1 Introduction and outline of this work

1.1 Introduction

Natural sciences often address problems that, due to the size of their functional units, cannot be intrinsically perceived by human beings. In life sciences, this scale is often defined by their most relevant object which is a single cell, too small in most cases to be observed by the unaided eye. Any observation of cellular processes therefore relies on instrumentation that magnifies cells to a detectable scale.

The most relevant tool is the microscope, in its simplest form consisting of one single lens. Microscopy has become an independent discipline, providing dozens of methods for nearly any demand. Very high sensitivity—in fact at the ultimate limit of single–molecule detection—and image contrast is achieved in fluorescence microscopy. In addition, fluorescence microscopy provides a diffraction–limited resolution in the sub–micrometer regime, allowing for subcellular localization of a signal. In contrast to other methods, fluorescence originating from chemically different emitters can often be distinguished which allows for multiplexed imaging of cells. Furthermore, fluorophores are sensors of their nanoenvironment [1]. Thus, the fluorescence properties of an emitting species depend on its immediate surrounding such as e.g. local pH, refractive index and ion strength. A readout of the spectral information therefore provides additional information to the spatial origin of a fluorescence signal. This potential of functional imaging in living cells is a unique feature to fluorescence microscopy. The readout of spectral information on a microscopic scale, either time– or frequency–resolved, is summarized in the field of spectro–microscopy.

Within a cell, most structures show either no or only weak fluorescence. Therefore, many investigations employing fluorescence rely on attachment of a fluorescent label to the structure under investigation. Chemical fluorophores often provide favorable photophysical properties such as a high brightness and photostability. However, they need to be introduced externally and are often—to a certain extent—toxic to living cells. Moreover, the exact stoichiometry of the labeling is difficult to control and the behavior of a living cell to such a treatment hardly predictable. Non–toxic labels that offer stoichiometric labeling, best produced by the host organism itself, are therefore desperately required. These demands are met by autofluorescent proteins (AFPs), a class of proteins represented by the widely known green fluorescent protein (GFP), first identified in the jellyfish Aequorea victoria [2]. AFPs form their fluorophore in an autocatalytic way [3] with no cofactor other than molecular oxygen required. By attachment of the DNA that encodes for an AFP to the DNA of any cellular protein, the fluorescent label is produced during translation of the genetic information. The labeling is typically non–toxic and permanent, allowing to monitor cells over a long time. While 20 years ago only one AFP was available, namely GFP,
a whole family of autofluorescent proteins covering the full visible spectrum has been designed [4], both by targeted and by random mutagenesis as well as by discovery of ever new variants in other maritime organisms. Constant engineering of these proteins has led to functional fluorescent reporters exhibiting a spectral response in dependence of their specific environment [5,6]. This makes AFPs a powerful tool in spectro–microscopic imaging of living cells.

A completely label–free readout is provided when the structure under investigation is fluorescent on its own. In plants, the chlorophylls in chloroplasts efficiently collect light which is used for photosynthesis. A small portion of the absorbed light, however, is released as fluorescence, providing a signal in the far–red and near–infrared region of the electromagnetic spectrum [7]. Since chloroplasts are fully packed with pigments, the signal they provide is strong enough to allow for spectro–microscopic imaging.

AFPs and chlorophylls thereby represent autofluorescent biomolecules that can be employed for functional studies in vivo. A robust interpretation of the experimental results, however, relies on a precise knowledge of the behavior of these biomolecules in different environments. Furthermore, the young field of spectro–microscopy still requires an expansion of spectral modalities that can be read out.

This thesis therefore addresses different aspects of spectro–microscopy of autofluorescent biomolecules. In a first part, different autofluorescent proteins are thoroughly analyzed in vitro. After that, the potential of spectro–microscopy is utilized in an exemplary study where protein–protein interactions in living plant cells are under investigation. Subsequently, a novel spectro–microscopic method is presented, allowing for probing the excitation spectrum of a single emitter. Chloroplasts of plants exhibiting different external and internal conditions are analyzed by means of emission and excitation spectroscopy in the next section, and the potential of combining different spectro–microscopic readout techniques at the same photophysical system is discussed. The last section gives an outlook on the potential of implementing spectroscopy in novel super–resolution techniques called nanoscopy.