as a spectro–microscopic readout of this energy migration that only occurs when these AFPs are in very close contact to one another. Thereby, different interactors of the cellular calcium–dependent protein kinase 3 (CPK3) are confirmed.

Section 7 presents a novel spectro–microscopic modality. In more detail, a microscope is presented, capable of recording the fluorescence excitation spectrum in situ from a spot with diffraction–limited spatial resolution. The potential of this approach is demonstrated by recording the excitation spectra of semiconductor nanocrystals with single–molecular sensitivity. This concept is shown to be very useful in studying complex energy transfer cascades, for example in chloroplasts.
Introduction and outline of this work

The evidence of a flexible adaptation of the photosynthetic capacity in living plants, obtained by this technique, is complemented by emission spectro-microscopy. It is shown that the emission spectra reveal minor differences between populations of living plants exhibiting different growth conditions and different extents of carbon starvation. Furthermore, the excitation spectra of plants exhibiting different internal degrees of fitness show that the photosynthesis apparatus is highly dynamic within plants.

The resolution achieved in light microscopy has been limited for centuries by the diffraction limit, quantitatively described by Ernst Abbe. However, over the past two decades, an increasing number of far-field techniques were conceived that break the diffraction barrier by switching molecules between distinguishable states, summarized as “nanoscopy”. Section 9 addresses the implementation of spectroscopic techniques into these novel techniques, estimating the potential of expanding spectro-microscopy to an unprecedented spatial resolution for in vivo sensing.
2 Theoretical background

2.1 Optical spectroscopy

2.1.1 Absorbance and relaxation

All experiments presented in this work are based on spectroscopic methods. Therefore, the basics of optical spectroscopy shall shortly be given in this section. The energy levels of a quantum mechanical emitter (QME) are not continuous but discrete. Therefore, any transition that is promoted by electromagnetic (EM) radiation has to fulfill the resonance condition:

$$\Delta E = h \cdot \nu.$$  \hspace{1cm} (2.1)

The biomolecules that are investigate in this thesis exhibit their transition energies within the visible region of the electromagnetic spectrum.

The transitions between different energetic states of a molecule are best depicted in a Jablonski term diagram, shown in Fig. 2.1 in a simplified form. After absorbance from the electronic singlet ground state $S_0$ to a vibronic substate of the excited state $S_1$, a molecule thermally relaxes within a very short time (femtoseconds to picoseconds) to the metastable vibronic ground state of $S_1$ (Kasha’s rule [8]), from where it further relaxes following different pathways.

![Jablonski term diagram of a typical dye molecule. Radiative processes are colored. Description: see text. The spin orientations are shown as boxed arrows.](image)

These pathways are roughly classified as radiative and non-radiative. Non-radiative pathways include the isoenergetic internal conversion (IC) into a vibronically excited state of $S_0$ with subsequent thermal relaxation to the vibronic ground state. Furthermore, a molecule may may change its spin multiplicity by intersystem crossing (ISC) while keeping its total energy. The molecule therefore ends up in a
triplet state. Since the total angular momentum has to be conserved, the change in spin has to be compensated by a change in the orbital angular momentum. Radiative transitions are fluorescence and phosphorescence where the former designates transitions between states of identical spin multiplicity whereas the latter involves a change in spin multiplicity. Typical lifetimes of the excited states are in the region of nanoseconds for $S_1$ and micro- to milliseconds for $T_1$. Radiative deactivation of excited states can be both spontaneous or stimulated by light.

The vibronic states involved in optical transitions are not random but determined by the potential energies in the ground and excited states. The exact explanation is called the Frank Condon principle and is depicted in Fig. 2.2.

![Fig. 2.2: The Frank Condon principle. Optical transitions occur very quickly and therefore at a constant nuclear distance (left). The probability of a specific transition is given by the overlap of the vibronic wave functions in the ground and excited states. The spectrum of the transitions is depicted on the right side.](image)

The vibronic structure of optical spectra can be understood by taking into account that optical transitions involve the promotion of electrons between different states, triggered by an external, oscillating EM field. The nuclei of a molecule, being about four orders of magnitude heavier than the electrons, cannot follow such a fast reorienting EM field. Therefore, electronic transitions occur at roughly constant nuclear separation (Born Oppenheimer approximation). However, the potential curves of the nuclei is often shifted to altered nuclear separations in the excited electronic state due to a loosened chemical bond. The probability of a specific transition is therefore proportional to the overlap of the squared vibrational wavefunctions of the
initial and final states:

\[ R_{fi} \propto \left| \int \psi'_{vib}^* \psi''_{vib} \, d\tau \right|^2 \]  \hspace{1cm} (2.2)

where \( \psi'_{vib} \) and \( \psi''_{vib} \) designate the vibrational wavefunctions of the final and initial states, respectively. The squared expression in eq. 2.2 is called Frank-Condon factor. It has to be noted that the probability \( p \) of an optical transition is proportional to the squared scalar product of the electronic contribution of the EM field \( \vec{E} \) and the unit vector of the transition dipole moment \( \vec{e}_p \):

\[ p \propto |\vec{E} \cdot \vec{e}_p|^2. \]  \hspace{1cm} (2.3)

The scalar product of two vectors becomes zero in case the angle between the vectors is \( \pi/2 \); it thereby follows that for an optical transition, the electric field and the transition dipole moment must not enclose an angle of \( 90^\circ \).

### 2.1.2 Förster resonance energy transfer (FRET)

Energy may migrate from an excited chromophore to a close-by acceptor non-radiatively if the absorbance spectrum of the latter overlaps with the emission spectrum of the former. The mechanism, established by Theodor Förster [9], involves dipole–dipole coupling. The transfer occurs without emission of a photon to the far-field, with the energy transfer rate constant

\[ k_{ET} = \frac{\Phi_D \cdot \kappa^2}{\tau_D \cdot r^6 \cdot \left( \frac{9 \cdot \ln 10}{128 \cdot \pi^5 \cdot N_A \cdot n^4} \right) \cdot J(\lambda)}. \]  \hspace{1cm} (2.4)

Here, \( \Phi_D \) and \( \tau_D \) denote the fluorescence quantum yield and the excited state lifetime of the donor in the absence of an acceptor, \( r \) is the interchromophoric distance and \( \kappa \) is a factor respecting the relative orientation of the transition dipole moments (see below). \( n \) is the refractive index of the medium between the chromophores and \( J(\lambda) \) is the overlap integral, defined as

\[ J(\lambda) = \int_0^\infty F_D(\lambda) \cdot \varepsilon_A(\lambda) \cdot \lambda^4 \, d\lambda, \]  \hspace{1cm} (2.5)

\[ F_D(\lambda) = \frac{f_D(\lambda)}{\int_0^\infty f_D(\lambda) \, d\lambda}, \]  \hspace{1cm} (2.6)

where \( f_D(\lambda) \) is the fluorescence intensity of the Donor at a wavelength \( \lambda \) and \( \varepsilon_A(\lambda) \) is the absorbance coefficient of the acceptor at the same wavelength.
The orientation factor, $\kappa^2$, is given by:

$$\kappa^2 = (\cos\vartheta_{DA} - 3 \cdot \cos\vartheta_D \cdot \cos\vartheta_A)^2.$$  \hspace{1cm} (2.7)

$\vartheta_D$ and $\vartheta_A$ are the angles between the vector that connects the centers of the transition dipole moments and the transition dipole moments themselves. $\vartheta_{DA}$ is the angle between the planes that are defined by the connecting vector and the transition dipole moments. Values for $\kappa^2$ therefore vary from four (collinear orientation), one (parallel or antiparallel orientation) and zero (perpendicular orientation). For rotating molecules in solution, the average orientation factor, $\langle \kappa^2 \rangle = 2/3$, is often employed.

FRET takes place in a highly distance-dependent manner (eq. 2.4), obeying the famous 6th power law

$$E_{ET} = \frac{R_0^6}{r^6 + R_0^6}$$  \hspace{1cm} (2.8)

where $R_0^6$ is the interchromophoric distance with 50% transfer efficiency. Typical values for $R_0^6$ are in the regime of one to ten nanometers, thereby offering a modality to determine interchromophoric distances which are normally not accessible by microscopic techniques. This is why FRET is often referred to as a “nanoruler” [10], making it particularly interesting for life sciences because this is exactly the range of macromolecular interactions in cells.

### 2.2 Single–molecule spectroscopy and confocal microscopy

Starting from the early 90s, single–molecule spectroscopy has become an emerging field in chemistry, physics and biology [1]. In single–molecule spectroscopy, exactly one copy out of a number of assumingly identical copies of a molecular species is investigated at a time. Whereas in bulk measurements, the mean of an observable quantity is probed, single–molecule spectroscopy provides the full distribution of this quantity (resulting in a histogram). This allows for the observation of phenomena that are averaged out in an ensemble, such as the observation of subpopulations of a molecular species [11,12]. Furthermore, single–molecule spectroscopy obliterates the need for synchronization of e.g. enzymes or molecular motors in dynamical studies, because only one functional entity is observed at a given time [13]. Moreover, single molecules are known to be sensors of their nanoenvironment [1].

Typically, a molecule of interest is surrounded by gazillions of host or solvent molecules. The general requirement for the observation of a single emitter in its surrounding is therefore that the signal by the molecule outperforms the inevitable background contributed by its surrounding. For fluorescent molecules, the first step to achieve this is to embed them in a non–fluorescent matrix. The background of this matrix then consists of Raman scattered light that reaches the detector.
along with the desired signal. The requirement for detection of the molecule of interest then is that the fluorescence signal of the single fluorescent molecule is more intense than the scattering by the matrix. A measure for the light emitted by a single molecule is its cross section\(^1\). For a highly efficient fluorescent dye such as Rhodamine 6G, the fluorescence cross section is in the region of \(10^{-15}\) cm\(^2\), fourteen orders of magnitude above typical scattering cross sections. This means that the detected light is dominated by the fluorescence signal when there are not more than \(10^{14}\) host molecules per one molecule of interest within the detection volume. This translates into picomolar concentrations in case of aqueous solutions. The main task to observe single molecules is therefore (a) to dilute samples sufficiently to assure that there is, at a given time, only one molecule of interest in the detection volume, and (b) to decrease the size of the detection volume in order to minimize the contribution from surrounding molecules. Ideally, the detection volume would have the size of a single molecule of interest; however, there is a physical limit to optical resolution.

Two point–like light sources cannot be perceived as separate objects down to an arbitrarily small distance and similarly, light cannot be focused yielding an arbitrarily small focal spot using lenses [14]. Rather, one has to account for the wave nature of propagating light. Briefly, the wavefront of a plane wave is converted by a lens into a converging spherical wavefront propagating towards the focus (Fig. 2.3a). This conversion is caused by the shape of the lens, being thicker in the center than at the borders. Thus, the wavefront in central areas is more retarded than at the lens’ borders. Every point on this spherical wavefront is itself treated as the source of a spherical wave. Because the focal length of a lens is typically much longer than the wavelength of the utilized radiation, these waves are treated as plane waves in the focal plane (Debye approximation). The incident waves form an interference pattern in the focal region, with a spatial region of non–zero size where the waves interfere constructively. The interference pattern formed by a circular aperture is best described by Fraunhofer diffraction as

\[
I(\vartheta) = I_0 \left[ \frac{2J_1(x)}{x} \right]^2. \tag{2.9}
\]

Here, \(\vartheta\) is the angle of inspection respective to the optical axis, \(I_0\) is the central intensity of the interference pattern, \(J_1\) is the first order Bessel function and \(x\) is given by \(x = ka \sin \vartheta\), with \(k = \frac{2\pi}{\lambda_0}\) being the wavevector and \(a\) being the radius of the aperture. This interference pattern consists of a central disc, surrounded by concentric rings with decreasing intensity. A cross section of such an interference pattern is given in Fig. 2.3b. The area enclosed by the first minimum is commonly

\(^1\)more precisely, the product of the absorbance cross section and the fluorescence quantum efficiency in case of fluorescence and the scattering cross section for scattering.
Theoretical background

2

b) 

Δx

a) spherical wavefront 

Fig. 2.3: a) An incident plane wave (left) is converted into a converging spherical wave (right) by a lens, yielding an interference pattern in the focus. b) Cross section of the light intensity across the focus in x direction, exhibiting a central maximum along with several minima with decreasing intensity. c) Rayleigh criterion for the optical resolution of two point–like light sources.

referred to as Airy disc. From equation 2.9, it follows the condition $x = ka \sin \vartheta = 1.22 \cdot \frac{\lambda_0}{a}$ for the first minimum, meaning the first minimum appears at an angle $\sin \vartheta = 0.61 \lambda/a$. For small angles, typical of imaging optics, this is equivalent to $\vartheta = 0.61 \lambda/a$. The resolving power of imaging optics is essentially delimited by the size of the interference pattern. According to the Rayleigh criterion, two objects in the object plane of a lens can be perceived as separate spots when they are separated by a distance larger than the distance between the central interference maximum and its first minimum [15] (Fig. 2.3c). This means the angular separation has to be at least $0.61 \lambda/a$.

For an objective lens, it follows accordingly that two points can be perceived as laterally separated spots if they are separated by at least:

$$\Delta x = 1.22 \cdot \frac{\lambda_0}{2 \cdot n \cdot \sin \vartheta}. \quad (2.10)$$

Here, $n$ is the refractive index of the immersion medium and $\vartheta$ is half the opening angle of the focusing optics. The product $n \cdot \sin \vartheta$ is the numerical aperture (NA) and is a characteristic of a lens. Similarly, the focus is extended along the z–direction, with the distance between the central maximum and the first minimum:

$$\Delta z = \frac{2n\lambda}{NA^2}. \quad (2.11)$$

Microscopy objectives used in microscopy typically have an NA between 0.7 (water immersion) and 1.45 (oil immersion), resulting in a lateral resolution of roughly 200 to 440 nm (for $\lambda_0 = 500$ nm) and a focal volume of approx 10 to 100 femtoliters. There is an immediate consequence of the focal volume to single–molecule spectroscopy: the prerequisite for single–molecule spectroscopy is that, at a given time,
only one (detectable) molecule is present within the focal volume. This means that the concentration of the molecular species of interest must not be higher than 1 molecule per 10 to 100 femtoliters, translating into a concentration of roughly $10^{-10}$ to $10^{-11}$ mole per liter.

In advanced microscopic studies such as single–molecule spectroscopy, high spatial resolution along with a high detection sensitivity are desired. Confocal microscopy, patented by Marvin Minsky in 1961 [16], meets these requirements imposed by small sample amounts. In a confocal microscope, the foci of the excitation, collection and detection beampaths are conjugated, hence its name. The use of objectives with a high numerical aperture ensures a high signal collection efficiency. The use of focused excitation furthermore prevents excessive sample photobleaching. An exemplary beampath of a confocal beampath is depicted in Fig. 2.4.

Fig. 2.4: Beampath of a confocal microscope. The foci of the excitation, collection and detection beampaths are conjugated. Only light originating in the focus (black) is transmitted through the pinhole whereas out–of–focus contributions (gray) do not reach the detector.

Since only a small spot is illuminated at a given time, no complete image is observed at any time. The image therefore has to be reconstituted by either scanning the focus over the sample or by scanning the sample over the spatially fixed focus. Either way, the light intensity is recorded from the focal region for every position and the image is constructed by recording the light intensity for every single beam
or sample position, corresponding to a single pixel in the final image. Light originating from positions outside the focus is rejected by insertion of a pinhole into the detection beampath (Fig. 2.4). This is particularly useful for blocking light originating from planes outside the focal plane, since this causes a blurred background in other microscopy techniques. Therefore, confocal microscopy combines highest spatial resolution with high image contrast and gentle sample treatment, making it ideal for single-molecule investigations.

2.3 Structure of plant cells

2.3.1 The plant cell

A part of the work presented here addresses scientific questions specific to plants. Therefore, this section gives a short overview of the organization of the plant cells and a few of its distinct properties. A schematic diagram of a plant cell [17] is shown in Fig. 2.5.

![Fig. 2.5: Schematic of a plant cell and its different compartments [17].](image)

Plant cells differ from their animal counterparts in a few compartments. The external boundary of plant cells is the rigid cell wall providing mechanical stability. Cells within a plant are connected by microscopic channels that penetrate the cell wall, the plasmodesmata. Furthermore, plant cells contain a central vacuole that occupies a large portion of the cellular volume and maintains the turgor. The demand of the vacuole for space often squeezes other cellular compounds to the plasma
membrane (syn. plasmalemma) and the cell wall. The vacuole is separated from the cytoplasm by a membrane, the tonoplast. The third compartment differing from animal cells are the chloroplasts, site of photosynthesis and thereby the energetic fundament for life as we know it in general.

2.3.2 Chloroplasts and photosynthesis

Chloroplasts are bordered by a system consisting of two membranes (Fig. 2.6). These membranes enclose the stroma where the chloroplastidiary DNA, ribosomes and starch granules are found. A unique feature to chloroplasts is the branched network of membranes enclosing the thylakoid space. These thylakoids are found in two forms: stacked, in granathylakoids, and unstacked in stromathylakoids. The thylakoid membranes accommodate the protein complexes that carry out the light reaction of photosynthesis. These are the light harvesting complexes (LHCs) and the photosystems (PSs) along with the plastidiary ATP synthase.

Photosynthesis, with the net chemical equation

$$6 \text{CO}_2 + 18 \text{ATP} + 12 \text{NADPH} + 12 \text{H}^+ \rightarrow C_6\text{H}_{12}\text{O}_6 + 6 \text{H}_2\text{O} + 18 \text{ADP} + 18 \text{P}_i + 12 \text{NADP}^+,$$

is the basic process that stores energy by light collection and energy conversion. The photosynthetic reaction is split into two processes: light is collected and its energy is stored in the light reaction of photosynthesis. The key players that carry out the reaction are the photosystems PSI and PSII. The end products of the light reaction are ATP and NADPH. In the dark reaction (Calvin cycle), this energy is used to reduce CO$_2$ to glucose. Here, ATP supplies the energy for the formation of the chemical bonds whereas NADPH provides the electrochemical potential for the reduction of CO$_2$. The light reaction takes place in the thylakoid membranes.

![Fig. 2.6: Schematic of a chloroplast. Description: see text.](image-url)
In contrast to that, the enzymes that catalyze the reactions of the Calvin cycle are located in the stroma of the chloroplasts.

Collection of light is mainly carried out by pigment–protein complexes, the light harvesting complexes (LHCs). The LHCs serve as antennas to collect light. The protein backbone accommodates a large number of pigments, in plants mainly chlorophyll a and b, but also the carotenoids lutein, neoxanthin and violaxanthin. In principle, light could be collected and converted by the photosystems themselves. However, the LHCs are highly specialized compounds, optimized for light absorbance. Thereby, they enhance the light collection capacities in chloroplasts. Electromagnetic radiation absorbed by one of the pigments is transferred with high efficiency to the reaction center of one of the photosystems. There, an energy transfer cascade is started that is often depicted in a z–shaped energy diagram, the z–scheme (Fig. 2.7).

A quantum of light absorbed at PSII (referred to as P680) promotes an electron of the reaction center to an excited electronic state. This electron is transferred to pheophytin (Pheo), basically a chlorophyll molecule lacking the central Mg$^{2+}$ ion. From there, the electron is stepwise transported along an electrochemical gradient involving plastoquinone (Q), the cytochrome b$_6$f complex and plastocyanine (PC). Likewise, an electron is driven to an excited state of PSI (P700) by light. From

![Fig. 2.7: The z–scheme of photosynthesis. P680 is the reaction center of PSII, P700 belongs to PSI. OEC: oxygen evolving complex; Pheo: pheophytin; Q: plastoquinone; PC: plastocyanine; FeS: iron sulfur complex; Fd: ferredoxin; FNR: ferredoxin NADP$^+$ oxidoreductase](image-url)
there, an electron transport chain starts, transferring the electron via intermediate iron sulfur redox centers to ferredoxin (Fd). There, the electron may undergo two different fates: in linear electron transport, it is transferred to NADP$^+$ by the enzyme complex ferredoxin:NADP$^+$ oxidoreductase (FNR), yielding the redox carrier NADPH. In cyclic electron transport, it is transferred back to plastoquinone, from where it is further transported to the cytochrome b$_{6}$f complex. In total, this serves for an adjustment of the ATP to NADPH yield in the light reaction, necessary because three molecules of ATP and two molecules of NADPH are used to reduce one molecule of CO$_2$ in the Calvin cycle. Excitation and transfer of an electron of P680 and P700 leads to the reaction centers of PSI and PSII lacking an electron. These holes are filled, in case of P700 by an electron of plastocyanine, in case of P680 by an electron from water, provided by the oxygen evolving complex (OEC). This complex cleaves water in the reaction

$$2\text{H}_2\text{O} \rightarrow \text{O}_2 + 4\text{H}^+ + 4\text{e}^-$$

and thereby generates the byproduct of photosynthesis, namely oxygen.

Water cleavage at the OEC, electron transport at the cytochrome b$_{6}$f complex and NADP$^+$ reduction at FNR are all accompanied by the net transfer of protons from the stroma to the thylakoid space of the thylakoid membranes. As a consequence, a proton gradient is established. This electrochemical potential is then used to generate ATP by ATP synthase.

### 2.4 Autofluorescent proteins

Over the past 20 years, autofluorescent proteins have gained enormous importance in life sciences. This is mainly due to the fact that they are genetically encodable fluorescent labels. As a consequence, virtually any desired cellular protein of interest can be labeled by simply fusing the encoding DNA of an AFP to the DNA encoding the specific protein under investigation. Thereby, the fluorescent label is generated in vivo, with a one-to-one stoichiometry, enabling for monitoring dynamic processes in living cells. The fact that autofluorescent proteins form their chromophore in an autocatalytic way in any organism except obligate anaerobes makes them a versatile tool in modern biology.

The success story began back in the early sixties when Osamu Shimomura and coworkers reported on the green fluorescent protein (GFP), actually as a purification byproduct of the luminescent protein Aequorin from the jellyfish Aequorea victoria [2]. This was three years before the genetic code was cracked [18] and molecular biology was not a discipline on its own yet. It is therefore no wonder that it took a while before the potential of GFP was fully unleashed.

Shimomura proposed a chemical structure for the chromophore, determined from a hexapeptide obtained by proteolytic breakdown of GFP with papain [19]. His
structure revealed the chromophore being a derivative of imidazolinone and was close to what structural investigations revealed much later. It was, however, not before 1992 that the sequence and primary structure of GFP were determined by Prasher and colleagues [20]. They argued in their report that the chromophore of GFP is an integral part of the protein. This was a remarkable difference to the popular Aequorin which requires a cofactor, coelenterazine, for generating bioluminescence in a Ca$^{2+}$–dependent manner [21]. Therefore, GFP promised to offer fluorescent labeling of cellular proteins by the insertion of one single gene into a host organism. The alternative, Aequorin, would have required at least two genes: one for the Aequorin itself, and one for providing the cofactor, coelenterazine. The GFP gene, leading to fluorescence peaking at around 510 nm, proved to be sufficient to mark organisms shortly after [22].

![Diagram](image.png)

**Fig. 2.8:** a Three dimensional structure of the wildtype green fluorescent protein (wt-GFP). Structure data from [23]. b Structure of the GFP chromophore, an imidazolinone derivative, formed from amino acids Ser$^{65}$, Tyr$^{66}$ and Gly$^{67}$. c Structure of the CFP chromophore, obtained from GFP by mutations S65T and Y66W. d Structure of the YFP chromophore, obtained from GFP by mutations S65G and T203Y.
The structure of the chromophore was revealed in 1993 [24] and the structure of the green fluorescent protein [23] as well as of an engineered variant called “enhanced GFP” (EGFP) [25] was determined shortly after. The three dimensional structure of GFP is shown in Fig. 2.8a. The structure consists of 11 β–sheets forming a so–called β–barrel. The chromophore resides in its center, making it quite inert to environmental disturbances. The structure of the GFP chromophore, formed from amino acids Ser$^{65}$, Tyr$^{66}$ and Gly$^{67}$ is shown in Fig. 2.8b.

The fact that the chromophore is part of the protein and formed from its primary structure opened the way to random and targeted alterations, leading to an ever increasing palette of fluorescent proteins. The first set of mutations involved replacement of the chromophore’s Tyr$^{65}$ by His or Trp [26], resulting in blue (BFP) or cyan (CFP) fluorescent proteins. The latter, however, requires some additional mutations to accommodate the bulkier chromophore [27]. The structure of the CFP chromophore is given in Fig. 2.8c. The structure of GFP also revealed positions that could be employed for targeted alterations. The most relevant of these concerns a pocket within the protein, close to the chromophore, at position 203. Mutation of this Thr for Tyr leads to a bathochromic shift of the absorbance and emission maxima [26], most probably due to a lowering of the energy of the excited state by π–π stacking [28]. The structure of this yellow emitting chromophore is given in Fig. 2.8d. In parallel, the red fluorescent protein DsRed, found in the reef coral Discosoma sp., was also engineered, yielding a set of yellow to red fluorescent proteins [29]. Targeted mutations have led to creation of a whole set of fluorescent proteins.

![Fig. 2.9: Autocatalytic chromophore formation in the green fluorescent protein.](image-url)