1 Introduction

1.1 Scope of this Thesis

Fluorescence microscopy is a powerful tool to study the subcellular localisation, interaction and intracellular dynamics of fluorophore tagged proteins in living cells [1–3]. The use of green fluorescent protein (GFP) and its derivates to create fluorescent fusion proteins as well as of the labelling of compartments with organic dyes facilitates the in vivo analysis of cell biological processes [1–3].

In the past years, many efforts have been done to improve the spatial resolution of confocal fluorescence microscopes below diffraction limit allowing for the investigation of subcellular compartments and responses on a nanometer scale. This has become possible by combining fluorescence microscopy with new time–resolved laser spectroscopic methods like higher–order pulsed laser irradiation (Stimulated Emission Depletion microscopy, STED) [4, 5]. Different approaches use single molecule blinking statistics (Photo Activated Localisation Microscopy, PALM) [6] and high resolution colocalisation of single molecules (STORM) [7] to obtain ultra–high spatial resolution. For observing dynamic processes associated with single molecules, techniques such as fluorescence correlation spectroscopy (FCS) are available [8–13].

However, live–cell imaging is still hampered due to several inherent problems, which will be described shortly in the following and will get even more obvious in the main text of this thesis.

The major problem in live–cell imaging using fluorescent labels is the interference of unspecific autofluorescence exhibited to a certain degree by every living organism. Therefore, high resolution and sensitive fluorescence microscopy is difficult to carry out in all living systems ranging from single cell organisms like bacteria to cells of higher organisms (mammalian cells). Extreme conditions are given in living plants, due to the additional autofluorescence of cellular compartments typical for plants such as the cell wall. This problem is usually circumvented by studying proteins of higher plants in mammalian cells [14], plant protoplasts [15, 16] or...
Plant cells after plasmolysis [17]. However, protoplast preparation and plasmolysis induce stress responses and may modify the subcellular partitioning, intracellular dynamics and activity of the receptors. Furthermore, expression of plant receptors in heterologous systems may lead to artificial results. Due to these limitations, the knowledge of cell biological mechanisms involving for instance, membrane–associated receptors, which act at the plant cell surface, traffic inside the plant cell and induction of early cellular responses, is restricted.

Additionally, even if the autofluorescence interference is circumvented in any way, fluorescence intensity measurements provide live–cell images with spatio–temporal resolution but usually no information about the physico–chemical parameters in the very close environment of the fluorescence tagged fusion protein. Thus, it is difficult to observe effects of internal or external effects on a GFP fusion protein, which do not result in a change of its localisation and stability but in a change of its nearby environment. Recent progress in fluorescence microscopy, using autofluorescent proteins which show a spectral shift upon changes in the physico–chemical environment such as pH or redox state [18–21], has opened new possibilities in live–cell imaging. Although these mutants have extended the field for intracellular environmental studies, their use has some intrinsic limitations. For instance, it is often not feasible to perform spectral mapping, a method which records fluorescence spectra spatially resolved for every image spot, due to extraordinary long measuring times. In addition, spectral filtering using band pass filters always suffer from spectral “bleed–through” (leakage of a particular signal into a different detection channel), which causes interference of the spectral channels, hampering a quantitative data analysis [22]. Background fluorescence with a broad spectral emission of differential wavelength–specific intensity such as that of the plant cell walls [23] also interferes with the quantitative analysis of environmentally induced spectral fluorescence shifts.

A complex system such as a living organism contains a manifold of components often coupled with each other e.g. in signal transduction or energy transfer pathways. Due to this interplay of several components, it often is highly complicated to investigate these components separately, particularly with
regard to their exact amount and relative concentration. This can be done by extracting the components in question from their native environment but this obviously results in the destruction of the intact organism. For the investigation of variations in the constitution of a coupled system due to changes in external parameters, it is essential to keep the system alive and therefore, one has to develop methods to monitor the constitution of such a coupled system \textit{in vivo}.

This thesis deals with several approaches to circumvent all specified problems inherent in fluorescence microscopy of living cells.
1.2 Outcome of this Thesis

Optical and spectroscopic technologies working at subcellular resolution with quantitative output are required for a deeper understanding of molecular processes and mechanisms in living cells. Such technologies are a prerequisite for the realization of predictive biology at a cellular and subcellular level. However, although established in the physical sciences, these techniques are rarely applied to cell biology in the plant sciences.

This thesis deals with several approaches to circumvent all specified problems inherent in fluorescence microscopy of living cells. A combined application of one–chromophore fluorescence lifetime imaging microscopy (ocFLIM) and wavelength–selective fluorescence microscopy is presented to analyse the subcellular localisation and intracellular dynamics of fluorophore tagged proteins in living plant cells resulting in the revelation of a ligand induced response pathway in living plants.

The experiments also show the potential of one–chromophore fluorescence lifetime imaging microscopy (ocFLIM) for the in vivo monitoring of the biochemical and biophysical subcellular environment using GFP fusion proteins as probes. The excited state lifetime of fluorescent proteins varies in response to alterations in the physico–chemical environment such as the pH–value, the refractive index and the electric field of the surrounding medium. Thus, alterations in the fluorescence lifetime of GFP–fusion proteins have been used to measure these physico–chemical parameters in living plant cells. One–chromophore fluorescence lifetime microscopy, combined with wavelength–specific fluorescence microscopy, opens up new frontiers for dynamic and quantitative in vivo analysis of cellular processes at high resolution which are not addressable by standard imaging technologies or transmission electron microscopy.

Furthermore, a novel method, fluorescence intensity decay shape analysis microscopy (FIDSAM), to suppress autofluorescence interference in common fluorescence intensity images is presented. The method benefits from the fact, that the fluorescence decay shape of a fluorescent label differs significantly from the autofluorescence decay shape. The applicability of this method is demonstrated impressively in
several biological samples including some of the most challenging ones like AFP–fusion proteins in living plants expressed under their native promotor resulting in a very low label–to–autofluorescence ratio.

The photosystems of plants consists of a multitude of interacting components transferring energy from one component to the other. Thus, only the last component of the energy transfer chain can be observed by fluorescence microscopy at room temperature. By applying fluorescence microscopy at low temperature or by extracting the different components from their native environment, this energy transfer can be eliminated. However, due to this energy transfer, it is very difficult to investigate the components separately without destroying the intact, living system. This thesis presents two independent approaches for the investigation of the very complex photosystems and changes in their constitution in vivo.
1.3 Outline of this Thesis

All experiments presented in this thesis have been carried out using confocal optical microscopy combined with time- and frequency resolved spectroscopy. The principles of the applied techniques are provided in Chapter 2. The biological background information for the studies described in this work will be presented in Chapter 3.

The thesis will be divided in five main parts:

PART I: ocFL(I)M
Part I will be concerned with one–chromophore fluorescence lifetime (imaging) microscopy (ocFL(I)M) and its application to use a label protein as probe for its physico–chemical environment. It can be used to monitor cell–specific responses to a corresponding ligand, which do not result in a change of localisation and stability but in a change of the nearby environment of the fusion protein and enables the revelation of a ligand induced response pathway in living plants. Furthermore, a novel application of BRI1–GFP as a probe for the non–invasive determination of the membrane potential and the local P–ATPase activity is introduced.

PART II: FIDSAM
Part II deals with a novel method to suppress the autofluorescence in common fluorescence intensity images and therefore to enhance the signal–to–background ratio. The method is based on the well–known Fluorescence Lifetime Imaging Microscopy (FLIM) technique and is performed by decay shape analysis of the recorded fluorescence decays. The applicability of this method will be demonstrated in several biological samples.

PART III: PSI/PSII Ratio
In Part III fluorescence spectroscopy will be applied to the photosystems of living plants at room temperature. The photosystems are located in the chloroplasts, the energy factories of plants. Their composition will change under extreme external conditions like a deficit of light or carbon, to optimize chloroplast efficiency. However, energy transfer between the different components results in the main detection of photons emitted by PSII and consequently, changes in the composition of the chloroplasts due to changed external parameters will
result only in slight changes of the fluorescence spectra. By accomplishing statistical analysis, it is possible to single out marginal differences in the fluorescence spectra and to determine a relative PSI/PSII ratio in living cells.

**PART IV: FExS**
The method presented in PART III enables the determination of the relative ratio of two main components. However, the photosystems of plants consist of a multitude of components coupled via energy transfer. Therefore these components cannot be investigated separately without destruction of the native environment. One approach to overcome this problem is Fluorescence Excitation (FEx) microscopy. Each component of an energy transfer chain such as the photosystems can be efficiently excited with different wavelengths. Thus, scanning the excitation wavelength continuously allows not only for the determination of the amount of components but even for their relative concentration with high spatial resolution.

**PART V: smSERS on AFPs**
In the last part of this thesis single molecule Surface Enhanced Raman Scattering (smSERS) on autofluorescent proteins (AFPs) will be performed. SERS spectroscopy provides insight into the chemical structure of the investigated molecules and therefore is a competing method to fluorescence spectroscopy. However, SERS spectra of biological samples are often very complex and thus, the correct interpretation of the obtained data is very difficult. On the track to SERS in living cells it therefore is essential to initially focus on individual biological molecules instead of using a great variety of biological molecules as existent in living cells. Consequently, it is suggestive to investigate the AFPs used for labelling as this will later on enable the differentiation between label Raman bands and Raman bands deriving from native cellular components. Furthermore, the SERS spectra presented in this thesis confirm findings about the chemical structure of AFPs obtained by x–ray structure analysis and offer possible future applications of AFPs.