10 Summary

In this work, spectro–microscopic investigations were carried out with different auto–emitting biomolecules. Two different classes of molecules were explored: autofluorescent proteins (AFPs) and chloroplast photosystems (PSs). The former are composed as core–shell systems, with a central chromophore largely shielded from the environment by the protein shell. The latter are complicated multichromophoric systems engaged in photosynthesis.

In a first part (sections 4 and 5), autofluorescent proteins (AFPs) were investigated in vitro for determination of their fundamental photophysical characteristics and parameters. The redox–sensitive green fluorescent protein roGFP2 exhibits two absorbance bands, attributed to the protonated and the deprotonated form of the chromophore. It was found that this equilibrium shifts in dependence of the oxidation state of the protein. In context with ab initio calculations, one amino acid, His\textsuperscript{148}, was identified that was postulated to play a major role in the protonation equilibrium. Mutation of this amino acid by neutral glycine indeed revealed an altered protonation behavior of roGFP2. Furthermore, it was found that the fluorescence lifetime of the deprotonated chromophore also depends on the oxidation status of the protein. It could be shown that the key factor is an altered fluorescence quantum yield. Single–molecule studies indicate that the mechanism underlying these observations is due to altered molecular dynamics, more precisely a change in the rigidity of the protein structure in dependence of roGFP2's redox state.

In section 5, complexes that form by bimolecular fluorescence complementation (BiFC) were under investigation. BiFC relies on the fact that two non–fluorescent fragments of an autofluorescent protein reconstitute their fluorescence when brought into close proximity. For instance, this is the case when fusing the fragments to two different proteins which closely approach each other by an interaction event. BiFC complexes also form from fragments originating from different AFPs, however with unknown photophysical parameters. An investigation of these parameters is hindered because the AFP fragments used in BiFC are insoluble ex vivo. The problem was successfully addressed here by directly fusing the coding DNA for the AFPs together, yielding soluble chimeric AFPs. Nine different fluorescent proteins were purified and thoroughly analyzed. In more detail, these proteins show different spectra, depending on the distinct composition. Furthermore, the chromophores' protonation equilibria in the ground and excited states were identified to depend on single amino acid mutations. The fluorescence lifetimes and the fluorescence quantum yields were determined along with the radiative and non–radiative excited state decay–rate constants. This investigation revealed that, depending on the chromophore, single amino acid substitutions greatly enhance or inhibit fluorescence. Finally, the rigidity of these proteins was probed by means of single–molecule spectroscopy. This disclosed that, in general, very bulky amino acid substitutions...
lead to a reinforcement of the AFP shells. However, single mutations turned out to exhibit more complicated effects that can only be understood when taking into account the full AFP context.

Whereas AFPs were thoroughly analyzed in vitro in sections 4 and 5, they were utilized in a typical in vivo application in chapter 6, namely in order to show protein–protein interactions. Eight putative interactors of the A. thaliana protein kinase CPK3 that were identified in a screen based on bimolecular fluorescence complementation were checked for interaction in vivo. Here, AFPs forming FRET–pairs were fused to the interaction pairs. The reduction of fluorescence lifetime of the FRET donor upon energy transfer was shown by fluorescence lifetime imaging (FLIM). In the study presented here, labeled CPK3 and its putative interactors were heterologously expressed in Tobacco epidermal leaf cells. Four proteins could be fully confirmed to interact with CPK3, others were not continuously found to interact, depending on the applied method and the chosen expression system. The results showed that fluorescence lifetime spectroscopy is a valuable complementation to the pool of biological methods, however with the benefit of being accessible in vivo. Overall, the data obtained with different methods demonstrates that FRET–FLIM, BiFC and localization studies represent complementary methods and often need to be applied simultaneously.

In section 7, a novel type of microscope allowing for measuring the excitation spectrum using a confocal beam path was established. To this end, a confocal microscope capable of detecting single molecules was combined with a supercontinuum white light laser for excitation and a grating monochromator for wavelength selection. The capability of this approach was demonstrated by measuring the excitation spectra of single semiconductor nanocrystals, often referred to as quantum dots. The spectra revealed the typical features indicative of single–molecule observation, such as blinking and bleaching in a single step. The observation of single emitters was furthermore confirmed by analyzing the photon stream emitted by the single Qdots in antibunching studies. The Qdots were shown to exhibit very similar spectral features when excited at energies considerably beyond the band gap. However, decided differences were found at the band gap region, originating from the individual size of the Qdots. Furthermore, several wavelengths were identified where the Qdots exhibited an enhanced probability for a transition into a dark state. The exact reason for this, however, still remains speculative.

The microscope described in section 7 was further employed in chapter 8. In more detail, spectro–microscopic studies were performed on chloroplasts of living A. thaliana plants. The plants differed in their growth conditions (more precisely, in the duration of the photoperiod) and in the expression level of the NADP malic enzyme (NADP–ME). Overexpression of NADP–ME led to a shortage of carbon supply and a dwarfed growth phenotype when these plants were grown at short photoperiods. By emission spectro–microscopy, it was shown that these phenotypes are accompa-
nied by a disparate ratio of the plant photosystems I and II. A careful statistical analysis revealed these differences even in plants that showed no different physiological phenotype. The interior of the light harvesting apparatus in the photosynthetic systems was probed by means of fluorescence excitation spectroscopy. It was shown that plants with different NADP–ME expression levels exhibited a different light harvesting capacity. This is attributed to a different embedding of the photosynthetic pigments into the protein backbone which alters the energy transfer efficiency within the energy transfer cascade. This insight purely relies on in vivo studies because any method that investigates photosynthesis in vitro inevitably destroys the cellular context.

Chapters 4 to 8 employed confocal microscopy with a diffraction–limited resolution. Novel techniques make use of the saturable light–induced switching of molecules between different states to break the diffraction barrier. These methods are occasionally referred to as nanoscopy. Up to now, nanoscopy has been successfully used in imaging; however, the spectral information intrinsically present in fluorescence studies has not yet been employed. In this chapter, the applicability of spectro–nanoscopy has been discussed. A way to combine fluorescence lifetime imaging and stimulated emission depletion microscopy (STED) has been proposed. In more detail, the fluorescence evolution after pulsed fluorescence excitation and depletion has been analyzed. It has been shown that the length of the light pulses that is typically used in STED is compatible with an accurate determination of the fluorescence lifetime. Furthermore, it was shown that the stimulated emission efficiency of fluorophores in solution depends on the size of the used fluorophore with small molecules exhibiting a lower efficiency than bigger ones because rotational diffusion is faster for molecules with a small hydrodynamic radius. This is expected to be useful for live cell imaging because autofluorescent proteins with a comparably large size are often employed. The determination of the relevant imaging parameters requires a detailed knowledge of the photophysical parameters of the employed chromophore, in essence closing the circle to chapter 4.