
# FLUORESCENČNÍ SPEKTROSKOPIE A MIKROSKOPIE: PRINCIPY, APLIKACE A SOUČASNÝ VÝVOJ

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# BASICS OF FLUORESCENCE SPECTROSCOPY

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**Abbreviations:**

| | |
|---|---|
| BODIPY | Derivatives of 4-bora-3a,4a-diaza-*s*-indacene |
| DPH | 1,6-diphenyl-1,3,5-hexatriene |
| ET | Energy transfer |
| 2D FLIM | 2-dimensional Fluorescence Lifetime Imaging |
| FRAP | Fluorescence Recovery After Photo-bleaching |
| FRET | Fluorescence resonance energy transfer |
| NBD | Derivatives of 7-nitrobenz-2-oxa-1,3-diazol-4-yl |
| NFOM | Near-Field Optic Microscopy |
| SNOM | Scanning Nearfield Optic Microscopy |
| SR | Solvent Relaxation |
| STM/AFM | Scanning Tunnel Microscopy / Atomic Force Microscopy |
| SUV | Small Unilamellar Vesicles |
| TCSPC | Time-Correlated Single Photon Counting |
| TIRF | Total Internal Reflection Fluorescence |
| TIR-FRAP | Total Internal Reflection Fluorescence Recovery After Photobleaching |
| TMA-DPH | 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene |
| TRES | Time-Resolved Emission Spectra |

## 1. INTRODUCTION

After the pioneering works of Kasha, Vavilov, Perrin, Jabłoński, Weber, Stokes, and Förster (including the appearance of the first book of the latter on fluorescence of organic molecules in 1951 [1]), fluorescence spectroscopy became a widely used scientific tool in biochemistry, biophysics, and also in material sciences. Within the last years, however, several new applications based on fluorescence have been developed, promoting fluorescence spectroscopy from a primarily scientific to a more routine method. The phenomena of fluorescence is, for example, exploited in simple analytical assays in environmental science and clinical chemistry, in cell identification and sorting in flow cytometry, and in imaging of single cells in medicine. Though there is a rapid growth in the number of routine applications of fluorescence, the principles remain the same. This text aims at a condensed but comprehensive description of the principles and selected applications of fluorescence spectroscopy.

Fluorescence of a molecule is the light emitted spontaneously due to transitions from excited singlet states (usually $S_1$) to various vibrational levels of the electric ground state, i.e., $S_{1,0} \rightarrow S_{0,v}$. It can be characterized by several parameters. The most important among them are the fluorescence intensity at a given wavelength, $F(\lambda)$, the emission spectrum (i.e., dependence of emission intensity on the emission wavelength), quantum yield ($\Phi$; see par. 4.1), lifetime ($\tau$; see par. 4.1), and polarization ($P$; see par. 3). These parameters can be monitored in a steady-state or in a time-resolved manner. They carry information on both photophysical properties of the fluorescing molecule and the chemical and physical nature of its microsurrounding. The following section will specify such parameters and describe how they are influenced by intra- and inter-molecular processes.

# 2. FLUORESCENCE AND ITS MEASUREMENT

## 2.1 Molecular electronic relaxation

Schematic representation of spontaneous molecular relaxation processes that follow any excitation of a molecule to a higher electronic excited state (e.g., by absorption of a photon) is depicted at Fig. 2.1 in the form of a Jabłoński diagram.

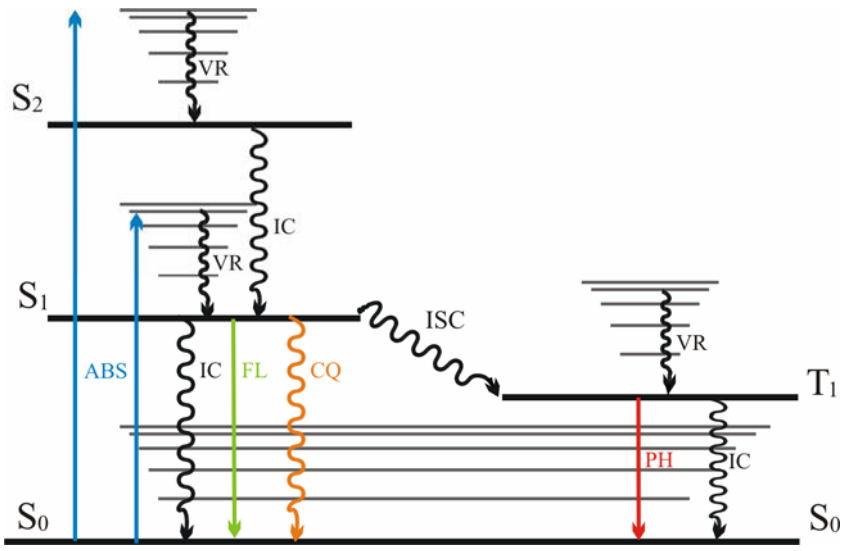


***Fig. 1:*** ***Jabłoński diagram illustrating the creation and fate of a molecular excited single-state, including absorption (ABS), fluorescence (FL),phosphorescence (PH), internal conversion (IC), intersystem crossing (ISC), vibrational relaxation (VR), and collisional quenching (CQ). Not included are processes like solvent relaxation, energy transfer, and photochemical reactions***

Fluorescence emission is clearly one of the several possible, mostly non-radiative processes that compete with each other. Thus, fluorescence intensity, emission wavelength, time-behaviour, and polarization can be indirectly influenced by every interaction of the fluorescing molecule that can either change probability of any of the competing relaxation processes (as of internal conversion – IC, or intersystem crossing – ISC, in particular), or that can introduce a new relaxation pathway as, e.g., photochemical bonding or a simple proximity of a heavy atom or a particular chemical group. Many biochemical and biological applications of the fluorescence are based on those phenomena, e.g., the widespread usage of a broad variety of fluorescent probes, just to name one.

## 2.2 Detecting fluorescence

The principal fluorescence measurement arrangement is depicted at Fig. 2, where the most important properties (parameters) are listed for both exciting radiation and fluorescence emission. Not all of those parameters are necessarily known or well specified for every spectrofluorometric instrument; but any attempt on sophisticated analysis and interpretation of the fluorescence data should be accompanied by a rigorous measurement of all those from the listed parameters that are relevant to the interpretation. The following examples of fluorescence spectroscopy applications will also indicate this aspect of practical fluorescence measurements.

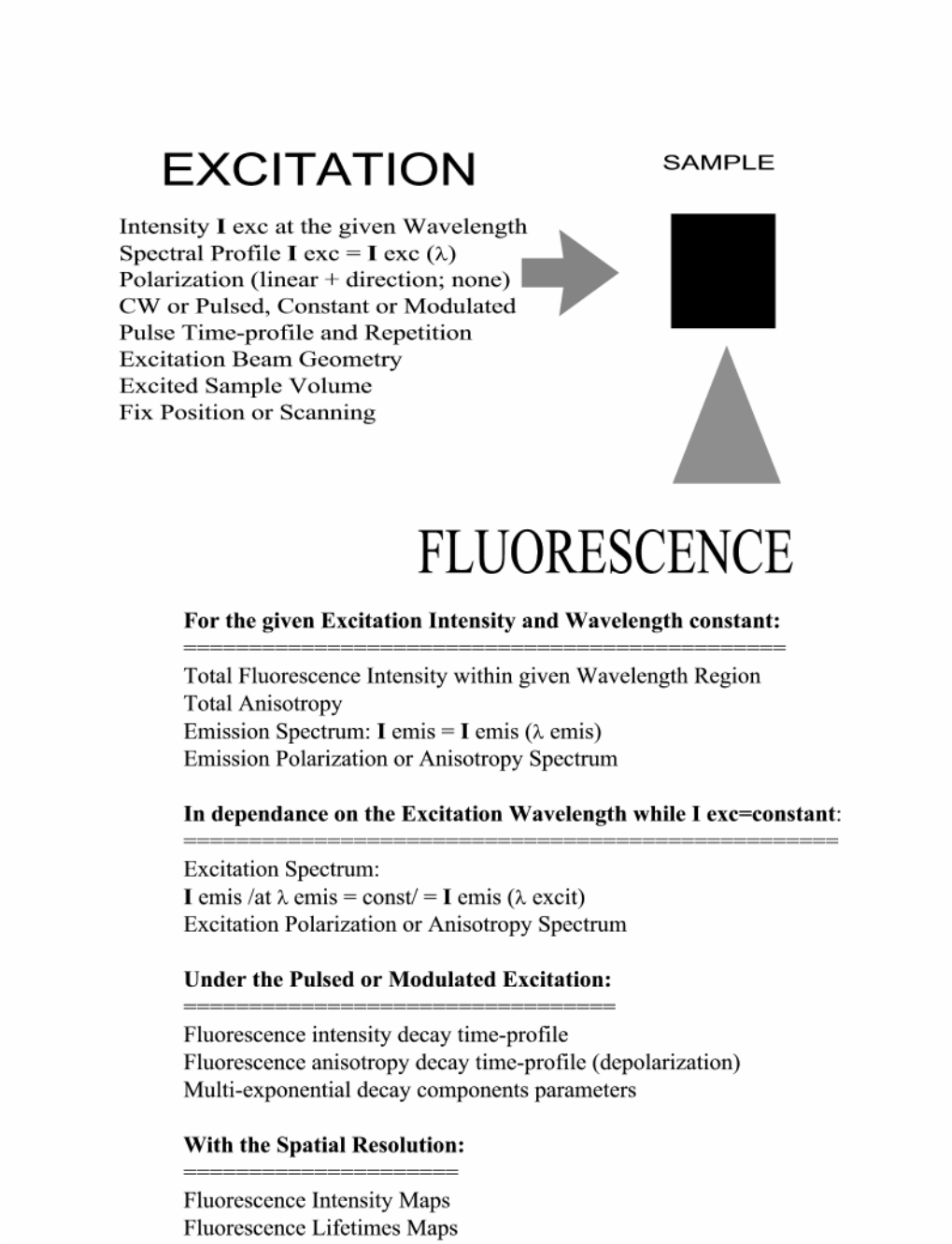


***Fig. 2: Summary of main variables and read-out parameters of fluorescence experiments***

Fluorescence of an object of interest can be detected in various ways. Beside of the classical solution fluorescence measurement in different types of cuvettes, there are several advanced ways of detecting the fluorescence signal. The use of fiber optics allows for measuring fluorescence even in biological organs *in vivo*. When looking at cells, one can use cell culture plates or flow cytometry in combination with optical microscopy. Selected spots within a cell can be monitored using classical, confocal, or multiphoton microscopy. Advanced techniques of single molecule spectroscopy, total internal reflection fluorescence microscopy, fluorescence correlation

spectroscopy, and other advanced techniques will be briefly introduced on the workshop. Two trends in recent developments of fluorescence techniques, often both combined within one instrument, should be mentioned here:

(1) high spatial resolution (extremely small volume probed or, e.g., a combination of local fluorescent probe FRET with SNOM techniques as in [2]);

(2) high time resolution, performed simultaneously [3].

An illustration of such instrumental development is the space-resolved TCSPC detector used by [4] for 2D FLIM with 500 nm spatial and 100 ps time resolution. New technology combining, e.g., NFOM or STM/AFM with high resolution photon timing when each detected photon is tagged with all other information related to it allows for multi-dimensional fluorescence lifetime and fluorescence correlation spectroscopy to be preformed during one measurement [3]. Single molecule fluorescence characterization thus can now be done with unprecedented accuracy and depth [3, 5].

## 2.3 Data evaluation

For the primary spectroscopic raw data treatment relevant to the technique used for a particular fluorescence measurement (such as correction of spectral intensity for the sensitivity of a detector, etc.), we refer to the instrument producer manuals, to basic physics textbooks, and to comprehensive books on fluorescence [as 22, 23, 24]. Also topics as fluorescence quantum yields evaluation and steady-state spectra analysis (e.g., decomposition) are covered by such literature [24]. Mathematically much more complicated is the fluorescence kinetics data treatment necessary for fluorescence lifetime and rotational correlation time calculation. Furthermore, mathematical models differ substantially with the detection technique used: time-correlated single photon counting [e.g., 23] or phase-shift measurement [e.g., 22]. For data evaluation methods in fluorescence correlation spectroscopy (such as number of particles and diffusion time calculations) see, e.g., [25], for fluorescence recovery after photobleaching (rate and extent of recovery calculations) see, e.g., [26, 27], internal reflection fluorescence parameters see, e.g., [28]. Moreover, there are many comprehensive books on optical spectroscopy covering aspects of techniques and data analysis of the fluorescence spectroscopy as well, e.g., [29, 30], just to name two of those.

# 3. POLARIZED FLUORESCENCE

Interaction of the exciting light with a molecule can be described as an interaction of the electric field component of the light with the relevant transition (electrical) dipole moment of the molecule. Thus, the absorption of the light quantum is proportional to the cosine of the angle between the two directions, i.e., between the excitation light polarization plane and the transition moment vector. Consequently, excitation by linear polarized light leads to an anisotropic spatial distribution of the excited molecules: those with transition dipole moment parallel to the light polarization can be excited, those in perpendicular position can not (this phenomenon is called **photoselection**). Resulting anisotropy can persist even up to the later moment of fluorescence emission, yielding partially polarized emitted light. Such fluorescence polarization will decay faster with higher rotational diffusion of the excited molecule, and it can be diminished further by, e.g., an excitation energy transfer. The rotational diffusion depends on the (micro)viscosity of the environment, and on the size and shape of the excited molecule. This connection represents the basis for applications

of fluorescence polarization studies. The depolarization by excitation energy transfer is often an undesirable process. However, it occurs only in concentrated solutions when the average distance between molecules is not much above 5 nm. Thus, this kind of depolarization can be avoided by the use of dilute solutions.

## 3.1 Definition of polarization and anisotropy

Direction of light polarization is conventionally specified with reference to a system of laboratory coordinates defined by the propagation directions of the excitation beam and of the fluorescence beam. It is customary to observe the fluorescence beam resolved in directions parallel ($F_{II}$) and perpendicular ($F_{\perp}$) to the direction of the linear polarized excitation light ($E_{II}$). The degree of fluorescence polarization $P$ is defined as

$$P = (F_{II} - F_{\perp}) / (F_{II} + F_{\perp}) \tag{1}$$

An equivalent parameter used for the description of polarization of fluorescence is the anisotropy, $a$:

$$a = (F_{II} - F_{\perp}) / (F_{II} + 2F_{\perp}) \tag{2}$$

Though both parameters are equivalent for the description of polarized light, anisotropy is usually preferred because it leads to simpler equations for the time-dependent behaviour. Following a pulse excitation, the fluorescence anisotropy of a spherical particle in homogeneous isotropic medium decays exponentially, given by

$$a = a_0 \exp(-t/\tau_p) \tag{3}$$

where $\tau_p$ is the rotational correlation time of a sphere and $a_0$ is the anisotropy at $t = 0$. The anisotropy keeps constant at the initial value $a_0$ if the fluorophore is fixed in space. Thus, it can be experimentally determined by measuring the steady-state anisotropy of the dye in a rigid and homogeneous medium like vitrified solutions. The value of $a_0$ depends on the angle between the absorption and emission transition moments of the dye, $\beta$. Since the orientation of absorption and emission transition moments is characteristic for each corresponding electronic transition, the angle $\beta$ is a constant for every given pair of electronic transitions of a dye. As explained earlier, fluorescence usually arises from a single transition. Thus, $a_0$ is supposed to be invariant to the emission wavelength. However, the solvent relaxation (par. 5) occurring on a nanosecond time scale can result in a time dependent shift of the emitting $S_1$ state energy and lead to a decrease of anisotropy across the emission spectrum. Since the excitation spectrum might be composed of several absorption bands with different transition moments, the fluorescence anisotropy might change with the exciting light wavelength. Thus, polarization excitation spectra can be used to identify partially overlapping electronic transitions. Using linear polarized light under one-photon excitation conditions (for multi-photon excitation see [6]), $a_0$ for a randomly orientated molecule is

$$a_0 = 0.6 \cos^2 \beta - 0.2 \tag{4}$$

For colinear absorption and emission transition dipole moments, the theoretical initial anisotropy value $a_0$ is equal to 0.4.

## 3.2 Steady-state fluorescence anisotropy

In low-viscous solvents the rotational relaxation of low molecular weight compounds occurs on the picosecond time-scale [7]. Since in this case the rotation is much faster than the fluorescence (typically with ns decay-time), the steady-state emission is depolarized. If a fluorophore rotational motion is on the same time-scale as its fluorescence decay-time, steady-state fluorescence polarization is observed. In the simplest case, i.e. for a spherical-rotor-like molecule with a single-exponential fluorescence intensity decay ($\tau$), the expected steady-state fluorescence anisotropy is given by

$$a = a_0 / [1 + (\tau / \tau_p)] \quad (5)$$

The rotational correlation time of a sphere $\tau_p$ is given by

$$\tau_p = \eta V / R T \quad (6)$$

where $\eta$ is the viscosity, $T$ the temperature, $R$ the gas constant, and $V$ the volume of the rotating unit. It is important to note that these equations do only hold for spherically symmetrical molecules. Corresponding expressions for spherically unsymmetrical and ellipsoidal molecules can be found in the literature [8-11]. By combining equations (5) and (6) it can be seen that a plot of $1/a$ *vs.* $T/\eta$ should be linear, with an intercept equal to $1/a_0$ and with a slope/intercept that is directly proportional to $\tau$ and indirectly proportional to $V$. If one of the latter two parameters is known, the other can be calculated from such a plot. An absence of the viscosity dependence indicates that some other depolarizing process dominates. A nonlinear plot of $1/a$ *vs.* $T/\eta$ indicates the existence of more than one rotational mode.

Prior to the availability of time-resolved measurements, such so-called Perrin plots have been extensively used to determine the apparent hydrodynamic volume of proteins [12-14]. Since protein association reaction usually affects the rotational correlation time of the protein label, such reactions have been characterized by steady state anisotropy measurements [15, 16].

## 3.3 Time-resolved fluorescence polarization

As described by eq. (3), the anisotropy of spherical particles in homogeneous isotropic medium decays exponentially. Anisotropy decay, however, can be more complex. The three most important origins of a deviation from mono-exponential decay are as follows:

### A. Non-spherical particles in homogenous isotropic medium

Theory for rotational diffusion of non-spherical particles is complex; the anisotropy decay of such a molecule can be composed of a sum of up to five exponentials [17]. The ellipsoids of revolution represent a smooth and symmetrical shape, which is often used for description of the hydrodynamic properties of proteins. They are three-dimensional bodies generated by rotating an ellipse about one of its characteristic axes. In this case the anisotropy decay displays only three rotational correlation times which are correlated to the rotational diffusion coefficients $D_{II}$ and $D_{\perp}$. The indexes II and $\perp$ denote the rotation around the main and side axis, respectively [11]. The pre-exponential factors of the three exponentials depend on the angle between the emission transition moment and the main axis of the rotational ellipsoid. In practice, due to the limited time-resolution, one rarely resolves more than two exponentials [11].

### B. Segmental mobility of the chromophore

An important factor is that a typical chromophore is not rigidly fixed to the biopolymer and, thus, rotates around the bond linking it to the biopolymer. Consequently, the anisotropy decay kinetics is found to be double or triple exponential, due to the contributions from internal and global rotation of the macromolecule. The same concept applies for the rotational wobble of that portion of a biopolymer that is in proximity to the fluorophore or, in the more defined case, for the rotation of a molecular domain [18].

### C. Hindered rotors: fluorescent dyes in biological membranes

If isotropic rotors are imbedded in an anisotropic environment, like in phospholipid bilayers, the decay of fluorescence anisotropy can be complex. Let us consider a dye, such as 1-(4-trimethyl-ammonium-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) or 1,6-diphenyl-1,3,5-hexatriene (DPH), intercalated inside the bilayer. The polarization of its fluorescence depends on its motion dumping exerted by the molecular environment. In the case of a fixed hindrance to rotational relaxation motion, the anisotropy value decreases exponentially, not to zero, but to a finite value $a_\infty$, yielding formula:

$$a = (a_0 - a_\infty) \exp(-t/\tau_p) + a_\infty \qquad (7)$$

The time-resolved measurement of such membrane probes contains information on the dynamics of the hindered probe rotation, often interpreted as the micro-viscosity, and about the hindrance of this rotation, usually interpreted as the static packing arrangement of the lipids or the so-called membrane order [19, 20]. Fluorescence polarization studies in membranes, however, exhibit some major limitations: the experimentally determined steady-state and time-resolved anisotropies characterize the motional restrictions of the 'reporter' molecule itself and give therefore only **indirect** information on its environment. The consequence is that the fluorescence of a probe – namely when it is bound covalently to the lipid (like a phosphatidylcholine-fixed DPH) – might report more on this attachment than on the surrounding membrane. The membrane order parameters obtained from freely mobile probes, like DPH, result from a broad distribution of localisations within the hydrophobic interior, the detailed characterisation of which reveals inherent ambiguities [21].

Despite these drawbacks, among the fluorescence techniques employed so far, the determination of fluorescence anisotropy has certainly been the dominating method in studies of biological systems. For a detailed description of the theory and several examples for its application see review articles [11, 20].

# 4. Influence of fluorescence quenching

## 4.1 Fluorescence quantum yield and lifetime

In the gas phase or in non-interacting solvents, exactly speaking in the absence of intermolecular photophysical or photochemical processes (see Fig. 2.1), the fluorescence intensity $F$ decays after a short pulse excitation according to the mono-exponential law with an average fluorescence lifetime $\tau$. The rate constant of this fluorescence decay, $k$ (= $1/\tau$), represents the sum of the emission rate constant of the unperturbed fluorophore, $k_0$ (= $1/\tau_0$), and the rate constants of its internal

radiationless processes: internal conversion and intersystem crossing, $k_{ic}$ and $k_{isc}$, respectively. The radiative lifetime $\tau_0$ can be correlated to the emission transition dipole moment $\boldsymbol{M}$ by

$$\tau_0 \approx \text{constant} / k_{ave}^3 \, n^2 \, |\boldsymbol{M}|^2 \tag{8}$$

where $n$ is the refractive index of the solvent and $k_{ave}$ the wavenumber of the center of gravity of the fluorescence emission spectrum. The radiative lifetime $\tau_0$ can be considered as a photophysical constant of a chromophore surrounded by a solvent shell with the refractive index $n$. In the case of planar aromatic systems it appears to be temperature-independent [31]. Since the internal conversion and intersystem crossing processes compete with fluorescence for deactivation of the lowest excited singlet state, not all molecules will return to the ground state by fluorescence. The fraction of the excited molecules that do fluoresce is called the quantum yield $\Phi$. In terms of the above defined rate constants and lifetimes, $\Phi$ is given by equation

$$\Phi = k_0 / ( k_0 + k_{ic} + k_{isc} ) = \tau / \tau_0 \tag{9}$$

The fluorescence lifetime $\tau$ can be determined directly by monitoring the decay curve of fluorescence intensity following a short excitation pulse [23] or by detecting the emission response delay (phase shift) to the intensity modulated excitation light [22]. When a standard steady-state spectrofluorometer is used for the fluorescence quantum yield measurement, $\Phi$ is usually determined by fluorescence intensity and spectra comparison with standard compounds of known quantum yield [32].

## 4.2 Fluorescence quenchers

A fluorescence quencher is a compound, presence of which in the vicinity of a fluorophore leads to a decrease of the fluorescence quantum yield and lifetime of the latter. For example, those molecules or ions can function as a quencher that are added to the solution and introduce new or promote already existing non-radiative deactivation pathways (solute quenching) by molecular contact with the chromophore. Further possibilities are the self-quenching by simply another fluorophore molecule of the same type, and the quenching by solvent molecules. In any case, the quenching term $k_Q$ [Q] has to be added to equation (9) yielding

$$\Phi = k_0 / ( k_0 + k_{ic} + k_{isc} + k_Q [Q]) \tag{10}$$

where $k_Q$ is the bimolecular quenching constant and [Q] the concentration of the quencher.

### Solute quenching

Solute quenching reactions are a very valuable tool for studies of proteins, membranes, and other supramolecular or macromolecular assemblies providing information on the location of fluorescent groups in the examined molecular structure. A fluorophore that is located on the surface of such a structure will be relatively accessible to a solute quencher (for a list of common quenchers see Table 1). A quenching agent will quench the chromophore that is buried in the core of the molecular assembly to a lesser degree. Thus, the quenching experiment can be used to probe topographical features of the examined structure and to detect structural changes that may be caused by addition of external compounds or changed physical conditions. In usual quenching experiments, the quencher is added successively to the fluorophore-containing solution. The analysis of the dependence of fluorescence intensity $F$, quantum yield $\Phi$, or lifetime $\tau$, on the

quencher concentration yields quantitative information on the accessibility of the chromophore within the macromolecular or supramolecular structure.

Depending on chemical nature of both the quenching agent and the chromophore, one has to distinguish between two forms of quenching: the dynamic and the static quenching.

***Table 1: List of selected solute quenchers***

| Type of fluorophore | Used quenchers | References |
|---|---|---|
| Indole | carboxy groups, chlorinated compounds, dimethylformamide | 33-35 |
| Tyrosine | disulfides | 36 |
| Tryptophan | acrylamide, histidin, succinimide, trifluoroacetamide, iodide, disulfides | 37-42 |
| Naphtalene | halogens, nitroxides | 43,44 |
| Anthracene | amines, halogens, thiocyanate | 45-47 |
| Anthranoyloxy probes | tetracain | 48 |
| Quinolinium ions and their betains | chloride, bromide, iodide | 49-51 |
| Pyrene | halothane | 52 |
| Carbazole | amines, chlorinated compounds, halogens | 53-56 |
| Commom quencher for almost all dyes | oxygen | 57,58 |

The static quenching results from the formation of a non-fluorescent fluorophore-quencher complex, formed already in the fluorophore´s ground state. Characteristic for this type of quenching is that increasing quencher concentration decreases the fluorescence intensity or quantum yield but does not affect the fluorescence lifetime. An important feature of the static quenching is its decrease with increasing temperature, as the stability of the fluorophore-quencher ground state complexes is generally lower at higher temperatures.

If quenchers act (e.g., through collisions) by competing with the radiative deactivation process – see eq. (10) and Fig. 1 – the ratio of the quantum yield in the absence of the quencher, $\Phi_a$, and in its presence, $\Phi$, will be equal to the ratio of the corresponding lifetimes, $\tau_a/\tau$, see eq. (9). The concentration dependence of this so-called dynamic or collisional quenching is described by the Stern-Volmer equation, where the Stern-Volmer constant, $K_{sv}$, is equal to $k_Q \tau_a$:

$$\Phi_a / \Phi = \tau_a / \tau = F_a / F = 1 + K_{sv}[Q] = 1 + k_Q \tau_a [Q] \qquad (11)$$

Thus, from a plot of one of those ratios versus the quencher concentration, and by knowing $\tau_a$ independently, the bimolecular quenching constant, $k_Q$, can be determined. From physical considerations, the $k_Q$ magnitude can be expressed as follows:

$$k_Q = 4 \gamma \pi D r N' \qquad (12)$$

where $\gamma$ is the efficiency of the quenching reaction, $D$ is the sum of the diffusion coefficients of quencher and chromophore, $r$ is the sum of the hydrodynamic radii of quencher and chromophore and $N_A$ is the Avogadro's constant. The diffusion coefficient for each species $i$ can be expressed by using the Stokes-Einstein relationship:

$$D_i = b\ T / 6\ \pi\ \eta\ r_i \qquad (13)$$

where $b$ is Boltzmann's constant and $\eta$ is the viscosity of the solution. It follows that the quenching constant increases with increasing temperature $T$ due to the diffusion control of the dynamic quenching.

Another mechanism of the dynamic fluorescence quenching is connected with the chemical nature of the chromophore and the solute quencher: quenchers containing halogen or heavy atoms increase the intersystem crossing rate (generally induced by a spin-orbital coupling mechanism). Acrylamide quenching of tryptophans in proteins is probably due to the excited state electron transfer from the indole to acrylamide. Paramagnetic species are believed to quench aromatic fluorophores by an electron spin exchange process.

In many instances a fluorophore can be quenched both by dynamic and static quenching simultaneously. The characteristic feature for mixed quenching is that the plot of the concentration dependence of the quantum yield or intensity ratios, see eqn. (11), shows an upward curvature. In this case the Stern-Volmer equation has to be modified, resulting in an equation which is second order in [Q]. More details on theory and applications of solute quenching can be found in an excellent review by Maurice Eftink [59]. An overview of typical fluorophore-quencher-pairs is given in Table 1.

## Solute quenching in protein studies: An application example

One of the main aims in biophysical studies of structure and function of proteins is to identify the protein domains that are responsible for interaction of the entire protein with physiologically relevant binding partners. Proteins usually contain several tryptophan residues which might be distributed over the different protein domains. Since each of those tryptophan residues is located in a distinct environment, each residue might exhibit different fluorescence decay profile as well as a different accessibility to quenching molecules. Using picosecond time-resolved fluorescence spectroscopy, the tryptophan fluorescence lifetimes in proteins containing up to three tryptophan residues can be determined with high accuracy [37]. A picosecond tryptophan study of prothrombin fragment 1 (BF1) which is the 1-156 N-terminal peptide of a key blood coagulation protein, prothrombin, may serve as an example. It is believed to be the region predominately responsible for the metal ion and membrane binding properties of prothrombin. An important question to answer has been to what extent the conformation of the two protein domains, the so-called Gla and kringle domain, is altered by the interaction with calcium ions and with negatively charged phospholipid surfaces (see Fig. 3).

The analysis of the fluorescence decay of the three tryptophan residues (Trp42, Trp90, Trp126) in apo-BF1 resulted in a five-exponential decay model, where the five fluorescence lifetimes are wavelength independent. Since structural data show a huge difference in solvent accessibilities for the kringle tryptophans ($4 \times 10^{-20}$ m$^2$ for Trp90 and Trp126) and the Gla tryptophan ($133 \times 10^{-20}$ m$^2$ for Trp42), acrylamide quenching studies were performed to assign the five lifetimes to the two types of tryptophans. Acrylamide was added successively up to a concentration of 0.7 mol l$^{-1}$. The Stern-Volmer analysis of the fluorescence decays showed that the five lifetimes are basically due to two different types of tryptophans characterised by two different $k_Q$ values ($0.2 \pm 0.2 \times 10^9$ mol$^{-1}$ l s$^{-1}$ and $1.1 \pm 0.3 \times 10^9$ mol$^{-1}$ l s$^{-1}$ for the kringle and Gla tryptophan components, respectively). Note that the theoretical $k_Q$-value for a fully exposed polypeptide-tryptophan is about $3 \times 10^9$ mol$^{-1}$ l s$^{-1}$. The resulting assignment of the lifetime compounds to the two types of tryptophan allowed for a

separate investigation of conformational changes in the two protein domains without cleaving BF1 into the isolated Gla (containing Trp42) and kringle domains (containing Trp90 and Trp126) or modifying the protein by site-directed mutagenesis. After the assignment of the lifetimes to the two tryptophan types in BF1, further experiments led to the conclusion that the Gla domain is exclusively responsible for the interaction with calcium ions and negatively charged phospholipids. Moreover, the first experimental evidence for a lipid specific conformational change in the Gla domain of prothrombin was found, indicating an important role of this domain in the regulation of blood coagulation [60].

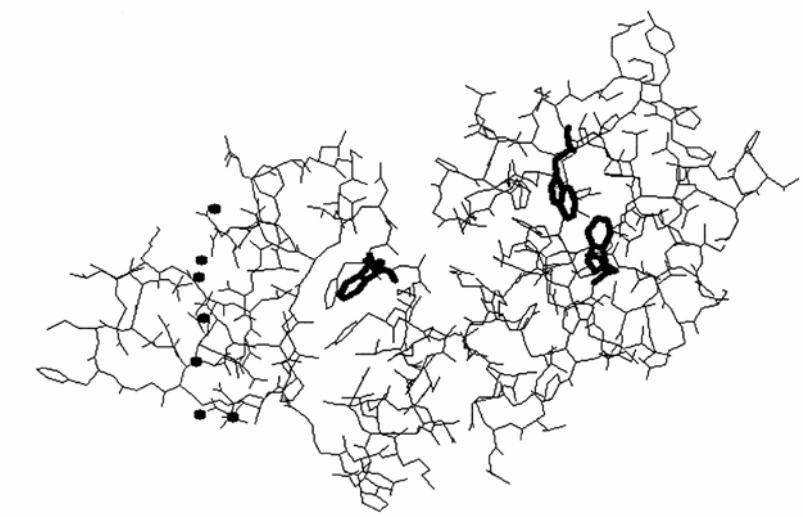

***Fig. 3:*** ***A depiction of the X-ray structure of Ca-BF1***

The right part of the protein is the kringle-domain, where the solvent inaccessible tryptophan residues Trp90 and Trp126 are located. The Gla-domain is the left part of the protein, containing the solvent and quencher accessible Trp42 and seven calcium ions (dots)

## Solvent quenching

The influence of solvent molecules on the fluorescence characteristic of a dye solute is certainly one of the most complex issues in fluorescence spectroscopy. Eventually, every chromophore shows some dependence of its quantum yield on the chemical structure of the surrounding solvent. This observation is to some extent due to the fluorescence quenching by the solvent. One possibility is that the interaction of the chromophore with its solvent shell can promote non-radiative pathways by changing the energy of the $S_0$, $S_1$, and $T_1$ states. Transition probabilities for the internal conversion and intersystem crossing processes are governed by the energy-gap law [61]. This law states that the rate constants $k_{ic}$ and $k_{isc}$ increase exponentially as the energy gap between the corresponding $S_1$, $S_0$, and/or $T_1$ states decreases [61]. Consequently, any change in those energy levels will strongly influence the fluorescence lifetime and quantum yield, see eq. (9). Some of the so-called hemicyanine dyes represent special cases for the promotion of non-radiative pathways by increasing solvent polarity [62]. These dyes undergo an intramolecular twist in their excited states. The intramolecular twist leads to an increase of the polarity, and the twisted form of the $S_1$ state is deactivated very efficiently by fast internal conversion. Increasing solvent polarity

promotes the intramolecular twist and, therefore, the non-radiative deactivation by internal conversion [62]. Moreover, evidence has been accumulated that quenching by interaction with solvent molecules can proceed by a vibrational mechanism. It has been speculated that the collision between dye and solvent molecules results in vibrational coupling favoring efficient internal conversion [63]. In this connection the solvent deuterium effect on the fluorescence lifetime, which has been observed for a variety of chromophores, should be mentioned [64-66]. It has been shown that the quantum yield increases substantially if $D_2O$ is used instead of $H_2O$ as the solvent. Interestingly, this effect appears to be independent on the chemical nature of the fluorophore. It is conceivable that the different energies of the OH versus OD stretching vibrations (3 657 $cm^{-1}$ and 2 670 $cm^{-1}$, respectively) are responsible for the more effective solvent quenching by $H_2O$ versus $D_2O$. Regardless of its physical nature, this "heavy" atom effect in solvent quenching proved to be a very smart tool for characterization of the water accessibility in supramolecular and macromolecular assemblies [64].

## Self-quenching

Self-quenching is the quenching of one fluorophore by another one of the same kind. It is a widespread phenomenon in fluorescence, but it requires high concentrations or labeling densities. The general physical description of the self-quenching processes involves a combination of trap-site formation and energy transfer among fluorophores, with a possibility of trap-site migration which results in quenching. Trap-sites may be formal fluorophore complexes or aggregates, or they may result from sufficiently high concentrations of fluorophores leading to close proximity of the dye molecules. A mathematical modelling of such processes is given in [67]. Self-quenching experiments are frequently performed by simply monitoring an increase in the fluorescence intensity $F$ due to a decrease in the local dye concentration. One such an example is the self-quenching assay for the characterisation of leakage of aqueous contents from cells or vesicles as a result of lysis, fusion or physiological permeability. This assay is based on the fact that carboxyfluorescein is > 95 % self-quenched at concentrations > 100 mmol $l^{-1}$ [68]. A concentrated solution of this water-soluble dye is encapsulated in liposomes. Upon addition of a fusogen or other permeabilizing agent, the dye release is accompanied by an increase in fluorescence. Other chromophores, the self quenching properties of which are exploited in biochemical assays, are NBD (derivatives of 7-nitrobenz-2-oxa-1,3-diazol-4-yl) [69,70], BODIPY (derivatives of 4-bora-3a,4a-diaza-*s*-indacene) [71], and DPH (derivatives of 1,6-diphenyl-1,3,5-hexatriene) [72].

## Trivial quenching

Trivial quenching arises from attenuation of the exciting beam and/or inability of the fluorescence photon to reach out of the sample, which occurs mainly when other compounds are added that strongly absorb in the UV region. Though the added concentration may be small, they might block the excitation light completely. Another reason for trivial quenching can be the turbidity of the sample. True and trivial quenching, however, are easily differentiated, since in trivial quenching lifetime and quantum yield remain constant.

# 5. INFLUENCE OF SOLVENT RELAXATION ON SOLUTE FLUORESCENCE

## 5.1 Basics of solvent relaxation

Electronic excitation from the ground state $S_0$ to a higher electronic excited state (such as $S_1$) is generally accompanied by a change of the permanent dipole moment, $\Delta\mu_c$, of the molecule, $\Delta\mu_c = \mu(S_1) - \mu(S_0)$. Since the time scale of the molecular electronic transition is much shorter than that of nuclear motion, the excitation-induced ultrafast change of the electron density happens virtually under fixed (original) positions and orientations of the surrounding solvent molecules. With the new dipol moment $\mu(S_1)$, the solute-solvent system is no longer in equilibrium. The solvation shell molecules are, thus, forced to adapt to the new situation: they start to reorient themselves into energetically more favorable positions with respect to the excited dye. This dynamic process, starting from the originally created non-equilibrium Franck-Condon state (FC) and leading gradually to a new equilibrium with the solute excited state (R) is called solvent relaxation (SR). This relaxation red-shifts the solute's fluorescence emission spectrum continuously from the emission maximum frequency corresponding to the Franck-Condon state ($\upsilon(0)$ for $t = 0$) down to the emission maximum frequency corresponding to the fully relaxed R-state ($\upsilon(\infty)$ for $t = \infty$). Since a more polar solvent typically leads to a stronger stabilization of the polar R-state, the overall shift $\Delta\upsilon$ , $\Delta\upsilon = \upsilon(0) - \upsilon(\infty)$, increases with increasing solvent polarity for a given change of the solute's dipole moment $\Delta\mu_c$. The detailed mathematical description of this relationship depends on the set of assumptions that each particular dielectric solvation theory formulates [73-78]. The fundamental "dielectric continuum solvation model" [76-78] predicts a linear proportionality between $\Delta\upsilon$ and a dielectric measure of the solvent polarity for a large variety of solvents [79]. According to this model, changes in $\Delta\upsilon$ directly reflect polarity changes in the dye environment – which can be major desired information thus accessible through solvent relaxation studies. Another important information that can be obtained from a solvent relaxation investigation follows from the fact that SR kinetics is determined by the mobility of the dye environment. The response of solvent molecules to a dye's electronic rearrangement is the fastest in the case of water: more than half of its overall solvation response occurs within 55 femtoseconds (fs) [80]. If the dye is located in a viscous medium, the typical solvent relaxation takes place on a nanosecond (ns) time scale [81]. In vitrified solutions, on the other hand, the dye may fluoresce before the solvent relaxation towards the R-state is completed [82].

## 5.2 Influence of solvent relaxation on steady-state spectra

### Non-viscous solvents

At ambient temperature, non-viscous solvents respond to the photoinduced ultrafast change of the solute dipole moment by a fast inertial (librational) motion in the range from 50 to 500 fs. After this initial stage of the solvation response, diffusion of the solvent molecules, occurring typically on pico- to sub-nanosecond time scales, leads to further relaxation towards the R-state [79, 80, 83, 84]. Fluorescence decay times, $\tau$, of common chromophores are usually 1 ns or longer. In such a case, most of the fluorescence detected in a steady-state experiment occurs from the equilibrium state R. Based on the above described relations between $\Delta\upsilon$, the dipole moments of the solute, $\Delta\mu_c$,

and the solvent polarity, there are two basic consequences for the spectral position of the steady-state fluorescence spectrum:

(1) Increased solvent polarity generally leads to a red-shift of the emission spectrum.

(2) As larger $\Delta\mu_c$, as more pronounced is the solvent polarity effect on the emission spectrum position.

### Viscous and vitrified solutions

If the dye is located in a viscous medium, the solvent relaxation might take place on the nanosecond (ns) time scale. Thus, emission occurs, to a substantial extent, during solvent relaxation, and the steady-state emission spectrum represents a time-average of the emissions from different partially relaxed states. In this case, the maximum of the emission spectrum in not any longer directly correlated with the polarity of the solvent. Any increase of temperature leads to a faster solvent reorientation process and, in this case, to a red-shift of the emission spectrum peak. Moreover, the emission band maximum for a polar fluorophore placed in motionally restricted media (such as very viscous solvents [85, 86] or membranes [81]), shifts to a longer wavelength when the used excitation wavelength approaches the red-edge of the absorption band [87]. The observed shift should be maximal when the solvent relaxation is much slower than the fluorescence, and it should be zero if SR is fast enough. Thus, the red-edge excitation shift can serve as an indicator of the mobility of the probe's surrounding [85, 86, 88]. Usually, such a red-edge excitation shift value ranges from several nm up to 40 nm depending on the chosen solute/solvent system. The red-edge excitation shift is especially useful information for dyes, the absorption and fluorescence maxima of which hold linear correlations with the polarity of low-viscosity solvents [82, 89]: then the probed polarity as well as the hypothetical emission maximum of the fully relaxed R-state can be estimated from the behaviour of the absorption maximum. In vitrified solutions, like sol-gel matrices [82], solvent relaxation becomes much slower than the fluorescence most of which in that case arises from states close to the initial Franck-Condon state.

## 5.3 Quantitative characterisation of solvent relaxation by time-resolved spectroscopy

Although there have been several attempts to simplify the characterisation of the SR process, the determination of time-resolved emission spectra (TRES) is certainly the most general and most precise way to quantitatively describe the solvent response. The time-resolved emission spectra are usually determined by 'spectral reconstruction' [79, 80, 89]. The time-resolved emission spectrum at a given time t is calculated from the wavelength dependent time-resolved decays by relative normalization to the steady-state spectrum. By fitting the TRES at different times t by the empirical "log-normal" function, the emission maximum frequencies $\upsilon(t)$ (or $\lambda(t)$) and the total Stokes-shift $\Delta\upsilon$ (or $\Delta\lambda$) are usually derived [89]. Since $\upsilon(t)$ contains both information on polarity ($\Delta\upsilon$) and viscosity of the reported environment, the spectral shift $\upsilon(t)$ may be normalized to the total shift $\Delta\upsilon$. The resulting 'correlation functions' $C(t)$ (eq. 14) describe the time course of the solvent response and allow for comparison of the SR-kinetic and, thus, of relative micro-viscosities, reported from environments of different polarities [79, 80, 89, 90-95]

$$C(t) = [\upsilon(t) - \upsilon(\infty)]/\Delta\upsilon \qquad (14)$$

Solvent relaxation probes used for the characterisation of micro-viscosities and polarities are listed in table 2. They are characterised by a large change in their dipole moment $\Delta\mu_c$ upon electronic excitation.

***Table 2: List of solvent relaxation probes***

| Dye or chromophore | References |
|---|---|
| 1,8-ANS (1-anilinonaphthalene-8-sulfonate) | 96 |
| 2,6-ANS (2-anilinonaphthalene-6-sulfonate) | 96 |
| 2,6-TNS (2-(p-toluidinylnaphthalene)-6-sulfonate) | 97,98 |
| NPN (N-phenyl-1-naphthylamine) | 99 |
| Dansyl Lysin (N-e-(5-dimethylaminonaphthalene-1-sulfonyl)-L-lysine) | 100 |
| Prodan (6-propionyl-2-(dimethylamino)-naphthalene) | 93-95 |
| Laurdan (2-dimetylamino-6-lauroylnaphthalene) | 103 |
| Patman (6-palmitoyl-2-[[2-(triethylammonium)ethyl]-methylamino]naphthalene chloride) | 92-95 |
| NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl) | 81 |
| Coumarin 153 | 79,82,89 |
| Nile red | 101 |
| hemicyanine dyes | 88,102 |
| aminobenzanthrone-derivatives | 90 |

# 6. FLUORESCENCE RESONANCE ENERGY TRANSFER AS A SPECTROSCOPIC RULER

The fluorescence resonance energy transfer (FRET) is a nonradiative transfer of the excitation energy from a donor to an acceptor chromophore that is mediated by a long-range interaction between the emission and absorption transition dipole moments of the donor and the acceptor, respectively. The rate of energy transfer depends on the extent of the spectral overlap of the donor emission and the acceptor absorption spectra, on the donor fluorescence quantum yield, the relative orientation of their transition dipole moments, and the distance between donor and acceptor molecules. The distance dependence has resulted in widespread use of FRET to measure distances between donors and acceptors in macromolecular systems. The quality of a donor/acceptor pair is usually characterized by the parameter $R_0$ which is typically in the range from 2 to 9 nm. It is defined as the distance at which the efficiency of resonance energy transfer equals to 50 %. $R_0$ can be estimated as follows:

$$R_0\,[\mathrm{nm}] = 979\,(\kappa^2\, n^4\, \Phi_0\, J)^{1/6} \qquad (15)$$

where $n$ is the refractive index of the medium, $\Phi_0$ is the fluorescence quantum yield of the donor, $J$ the spectral overlap integral, and $\kappa$ an orientation factor. The energy transfer rate $k_{ET}$ is given by

$$k_{ET} = 1/\,\tau_d\,(R_0\,/\,r)^6 \qquad (16)$$

where $\tau_d$ is the decay time of the donor fluorescence in absence of an acceptor and $r$ is the distance between donor and acceptor. Thus the rate depends strongly on distance, providing a spectroscopic ruler for determining distances in macromolecular assemblies.

## 6.1 Donor-acceptor pairs at fixed distances

The magnitude of $k_{ET}$ can be determined form the efficiency of energy transfer, $E_{T'}$, via

$$k_{ET} = \frac{1}{\tau_d} \frac{E_T}{1 - E_T} \tag{17}$$

and $E_T$, in turn, can be established experimentally by measuring the decrease in the intensity $F$ or lifetime $\tau$ of the donor in the presence of the acceptor:

$$E_T = \frac{1 - F}{F_d} = \frac{1 - \tau}{\tau_d} \tag{18}$$

Thus, by determining $E_T$ and knowing $R_0$, the separation distance $r$ can be calculated. When distances are estimated this way, there is often some concern about the correct value of the orientation factor $\kappa^2$, which depends on the relative orientation of the donor emission transition moment and the acceptor absorption transition moment. The value of $\kappa^2$ varies from 4 (parallel orientation of the transition moments) to 0 (perpendicular orientation). Often, a value of $\kappa^2 = 2/3$ is assumed which corresponds to the situation of a rapid, isotropic rotation of the donor and acceptor molecules. Randomly oriented dipoles that remain fixed during the singlet lifetime give $\kappa^2 = 0.476$. When needed, the value of $\kappa^2$ can be estimated by polarization measurements [104]. A comprehensive discussion on the theory and effects of the orientation factor is given in [105].

## 6.2 Donor-acceptor pairs at variable distances

Lets assume the simplest case: a donor with mono-exponential fluorescence decay $\tau_d$, a fixed donor-acceptor distance $r$, and a dynamically random orientation factor $\kappa^2 = 0.476$, for which $k_{ET}$ has to be added to eq. 10. The energy transfer in this situation will simply result in a shortened but still mono-exponential decay of the donor $\tau_d$. In homogeneous solution, however, at low donor concentration and without any significant diffusion of the donor and acceptor within the fluorescence lifetime, the donor fluorescence intensity decay is given by [106-109]:

$$F = F_0 \exp\frac{-t}{\tau_d} \exp - \delta \left( \frac{-t}{\tau_d} \right)^6 ; \qquad \delta = \mathrm{dim}/6 \tag{19}$$

For randomly distributed donor and acceptor molecules the value for the dimension dim is equal to 3 and $\gamma$ is

$$\gamma = 4/3\ g\ \pi^{3/2}\ c_a R_0, \qquad \text{where} \quad g = (3/2\ \kappa^2)^{0.5} \tag{20}$$

with $c_a$ as the acceptor concentration. With knowledge of the acceptor concentration and provided that the donor fluorescence decays mono-exponentially in absence of the acceptor, the $R_0$ value and the dimension of the medium in which donor and acceptors are imbedded can be determined. Two-dimensional or so-called fractal energy transfer is of interest, if the dye molecules are bound to phospholipid membranes [110, 111] or imbedded in silicate networks [112]. One-dimensional energy transfer has been considered for dyes bound to DNA [113]. A detailed and up-to-date review of physics and theory of long-range resonance energy transfer in molecular systems was published recently by Scholes [114].

## 6.3 Some applications of fluorescence resonance energy transfer

An important class of FRET applications is represented by assays for the characterisation of fusion of cells or vesicles. Usually, their membranes are labeled either by donor or acceptor molecules. Fusion leads to an intermixing of these membrane labels in the same bilayer, allowing resonance energy transfer to occur. Examples can be found in the literature [115-119]. Another membrane application of energy transfer has been the demonstration of lipid asymmetry in human red blood cells [120]. Moreover, energy transfer has proved to be a very useful tool in elucidating the subunit structure of oligomeric assemblies in membranes. Examples are studies on the oligomerisation of ATPase of sarcoplasmic reticulum in phospholipid vesicles [121], on gramicidin A trans-membrane channels [122], and on the aggregation state of bacteriorhodopsin [123]. Finally, the combination of energy transfer with flow cytometry [124] and its use in immunoassays should be mentioned [125]. Further information on theory and application of energy transfer can be found, e.g., in [105, 126].

# 7. IRREVERSIBLE PHOTO-BLEACHING

'Fluorescence recovery after photo-bleaching' (FRAP) was introduced as a method to measure the local mobility of fluorescently labeled particles bound to the plasma membrane of living cells [26, 127, 128]. It has been used to study transport phenomena in a wide variety of biological membrane-bound systems, as well as to probe the photo-bleaching properties of fluorescent molecules [129]. FRAP is based on observing the rate of fluorescence recovery due to the movement of a new fluorescent marker into an area of the membrane which contains the same kind of marker but which has been rendered non-fluorescent via an intense photo-bleaching pulse of laser light. The two-dimensional diffusion coefficient of the fluorescent marker is related to both its rate and extent of fluorescence recovery. For a discussion of the photophysical mechanism of photo-bleaching see reference [130]. In order to create a finite observation area, usually both laser pulses – the single short pulse with rather high intensity leading to photo-bleaching and the less intensive pulse monitoring the fluorescence recovery – are focused by an epifluorescence or confocal microscope. A very elegant variant is the combination of FRAP with total internal reflection fluorescence (TIRF) technique (**T**otal **I**nternal **R**eflection **F**luorescence **R**ecovery **A**fter **P**hotobleaching; TIR-FRAP [28]). Here, a laser beam totally internally reflects at a solid/liquid interface, creating an evanescent field which penetrates only a fraction of the laser beam´s wavelength into the liquid domain. Using planar phospholipid bilayers and fluorescently labeled proteins, this method allows for the determination of adsorption/desorption rate constants and surface diffusion constants [28, 131, 132]. Figure 4 shows a representative TIR-FRAP curve for fluorescein-labeled prothrombin bound to a planar membrane. In this experiment the conditions are chosen in a way that the recovery curve is characterized by the prothrombin desorption rate. It should be mentioned that, in analogy with other fluorescence microscopy techniques, two- and three-photon absorption might be utilised for FRAP in the near future.

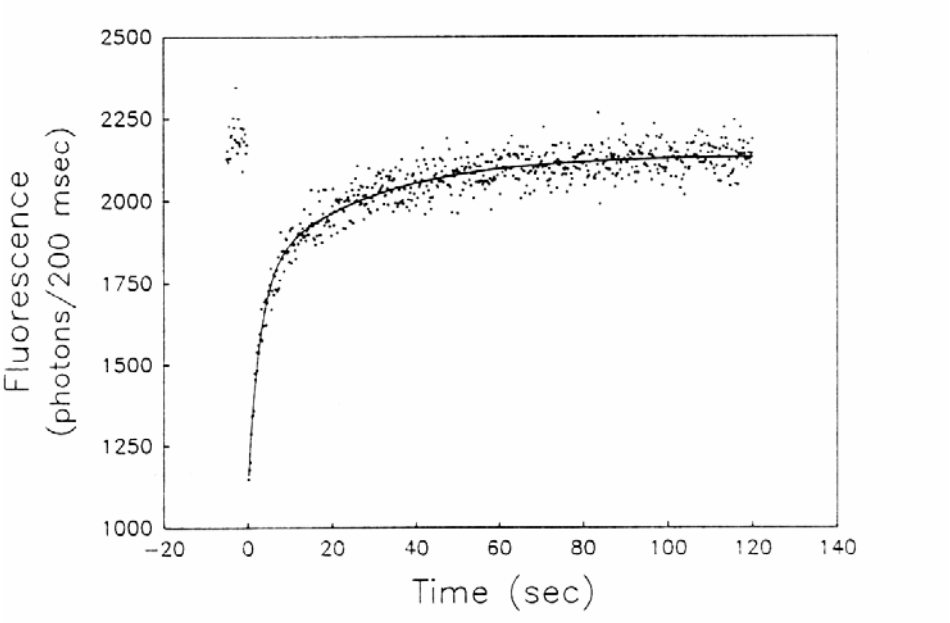


***Fig. 4:*** ***Representative TIR-FRAP curve for fluorescein-labeled prothrombin bound to planar membranes***

Shown is a typical recovery curve for the binding of 1μM-prothrombin (labeled with fluorescein) to a planar bilayer. The dotted points represent the experimental data and the line the best fit, yielding the desorption rate. Note, that the fluorescence intensity does not recovery fully. This effect is generally observed in photo-bleaching experiments and is one of the major drawbacks of this method

# 8. SINGLE MOLECULE FLUORESCENCE

Recent advances in ultra-sensitive instrumentation have allowed for the detection of individual atoms and molecules in solids [133, 134], on surfaces [135, 136], and in condensed phase [137, 138] using laser-induced fluorescence. In particular, single molecule detection in condensed phase enables scientists to explore new frontiers in many scientific disciplines, such as chemistry, molecular biology, molecular medicine and nanostructure material. There are several optical methods to study single molecules, the principles and application of which have been reviewed by Nie and Zare [139]. These methods are listed in table 3. A broader coverage of this topic is given in recent reviews [3, 5].

***Table 3: Methods for studying single molecules using laser-induced fluorescence***

| **Method for studying single molecules** | **References** |
|---|---|
| Solid matrices at low temperatures | 140, 141 |
| Liquid streams | 142-144 |
| Microdroplets | 145 |
| Near-field scanning optical microscopy | 136, 146-149 |
| Far-field confocal microsopy, including fluorescence correlation spectroscopy | 150, 151 |
| Microscopy combined with multi-photon excitation | 152-154 |
| Wide-field epi-illumination | 155, 156 |
| Evanescent wave excitation | 157 |

In contrast to the other single molecule techniques listed above, measurements based on fluorescence correlation spectroscopy (FCS) can be already performed both routinely and rapidly. Moreover, FCS is applied in many scientific disciplines and the number of applications of this technique is growing rapidly. In a FCS experiment fluorescence fluctuations due to diffusion, chemical reactions or flow are detected and analysed. Usually in FCS, a sharply focused laser beam illuminates a volume element of about $10^{-15}$ L by using confocal or multi-photon microscopy. This volume is so small that at a given time, it can host only one fluorescent particle out of many under analysis. The illuminated volume is adjustable in 1 μm steps in three dimensions, providing a high spatial resolution. If diffusion is the investigated process, the single fluorescent molecules diffusing through the illuminated volume give rise to bursts of fluorescence light quanta. Each individual burst, resulting from a single molecule, can be registered. The photons are recorded in a time-resolved manner by a highly sensitive single photon counting device. In the diffusion case, the autocorrelation function of the time-course of the fluorescence signal gives information about the number of molecules in the illuminated volume element and their characteristic translational diffusion time. Since the size of the illuminated volume is known, the concentration and diffusion constant of the fluorescent species can be determined.

# 9. OPTICAL SENSORS BASED ON FLUORESCENCE

An optical sensor for chemical analysis is part of the detector system that allows for continuous monitoring of a physical parameter or concentration of an analyte. Such sensors can detect changes in optical absorbance, reflectance, fluorescence, chemoluminescence, Raman scattering, refractive index, light polarization, and other optical properties. Due to the high sensitivity, selectivity, and versatility of fluorescence spectroscopy, optical sensors based on fluorescence are the most highly developed. Typically, the sensing probe (chemically interacting part) is placed on a carrier material, while the analyte can be either in gas phase or in solution. Interaction between the sensing probe and the analyte leads to a change in the sensor´s fluorescence properties. If needed, fiber optics allows one to perform the following sensor fluorescence measurement on a remote part of the detector, which is especially useful in clinical applications. Such a fluorescence change monitoring e.g. pH, $O_2$ pressure, or the concentration of ions in blood can occur in intensity, emission spectrum, anisotropy, or lifetime of the sensor probe. Mechanism for the fluorescence change can be e.g. collisional quenching, resonance energy transfer, photo-induced electron transfer, or analyte induced change of the state of the sensing chromophore. Table 4 gives some examples of analyte, sensing probe and sensing mechanism combinations.

Since intensity measurements are dependent upon the concentration of the fluorophore, they are often not applicable since they might be inaccurate if e.g. photo-bleaching occurs. Moreover, intensity-based systems suffer from other problems including turbidity, limited range of detection, low signal-to-noise ratio, and optical losses. Fluorescence lifetime-based sensing, on the other hand, does not suffer from these problems and it seems likely to become widely used in the near future. A comprehensive overview on this topic is given in reference [158].

*Table 4: Examples for fluorescence sensing*

| Analyte | Sensing dye | Sensing mechanism | References |
|---|---|---|---|
| Oxygen | Several Ru-complexes (e.g. $[Ru(Ph_2phen)_3]^{2+}$ | Collisional quenching | 159 |
| Chloride | Sultons (betains) of quinolinium and acridinium ions | Collisional quenching | 51, 160 |
| Calcium | Blue and green fluorescent proteins | Resonance energy transfer | 25 |
| pH | Fluoresceins | pH-dependent ionization | 161, 162 |
| Glucose | Fluorescein and rhodamine | Resonance energy transfer | 163 |

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