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Introduction

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1.1. Microspectroscopy

Spectroscopy is the study of the measurement and interpretation of spectra arising from the interactions of all types of radiation with matter. Many different forms of radiation can be used in order to perform spectroscopy and so it is divided into several groups according to the frequency of the radiation used. In this thesis, UV/Vis/Near-IR spectroscopy will play the main role, in which electromagnetic waves from the visible range together with near ultraviolet and near infrared are used. In general, the wavelengths used in UV/Vis/near-IR spectroscopy range from about 200 nm to 1100 nm. Radiation in this energy range causes transitions in molecules in which the electron distribution is changed, that is transitions to electronically excited states. In many cases, specific parts of the molecule can be identified in which the electronic transition is localized. These are commonly known as chromophores.¹⁻³ Compounds with absorption in the visible range of the spectrum are often referred to as dyes. Materials that emit light after excitation are known as luminophores. These are divided into two subcategories: fluorophores and phosphors based on the nature of the excited state from which the emission of photons takes place.

The physical and chemical properties of bulk materials have been analyzed using spectroscopic methods for several decades. Many processes governing the behavior of materials have been characterized and understood. Developments of optical and scanning probe microscopes have allowed observation and manipulation of individual particles, molecules or even atoms. From this point matter could be analyzed not only as bulk material, but also much more deeply, on the atomic or molecular level. The physics and chemistry of single molecules and particles (“single molecule spectroscopy”, SMS) have gained their position in current science because they offer additional understanding of bulk materials down to the level of individual molecules and particles. Measurements performed on ensembles of molecules/particles are statistical. The physical and chemical properties of populations of molecules/particles with different sizes, morphologies, chemical composition or spatial conformation and embedded in different nanoenvironments will be summed up and yield only averaged information. Many properties, however, cannot be explained as an average and in order to separate contributions of different species, it is crucial to be able to probe the behavior of individual molecules/particles. By doing so, the influence of individual parameters on the properties of molecular materials can be investigated and the nature of these materials can be fully understood. An analogous approach can be applied to surfaces, thin films, interfaces or monolayers of materials. Heterogeneities of systems can be revealed by isolating molecules or applying sensitive methodology and probing with a very high spatial resolution.

A big family of techniques which allow to probe properties of molecular materials with spatial and temporal resolution is collectively called microspectroscopy. The next sections in this chapter will be focused on the basic principles of UV/VIS/Near-IR spectroscopy.

1.2. Basic concepts in absorption and fluorescence

Absorption of radiation by molecules occurs due to the existence of electronic, vibrational and rotational energy levels. Most organic dye molecules at room temperature are in a singlet ground state, with spin-paired electrons. Vibrational and rotational levels are occupied according to the Boltzmann distribution, and the absorption of a photon can take place from these different levels to a large number of electronic/vibrational states.

A schematic representation of energy levels of molecules and the most important processes that can occur after excitation are shown in the Jabłoński diagram (**Figure 1.1**).¹

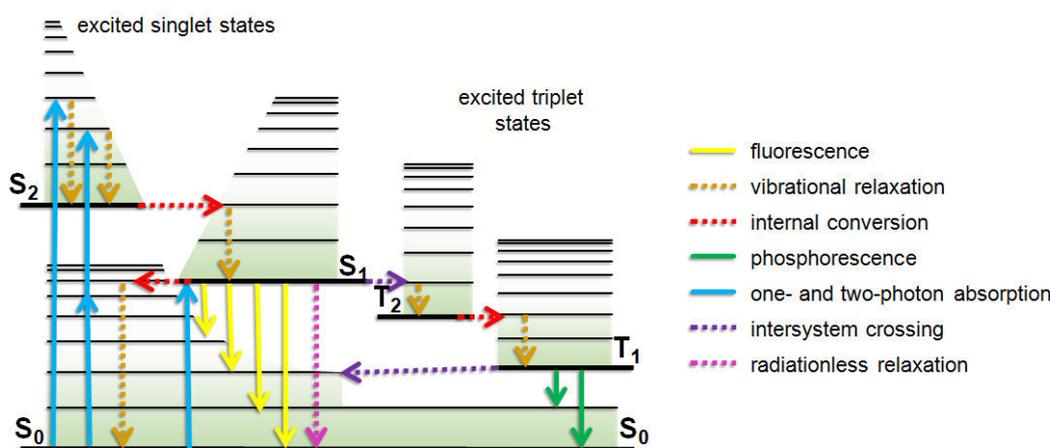


Figure 1.1. Jabłoński diagram.

The scheme shows energy levels of the ground state, two excited singlet states and two triplet states of a molecule. Horizontal lines in the ground as well as in excited electronic states represent vibrational energy levels; rotational are were ignored for clarity of the scheme. Continuous arrows represent processes involving absorption or emission of photons; dotted arrows indicate non-radiative processes.

Excitation of the molecule from its ground state to the singlet excited state takes place after absorption of a photon with proper energy. The energy of a photon is described by Planck's law:

$$E = h\nu = \frac{hc}{\lambda} \quad (1.1)$$

where h is Planck's constant, ν and λ are the frequency and the wavelength of the wave associated with the photon, and c is the speed of light. The photon can be absorbed only if its energy matches with a molecular vibrational/electronic transition. The bands in the UV-Vis absorption spectra of molecules typically correspond to a small number of electronic transitions, each with a set of closely spaced vibrational energy levels. The transition probabilities for electronic absorption, characterized by the molar absorption coefficient $\varepsilon(\lambda)$, vary widely.

The absorption bands corresponding to electronic transitions have a certain width due to the

associated vibrational levels. The highest transition probability corresponds to the vibrational level with the largest Franck-Condon factor. Similarly, emission spectra of polyatomic molecules are often broad due to the transitions to different ground state vibrational levels. The difference between the absorption maximum and the emission maximum, properly expressed in energy units (wavenumbers, cm^{-1}) is known as the Stokes shift.

As mentioned above, in order to excite a molecule the photon has to carry enough energy. However, in a dense photon flux a simultaneous absorption of two (or more) photons can bring a molecule to a higher energy electronic state. This phenomenon was theoretically predicted by Göppert-Mayer⁴ and verified after the development of lasers.⁵ In such a case, the difference of energies between the lower and upper state must be equal to the sum of the energies of the photons. Two-photon absorption is several orders of magnitude weaker than linear absorption.

Directly after absorption of a photon by a molecule in solution, several processes may take place. If the excitation was to one of the higher vibrational levels of the excited state, a relaxation process to give a Boltzmann distribution of the $S_{1,0}$ state will occur (orange arrows in **Figure 1.1**). This so-called vibrational relaxation process appears in a pico- or sub-picosecond time range. The excess energy is dissipated as heat.

The molecule stays in the lowest excited singlet state for a time typically in the nanosecond range, and it will subsequently relax to a state of lower energy. One of the possible relaxation processes to the ground state is emission of a photon (fluorescence). Due to the fast internal conversion and vibrational relaxation fluorescence almost always takes place from the lowest electronic excited singlet state and so the emission spectra are in principle independent on the excitation wavelength,⁶ a phenomenon which is known as the Kasha – Vavilov rule. There are, however, exceptions from this rule.⁷

Several other pathways of relaxation are possible. One is radiationless decay to the ground state, in which the energy is dissipated as heat. Another common possibility is the transition to the triplet state via intersystem crossing. In principle the transitions between singlet and triplet states are forbidden and therefore their rate constants are usually several orders of magnitude smaller than the transitions between states of the same multiplicity. In some cases, strong spin-orbit coupling can facilitate a spin flip leading to efficient intersystem crossing.³ Depending on the relative energies, the molecule can undergo transition from the triplet state back to the singlet excited state and then decay radiatively, a process known as delayed fluorescence. Emission of a photon from the triplet to the singlet ground state is called phosphorescence. Relaxation from the excited triplet state of molecules in solution mostly occurs by non-radiative pathways on the microsecond time scale.

Molecules in excited singlet or triplet states are often more chemically reactive than molecules in the electronic ground state. Any process that removes molecules from the excited state via an interaction with other species is called "quenching".¹ Some quenching processes may lead to photochemical reaction products, irreversibly removing the fluorescent molecule from the sample, but others may simply provide radiationless decay channels.

The typical time constants of all processes discussed here are collected in **Table 1.1**.

Table 1.1. Timescale ranges for photophysical processes.³

transition	process	rate constant	timescale / s
$S_0 \rightarrow S_1$ or S_n	absorption	-	10^{-15}
$S_1 \rightarrow S_1$	vibrational relaxation	k_{vr}	$10^{-12} - 10^{-10}$
$S_1 \rightarrow S_0$	fluorescence	k_f	$10^{-10} - 10^{-7}$
$S_1 \rightarrow T_1$	intersystem crossing	k_{isc}	$10^{-10} - 10^{-8}$
$S_n \rightarrow S_1$	internal conversion	k_{ic}	$10^{-11} - 10^{-9}$
$T_1 \rightarrow S_0$	phosphorescence	k_p	$10^{-6} - 100$

1.3. Quantitative measures of molecular photophysics

The molar absorption coefficient ϵ , the fluorescence quantum yield Φ_f and the fluorescence lifetime τ_f are the most important parameters describing the properties of fluorophores. Each of them is widely employed in spectroscopy and microscopy and allows to obtain quantitative information on photophysical properties of molecules.

The molar absorption coefficient is the direct measure of the ability of a given chromophore to absorb a given wavelength of light. It allows to connect the absorbance of the compound A into the molar concentration c in the solution according to the Beer-Lambert law:

$$A(\lambda) = \epsilon(\lambda)cl \quad (1.2)$$

where l is the light path length.

The absorption coefficient is also related to the radiative lifetime of the fluorophore, which is inversely proportional to it: compounds which have a high molar absorption coefficient have a high radiative rate k_f .⁸

The luminescence quantum yield is a measure of the probability of an excited fluorophore to emit a photon. It is defined as the ratio of the number of emitted photons to the number of photons absorbed. The quantum yield can be also expressed in terms of rate constants of radiative and non-radiative processes (see **Table 1.1**), eg. for fluorescence:

$$\Phi_f = \frac{n_{ph\ em}}{n_{ph\ abs}} = \frac{k_f}{k_f + k_{isc} + k_{ic}} \quad (1.3)$$

where $n_{ph\ em}$ and $n_{ph\ abs}$ are numbers of photons emitted and absorbed, respectively, k_f , k_{isc} and k_{ic} are the rate constants of fluorescence, intersystem crossing and internal conversion. The quantum yield may vary from 0 (non-luminescent compounds) to almost unity for the best fluorophores. Φ_f of given compound normally depends on environmental factors, for example solvent polarity, pH, concentration.

The measurement of the absolute quantum yield is difficult.⁹ In practice, the quantum yield is most often determined by comparison with a fluorophore of known quantum yield, a so-called quantum yield standard.¹⁰ The calculation of Φ_f of a compound requires comparison of the integrated fluorescence intensities of the compound and that of the standard (**Equation 1.4**).

$$\Phi_u = \frac{(1-10^{-A_u})I_u n_u^2}{(1-10^{-A_r})I_r n_r^2} \Phi_r \quad (1.4)$$

In **Equation 1.4** indexes u and r are the unknown and reference respectively, Φ is the luminescence quantum yield (fluorescence or phosphorescence), A is the absorption factor at the excitation wavelength, I is the integrated emission intensity and n is the refractive index of the solvent.

The fluorescence lifetime is a measure of the time a fluorophore spends in the singlet excited state. During this time, the molecule can change its conformation or collide with other molecules allowing energy transfer, electron transfer, or other processes to take place. For a population of fluorophores, the decay of the fluorescence intensity after a short excitation pulse is described by an exponential function of time, described by the model:

$$I(t) = I_0 \exp(-t/\tau) \quad (1.5)$$

where $I(t)$ is the fluorescence intensity at time t , I_0 is the initial intensity observed immediately after excitation, and τ is the fluorescence lifetime defined as the time for which the initial fluorescence intensity decays e times. The lifetime is the inverse of the excited state decay rate $k_f + k_{isc} + k_{ic}$.

In the case that there are more complex processes involved in the dynamics of the excited state the fluorescence decay curve may be properly modeled using multiple exponential decay functions. Multiple exponential decay also results naturally from a heterogeneous sample, in which otherwise identical fluorophores are in different nanoenvironments.

Most of the common fluorophores have their lifetimes on the time scale of nanoseconds but these values can strongly depend on the environment and the molecular structure. Lifetime measurements sometimes allow to distinguish fluorophores in different environments. Knowledge of the excited state lifetime of a fluorophore is very helpful for quantitative interpretation of various fluorescence measurements.

1.4. Solvent effects on fluorescence

Fluorophores in solution are surrounded by solvent molecules. Because each of these molecules has its dipole moment, the fluorophore and solvent molecules rearrange with respect to each other in a way to bring the system to its lowest free energy state. After excitation of the dye molecule to the higher vibrational levels of the excited state a vibrational relaxation on a picosecond time scale will take place. The molecule in its excited state often has a larger dipole moment (and possibly a different orientation) than in the ground state.

During the lifetime of the excited state the solvent molecules will stabilize the fluorophore (its dipole moment) by re-orientation to further lower the free energy of the excited state. The time of solvent relaxation processes is ca. $10^{-12} - 10^{-10}$ s in non-viscous fluid solutions. The different stabilization of the ground and excited states of fluorescent molecules causes a change in the energy gap between these electronic states depending on the polarity of the solvent. Thus, the spectral position of the emission (and sometimes also intensity and shape of the spectra) depends on the polarity of solvent. **Figure 1.2** shows schematically the mechanism of this so-called solvatochromism of dye molecules.

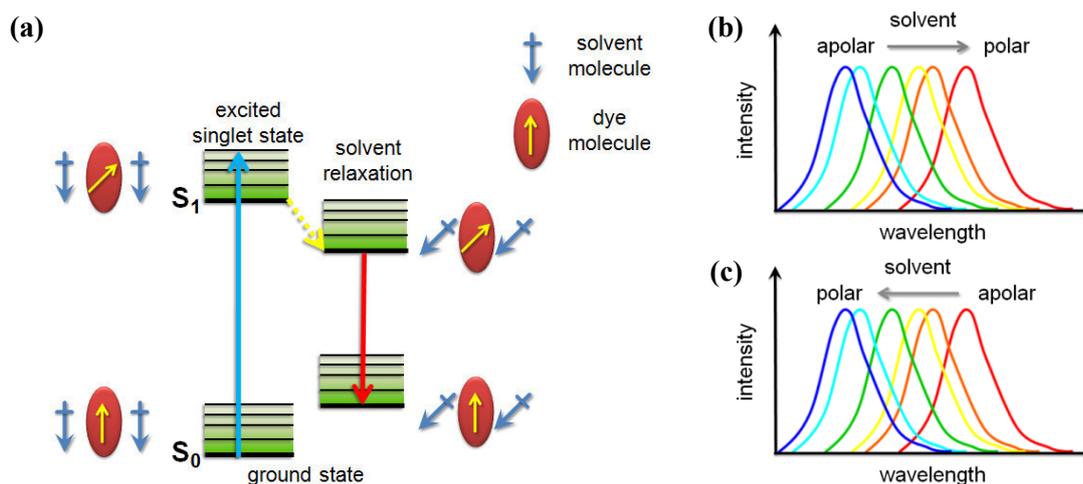


Figure 1.2. Principles of solvatochromism: (a) schematic representation of mechanism of solvatochromism; solvent relaxation around the fluorophore. An effect of (b) positive solvatochromism - bathochromic shift, (c) negative solvatochromism – hypsochromic shift with increasing solvent polarity.

The influence of the solvent polarity on the solvatochromic compound can be observed directly as a change of the color of the solution, depending of the solvent. There are two distinct kinds of solvatochromism: positive and negative. A positive solvatochromic compound gives a red (bathochromic) shift with increasing the polarity of the solution (**Figure 1.2 b**). A negative solvatochromism corresponds to a blue (hypsochromic) shift of the emission spectrum upon increasing the polarity of the solvent (**Figure 1.2 c**). The type of the solvatochromism depends on the difference between the dipole moments of fluorophore in its ground and excited states.

The solvatochromism of dye molecules has found many applications in sensing environmental changes of polarity.¹¹⁻¹³

1.5. Anisotropy

Excitation of a fluorophore by absorption of a photon is a process in which the oscillating electric component of the incident radiation interacts with the transition dipole moment of the molecule. If the light is linearly polarized, the probability of excitation of the fluorophore is proportional to the square of the scalar product of the transition dipole moment and the electric vector of the excitation radiation. The probability is maximal when the vectors are

parallel and zero in the case of perpendicular vectors. The efficiency of excitation of the molecule will be proportional to the cosine squared of the angle between the two vectors.

Illumination of a population of dye molecules with linearly polarized light will preferentially excite fluorophores with the absorption dipole moment oriented in a direction parallel to that of the electric vector of the incident photon. Because of the isotropic distribution of the fluorophores in most samples, only an anisotropic sub-population of them will be excited (photoselection) and the fluorescence emission can be expected to be partially polarized. Many processes can, however, cause depolarization of the fluorescence. First of all, the fluorescence emission of each molecule is polarized in a direction of the emission transition dipole moment. In general, the directions of absorption and emission transition moments are not parallel. Non-parallel absorption and emission transition moments are the first source of depolarization of the emission fluorescence.¹⁴

Besides that, the dye molecule stays in its excited state for a while and during this time Brownian rotational motion of the fluorophore will depolarize its emission. From the extent of fluorescence depolarization, one can obtain information on the molecular motion, which depends on the size and shape of the molecules and the viscosity of their microenvironment (**Figure 1.3**).¹⁴

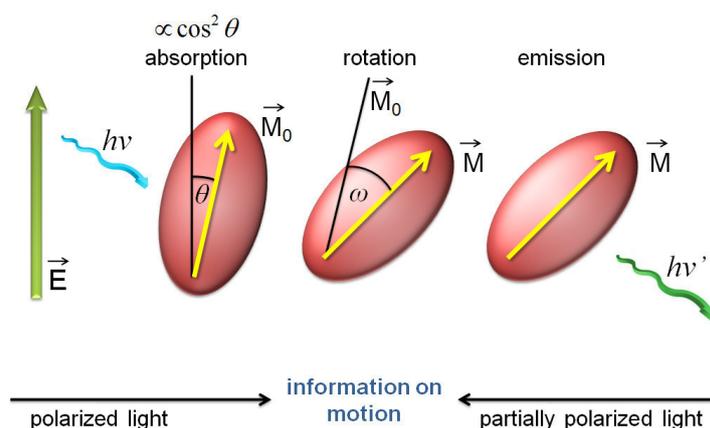


Figure 1.3. Depolarization of fluorescence caused by rotational motions of a fluorophore. \vec{E} is the electric vector of incident light, $h\nu$ and $h\nu'$ are the excitation and emission photons, vectors M_0 and M are the absorption/emission transition moments at $t = 0$ and $t \neq 0$. The absorption and emission transition moments were assumed to be parallel. Adapted from ref. 14.

The measurement of the polarization state of fluorescence relies on excitation of the fluorophores with polarized light and detection of the emission in two independent polarization channels: parallel and perpendicular with respect to the excitation light polarization. The degree of fluorescence polarization is characterized either by the polarization ratio:

$$p = \frac{I_{//} - I_{\perp}}{I_{//} + I_{\perp}} \quad (1.6)$$

or the emission anisotropy:

$$r = \frac{I_{//} - I_{\perp}}{I_{//} + 2I_{\perp}} \quad (1.7)$$

where $I_{//}$ and I_{\perp} are the intensities of the fluorescence emission with polarization parallel and perpendicular to the excitation light polarization, respectively.

In order to obtain quantitative information from the anisotropy, the timescale of rotational motions of molecules has to be of the order of their excited state lifetime. If the rotational correlation time of molecules exceeds the excited state lifetime (for example for fluorophores attached to larger macromolecules or embedded in rigid environment), no information on the motions can be obtained from emission anisotropy measurements because the motions occur outside of the experimental window. In the case of very fast rotations (significantly faster than the emission rate) the measured anisotropy will be zero due to completely random distribution of emission transition dipole moments of fluorophores and total depolarization of the emission light.

During sample preparation for anisotropy measurements one has to be careful not to use too high concentration because of the possible energy transfer between fluorophores, which also leads to depolarization of the emission.

1.6. Fluorescence microspectroscopy

Fluorescence methods, due to their high sensitivity and intrinsic selectivity, are a very important spectroscopic tool in natural sciences and technology. Fluorescence techniques are continuously improved in particular in combination with microscopy. The trend in the evolution is towards an increase of spatial and temporal resolution and contrast between signal and background.

The great progress of fluorescence microspectroscopy was possible thanks to development of modern, high power, often tunable pulsed lasers and ultra-sensitive detectors. Combining those with confocal microscopes or reflection optics initiated many new experimental approaches, for example: picosecond or sub-picosecond time-resolved fluorescence¹⁵ and absorption microscopy,¹⁶ picosecond time-resolved reflection fluorescence spectroscopy¹⁷⁻¹⁸ or femtosecond time-resolved diffuse reflectance spectroscopy.¹⁹⁻²⁰ Lasers have been also used for catching, transferring and fixing individual particles at a certain position. Combining the trapping method with time-resolved microspectroscopy allowed to develop for example trapping fluorescence spectroscopy.²¹

By far the most important application of fluorescence microspectroscopy, however, is in the field of biology. Cells, or cell components, can be visualized in real time with high spatial resolution. The use of single molecule methods allows to beat the diffraction limit (see **Chapter 2**) because a single molecule can be localized with nanometer precision as it is at the position of the maximum intensity of the point spread function. Sub-diffraction imaging requires a sufficiently high density of labels, but these labels can be observed one-by-one if they can be switched on and off. A variety of methods is now available to achieve this.²²⁻²⁵

Fluorescence spectroscopy requires that the measured sample shows fluorescence/luminescence. If the object of interest is not fluorescent, it must be labeled with a species that emits light upon excitation. In order to obtain high signal-to-background ratio, the chromophore must have specific photophysical properties such as high efficiency in absorption and emission, preferably large Stokes shift, and high photostability.

Efficient absorption of the excitation light by fluorescent species makes it possible to use low excitation powers, which in turn reduces the amount of unwanted, scattered light, which needs to be filtered out of the useful signal. Strong emission allows to accumulate enough photons from the sample in order to extract the desired information on photophysics of the chromophore in given surroundings.

A large Stokes shift is preferential for molecules used as chromophores from a practical experimental point of view. The difference between the maximum absorption and maximum emission makes possible excitation at the absorption maximum and recording the whole spectral range of emission. Capturing the maximal amount of fluorescence light will increase the ratio between desired signal and unwanted background. The ratio can be, however, very much decreased due to reduction of the emission intensity caused by quenching of the fluorescence. This process originates from existence of many inter- and intramolecular radiationless relaxation pathways competing with the fluorescence. Quenching of the fluorescence can be caused for example by forming non-fluorescent complexes between fluorophore and quencher, energy transfer, charge transfer or intersystem crossing to the triplet state.²⁻³

A high stability of the compound is very important. The number of photons captured before irreversible photobleaching of the molecule(s) must be large enough to extract desired information from the measurement. The average number of excitation – emission cycles depends also on the fluorophore itself. Some dye molecules are more immune to bleaching than others. The typical number of photons emitted by a single molecule during its life span in ambient conditions can be on the order of 10^6 .²⁶

Photobleaching of fluorophores is the main practical limitation of SMS. All molecules upon repeated electronic excitation will undergo an irreversible chemical reaction at some time. Depending on the dye and the light intensity survival times range from less than seconds to hours. Typically, single molecules at room temperature are observed for about a minute on average before photobleaching. Many reaction types can be responsible for photobleaching. For most cases the details are not known. Usually, exclusion of oxygen, to avoid oxidation reactions, is favorable, but in some cases oxygen has a stabilizing effect because it decreases the lifetime of the long-lived triplet state.²⁷

Although photobleaching is usually undesired, it can also be turned into use as for example fluorescence recovery after a photobleaching (FRAP) in a small volume can be a measure of anisotropy or diffusion of molecules in particular environments.³

At a single molecule level, fluorescence traces of individual molecules shows so-called blinking. This phenomenon is a switching of the fluorophore between fluorescent and non-fluorescent states. The switching is spontaneous and appears on time scales ranging from milliseconds up to hours.²⁸⁻²⁹ In principle, the blinking can be a serious obstacle for successful SMS, while at the ensemble level, the effect of blinking of individual molecules is averaged and causes decrease of the emission intensity. The fluorescence blinking can assigned to long-

lived dark states but the nature of these in many cases is still a subject of study.³⁰⁻³¹

1.7. Outline

This thesis presents a variety of applications of microspectroscopy. After an overview of fundamental phenomena governing molecular fluorescence in this introduction, **Chapter 2** will be devoted to the presentation of the experimental setups. It contains descriptions of instrumentation for steady-state and well as time-resolved spectroscopy and microscopy that were used for the measurements presented in the next chapters. **Chapter 3** and **Chapter 4** describe a molecular probe characterized by a photoinduced intramolecular electron transfer which was used as a tool for studying of the dynamics of polymer material around its glass transition temperature.

Chapter 5 presents and discusses photophysical properties of small silicon nanoparticles coated with a thin organic layer. **Chapter 6** deals with observations of a pyrrolidine substituted perylene imide which at the single molecule level shows spectra that are blue shifted compared to those taken on ensemble level.

Examples of applications of confocal and two-photon microscopy for studying a variety of systems are presented in **Chapter 7**. This chapter is a result of work in collaboration with other scientists from our department.

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