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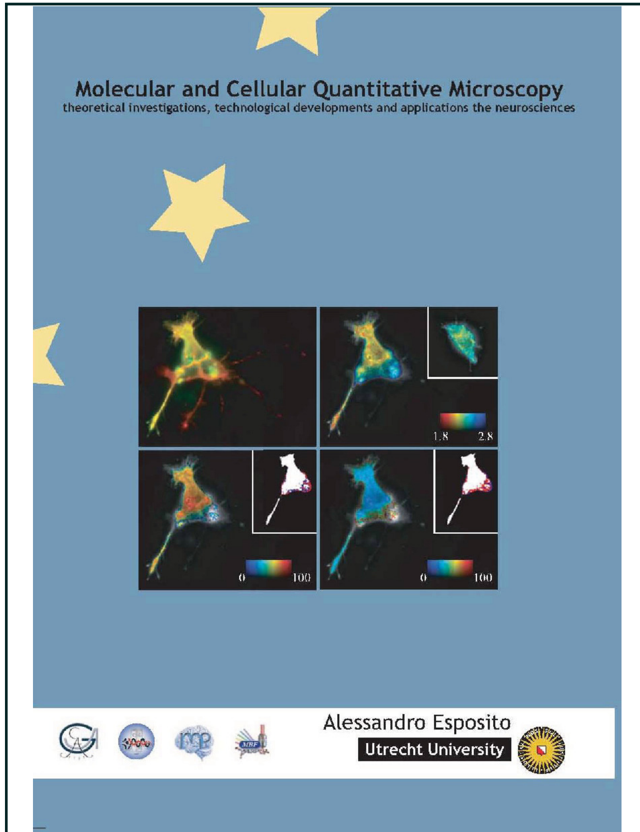
Molecular and Cellular Quantitative Microscopy

Theoretical investigations, technological developments and applications to the neurosciences



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Chapter 1 - Introduction

General introduction

Förster Resonance Energy Transfer (FRET) is the non-radiative transfer of energy between a donor fluorophore and an acceptor chromophore (see *FRET* section for a detailed description). Typically, FRET occurs at intermolecular distances shorter than 10 nm, *i.e.* distances comparable with the dimensions of biological macromolecules. Therefore, FRET imaging can be used to resolve events that occur at the molecular scale, *e.g.* protein-protein interactions and conformational changes. Fluorescence Lifetime Imaging Microscopy (FLIM) allows the quantitative detection of the excited state lifetime of fluorophores (see *FLIM* section for a detailed description). Because FRET reduces the lifetime of the donor fluorophore proportionally to the energy transfer efficiency, FLIM can be used for the quantitative detection of FRET. Furthermore, many fluorophores exhibit fluorescence lifetimes that depend on their biochemical environment, *e.g.* pH, ion concentration and oxygen content. Therefore, FRET imaging and FLIM, combined with the many available fluorescent probe molecules (see Bunt *et al.*, 2004 for a thorough review), enables the quantitative mapping of cellular biochemistry. Lifetime sensing is also used in material sciences and other branches of physics like photonic crystal research. However, this PhD dissertation is focussed on aspects more relevant for biological and biophysical applications.

Microscopy provides spatio-temporal information that allows the quantification of morphological features, spatial localization, and temporal responses that are initiated by biochemical or mechanical stimuli. Frequently, when microscopy is used for the investigation of biological models, the heterogeneity at both the cellular and molecular level cannot be neglected. A biological process is usually the result of many cooperating molecules; hundreds of proteins can be intimately involved in a biological phenomenon. Furthermore, heterogeneity in the cellular metabolism, cell cycle, or differences between cell types, can increase the heterogeneity of the response observed in an experiment. In fact, more accurate and quantitative experimental tools may benefit from higher throughput to describe the results with statistical relevance. Therefore, the wealth of information provided by quantitative microscopy can be proficiently exploited by higher throughputs and cell profiling.

Proteomics is a recently defined discipline which relies on the identification and analysis of the many protein ensembles (the proteomes) expressed by a cell or an organism. Similarly, cellomics studies the diverse cellular states and types that constitute a biological sample.

The aims of proteomics are the identification, the quantification, the determination of the structure, the characterization of post-translational modifications of all the proteins, and the study of their protein-protein interactions. The sub-discipline that studies protein-protein interactions is called interactomics. The human interactome is the ensemble of entire interaction networks and its determination is an insurmountable challenge for current methods and technologies. The human genome comprises ~25’000 genes, whose large majority codes for proteins. However, the entire human proteome accounts for ~400’000 proteins. This discrepancy is explained by alternative splicing of RNA and post-

translational modifications. On the other hand, the proteome of a human cell, *i.e.* the proteins actually expressed at a specific moment, is smaller (probably ~30% of the total). Dissimilar to the genome, the proteome is a dynamic entity whose constitutive elements are continuously changing with respect to cell cycle and environmental stimuli. Because a proteome defines the state of a cell and the cellular type, cellomics studies the cellular phenotype of a proteome. In other words, cellomics studies, at the cellular level, what proteomics investigates at the molecular level.

Proteomics mainly relies on biochemical separation and identification methods and mass spectroscopy. Their combination enables the separation and identification of the proteome of an entire organism, a tissue or an organ, a cell, or a sub-proteome localized in a specific cell fraction or organelle. Biochemistry further allows the study of protein-protein interactions. However, yeast-two-hybrid screening is one of the few techniques that provides a sufficiently high throughput to study interactomics. The very first studies in interactomics were carried out using such assays. Nevertheless, two-hybrid data has to be validated and cross-referenced with other techniques because frequently a high amount of false positive interactions are observed. One of the main challenges in proteomics is to increase the throughput of other techniques which could be useful for the validation or the cross-referencing of two-hybrid data. At the moment, screening instrumentation has been developed to perform more than 100’000 assays per day in microliter volumes. This is at present the standard speed for ultra high-throughput screening.

Cellomics relies on multi-parametric quantitative methods and cell profiling. Flow cytometry and Fluorescence Activated Cell Sorting are the leading high throughput technologies in this field. In this case, the challenge is to provide more information about the sample, *i.e.* to develop instrumentation for high-content screening. Automated imaging platforms are therefore the current primary technological confront in cellomics.

FRET imaging can be optimized and automated to enable large-scale screening of protein-protein interaction networks. The unsupervised operation of an automated FLIM system and data mining analysis combines the high information content of quantitative imaging with the high-throughput of automation. Therefore, we pursued the development of an unsupervised FLIM screening platform. Unfortunately, FLIM requires expensive instrumentation and specialized know-how to be operated. Therefore, the high economical and human costs associated with this technique are not compatible with the adoption of such screening platforms on a large scale.

All-solid-state technologies are rapidly diffusing in the microscopy field. Laser diodes, for instance, are routinely used in microscopy. Inexpensive light emitting diodes have also recently been used. These light sources can provide modulated or pulsed excitation, suitable for time-resolved detection. Charged-coupled devices (CCD) and complementary metal oxide semiconductor (CMOS) cameras are extensively used in microscopy, however, such detectors are not fast enough for reliable lifetime sensing in the nanosecond region. Fast solid-state detectors are available for use in laser scanning microscopes (LSM), *e.g.* (single photon counting) avalanche photo diodes (APD). However, the

comparatively high cost and low acquisition speed of LSMs do not allow the development of LSM-based high-throughput FLIM systems.

This PhD dissertation demonstrates that unsupervised fluorescence lifetime imaging can provide both high-throughput and information-content by the combination of automation and quantitative imaging. This novel methodology could offer a solution to the current challenges in proteomics and cellomics. Unit 2 describes the technological developments that resulted in: the establishment of an unsupervised FLIM screening platform (**chapter 5**) and the development of novel all-solid-state technologies capable of fast lifetime sensing (**chapter 6** and **chapter 7**). The integration of the unsupervised FLIM in a cost-effective, compact turnkey system could be a first step in the direction of large-scale protein-protein interaction screening. The spreading of simple and comparatively cheap technologies could make a substantial contribution to interactomics, proteomics and cellomics in the same way that cost-effective and user-friendly instrumentation favoured the success of the human genome project. Furthermore, all-solid-state FLIM technology could broaden the application of FLIM among the medical community, where FLIM can be used for diagnostic and histopathological purposes.

The methods and techniques developed here have their own limitations. Single-frequency frequency-domain lifetime detection possesses high-throughput at the cost of a lower photon-economy and information content, *i.e.* not all the collected photons are used efficiently and multiple lifetimes are not resolved. **Unit 1** describes the theoretical frameworks whose development was necessary to lift those restrictions (**chapter 2** and **chapter 3**, respectively). **Chapter 3** describes non linear effects present in the photophysics of the FRET pair: FRET incompetent fraction, FRET frustration, and sensitized acceptor photobleaching. A novel theoretical work on FRET quantification by molecular cross-correlation is also presented.

Unit 3 (**chapter 8**) describes biological applications of quantitative imaging techniques. The developed technologies have been used in combination with confocal microscopy, multi-photon imaging and conventional FLIM systems, for the study of the molecular mechanisms of neurodegenerative diseases like Parkinson's (PD) and Alzheimer's disease (AD). The focus of this thesis was on α-synuclein and its possible interactions with the cytoskeleton. α-Synuclein is a relatively small and natively unfolded protein whose function is not yet fully characterized. Particularly, the interaction between α-synuclein and the tau protein, a protein directly involved in Alzheimer's disease and hereditary tauopathies, was studied. Furthermore, the unsupervised FLIM was used to assess the ubiquitination of α-synuclein, a post-translational modification that mediates protein sorting and could target substrates for proteasomal degradation. Differences between wild-type α-synuclein and mutants causative for a familial form of Parkinson's disease are described.

In summary, this thesis deals with technological and theoretical challenges that limited the adaptation of state-of-the-art FRET methods and technologies for large-scale protein-protein interaction screening. A FLIM screening platform and an all-solid-state FLIM operating at video-rate are described. The photon-economy and the information content of

the adopted techniques are investigated and optimization strategies are proposed. The theoretical foundation for a new alternative microscopic technique based on the cross-correlation at the molecular level is also presented. Finally, state-of-the-art and newly developed tools of molecular biology and fluorescence lifetime imaging microscopy were combined for the investigation of the molecular physiology of two of the most prevalent neurodegenerative diseases.

In the near future, the all-solid-state FLIM will be integrated with the unsupervised screening platform. The prototyping of such an advanced but simple and cost-effective system could start the diffusion of this technology into the life sciences, the medical and the drug screening communities. Furthermore, the investigation of the molecular basis of α-synucleinopathies will be continued. Here, the working hypothesis is that α-Synuclein is an adapter protein whose main function is to increase the affinity of its interactome for lipid membranes.

Fluorescence Lifetime Imaging Microscopy and Förster resonance Energy Transfer

Nowadays, fluorescence microscopy plays a central role in the fields of biology, biophysics and in the life sciences in general. Its utility for imaging tissues, subcellular compartments and even single molecules with high contrast is expanded by the possibility to investigate different properties of fluorescence like intensity, polarization, lifetime and other spectroscopic parameters. Fluorescence lifetime in particular is a molecular property that offers a clearer fluorophore signature than intensity spectra and that contains information on the local molecular environment and chromophore photophysics of fluorescent labels. For these reasons, FLIM is used to enhance the image contrast of biological samples and to study molecular interactions of fluorescently labelled biomolecules by FRET. FRET is a photophysical phenomenon that allows the investigation of biochemical reactions in living cells. Under physiological conditions, FRET occurs exclusively between interacting molecules labelled with suitable fluorescent dyes with proper optical characteristics.

FRET imaged by FLIM exhibits the normal diffraction limited spatial resolution offered by the light microscopical system used, but it maps interaction events that are on the 10-100Å scale. Fluorescence microscopy flourished in the last decade with the advent of different detection techniques and the discovery of the green fluorescent protein and its different spectral variants. FRET imaging by FLIM contributed to the recent increase in sensing possibilities in cells.

FLIM – fluorescence lifetime imaging microscopy

Fluorescence emission is a property of molecules that can absorb photons and re-emit the accepted energy as photons with lower energy, *i.e.* at higher wavelengths. This process is described by the energy level (Jabłońksy) diagram presented in Figure 1. Thicker horizontal lines represent different electronic energy levels with their associated vibrational states (thinner lines). These are ordered with energy increasing from the bottom to the top of the graph. Transitions are plotted with straight lines for radiative processes and with wavy lines for non-radiative phenomena.

Only considering the ground state, the first two excited states and the first triplet state, the number of possible transitions between different electronic states is quite various:
photon absorption: $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_0 \rightarrow S_2$
radiative de-excitation between singlet states (fluorescence processes)
non radiative de-excitations (quenching)
radiative transition $T_1 \rightarrow S_0$ (phosphorescence)

Each transition is associated with a rate constant, a molecular property that, in general, can be perturbed by the environment. In fluorescence microscopy we are usually interested in the fluorescence transition $S_1 \rightarrow S_0$.

The excited state S_1 has a defined lifetime which depends on the rate constants of the different de-excitation pathways. After a photon is absorbed a fluorescence photon is emitted with a certain delay. One definition of lifetime is therefore the average time that a molecule spends in its excited state.

With average time is meant that the time decay is stochastic and follows a distribution that in general is exponential:

$$\text{(Eq.1)} \quad p(t) = \frac{1}{\tau} e^{-\frac{t}{\tau}}$$

The exponential decay constant τ of this probability distribution p(t) also represents the lifetime and it is easy to find that the two definitions match when one considers the average lifetime.

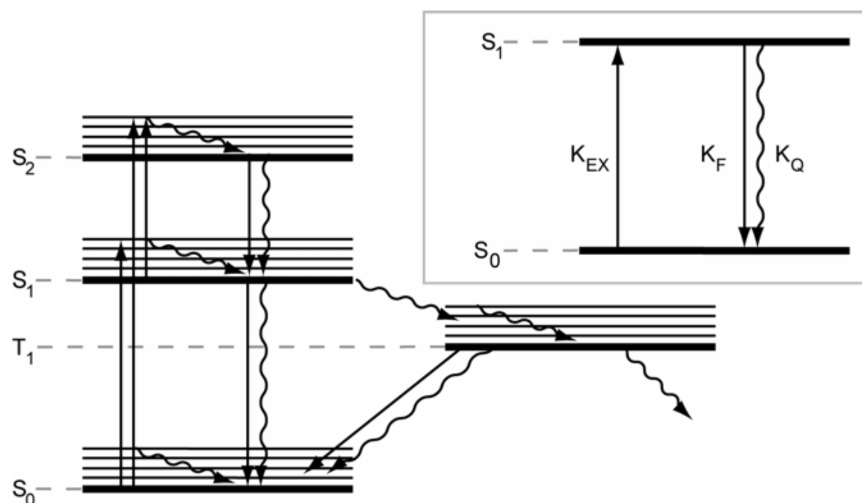


Figure 1. Typical depiction of fluorochrome photophysics (Jabłońksy diagram). Plotted are the first three singlet and the first triplet electronic states (more detailed description can be found in the text). Top inset: simplified Jabłońksy diagram, with photon absorption (K_{EX}), fluorescence (K_F) and internal conversion (K_Q, quenching) transitions indicated. Straight lines represent radiative transitions, wavy lines indicate non-radiative transitions.

The lifetime depends on the equilibrium of the different rate constants:

$$\text{(Eq. 2)} \quad \tau = \frac{1}{K_F + K_{NR}}$$

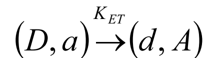
where K_F is the rate of photon emission, while K_{NR} is the rate of non-radiative de-excitation that could represent collisional quenching and/or energy transfer. An increase of these non-radiative processes due to interaction with the environment thus reduces the lifetime of a fluorophore that could be used by FLIM to probe these environmental influences. If the interactions with the environment are negligible or invariant, the lifetime represents a more reliable marker for a fluorescent species than its spectra. In fact, lifetimes distributions are considerably narrower than intensity spectra.

FLIM has been used successfully for the separation of different fluorophores in multiple-stained samples (Pepperkok *et al.*, 1999; Neher and Neher, 2004), to map endogenous autofluorescent cellular metabolites like NADH (Lakowicz *et al.*, 1992) or to measure ratiometric probes like the high-affinity calcium indicator dye Indo-1 (Szmacinski *et al.*, 1993). FLIM, performed on tissues, presents a growing and interesting diagnostic tool in the medical sciences (Tadrous *et al.*, 2003). A recent investigation (Eliceiri *et al.*, 2003) using FLIM on tissues stained with conventional histological dyes also demonstrated improved contrast and information content. Also, lifetime sensing has been recently applied for drug screening and, more in general, for high throughput applications (Eggeling *et al.*, 2003). In general, this thesis work developed on the technological and methodological challenges of the high-throughput and high-content screening by lifetime sensing.

FRET – Förster resonance energy transfer

FRET is the phenomenon by which a donor fluorochrome transfers energy to an acceptor molecule in a non-radiative manner (Förster, 1967; Clegg, 1996). This phenomenon can be better understood by a quantum physical mode; on the other hand, it is easier explained by the classical concept of coulombic dipole-dipole interactions, although without emission and consequent re-absorption of a real photon.

The simplified Jabłońksy diagram in Figure 2 presents the transitions between the donor and acceptor ground and excited states. The energy transfer transition is given by:



$$(D, a) \xrightarrow{K_{ET}} (d, A)$$

where D, A denotes excited states and *d, a* the ground states of donor and acceptor respectively. We can consider the donor-acceptor pair as a system with four quantum states (Figure 2):

1) *da*, both donor and acceptor are in the ground state;
2) *Da*, only the donor is excited;
3) *DA*, both donor and acceptor are excited;
4) *dA*, only the acceptor is excited.

The presented formalism will be thoroughly described in the appendix to this dissertation, where more complex aspects of a FRET pair photophysics will be reviewed.

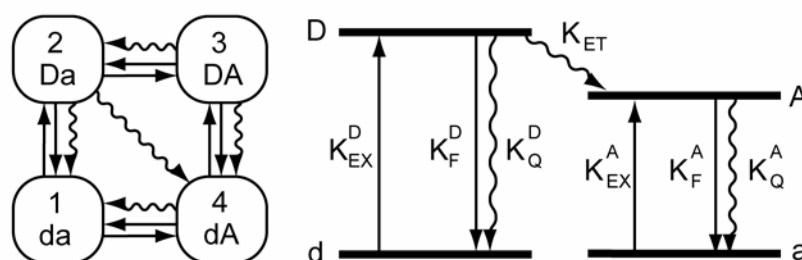


Figure 2 - Four-state transition diagram. The simplified energy level diagram (right) illustrates the transitions between ground and excited state of donor (D) and acceptor (A) fluorophores. The donor-acceptor pair can be considered as a unique photophysical system with four states (left) in which the diagonal radiation-less transition represents energy transfer. Small letters indicate the ground state, capitals indicate the excited state.

The energy or excitation transfer (ET) transition adds a de-excitation pathway to the single fluorochrome diagram (Figure 1 inset, 2) and hence reduces the lifetime of the donor excited state in accordance with equation 2, as it increases the probability per time unit of excited state depopulation. The quantum yield of this transition is called the FRET efficiency (ε). When the FRET efficiency is maximal (100%), *i.e.* when K_{ET} is very much greater than the other rate constants, all absorbed energy is transferred from the donor to the acceptor.

FRET is often called fluorescence resonance energy transfer. Because this name is ambiguous and could lead to the assumption that there is emission of a fluorescence photon by the donor or that the acceptor should be a fluorescent molecule, it is preferable to associate the first letter with the name Förster, the discoverer of this phenomenon. For the same reason, FRET is also referred to simply as RET or ET (energy transfer or excitation transfer) in literature.

One of the most interesting aspects of ET is its extreme dependence on the intermolecular distance. An ET event occurs typically between distances of 10-100Å, in the order of magnitude of protein size. Molar concentrations are required to reach these intermolecular distances in the absence of a specific interaction mechanism, a meaningless range under physiological conditions. This implies that if two molecules undergo FRET in a biological system, they are also interacting. The second interesting aspect is that the FRET efficiency falls with the sixth power of the intermolecular distance (

Figure 3):

$$\text{(Eq. 3)} \quad K_{ET} = \frac{1}{\tau_D}\frac{R_0^6}{R^6}; \quad R_0^6 = \frac{\kappa^2}{n^4}Q_D J; \quad \varepsilon = \frac{R_0^6}{R_0^6 + R^6}$$

Equation 3 illustrates some of the parameters of interest. R_0 is called the critical or Förster distance (or Förster radius). This parameter is a property of the FRET pair and is linked to the overlap integral J of the donor emission and acceptor absorption spectra, the

donor quantum efficiency Q_D, the refractive index of the medium n and an orientational factor κ. R_0 is the distance at which the two molecules transfer energy with 50% efficiency. The sixth-power dependence of FRET on fluorophore separation distance causes FRET to sense only short (<10 nm) distance interactions which diminishes the possibility for false positive detection of interactions. It also allows high sensitivity for intermolecular distance variations over a scale of a couple of nanometers where the efficiency goes from all to none.

κ is an orientational parameter whose most common definition is:

$$\text{(Eq. 4)} \quad \kappa^2 = \left(\cos\theta_T - 3\cos\theta_D \cos\theta_A\right)^2$$

As represented in figure 3, θ_T, θ_D and θ_A are the angles between donor-acceptor dipole moments, the donor dipole moment-intermolecular distance vector and the acceptor dipole moment-intermolecular distance vector. Another useful definition is:

$$\text{(Eq. 5)} \quad \kappa^2 = \cos^2\omega\left(1 + 3\cos^2\theta_D\right)$$

in which ω is the angle between the acceptor dipole moment and the electric field originating from the donor transition moment. This second relation illustrates that the FRET efficiency depends on two angular degrees of freedom and an intermolecular distance degree of freedom. κ^2 assumes a maximum value of 4 only when the donor and acceptor dipoles and the intermolecular distance vector are parallel or antiparallel as shown in table 1. This happens when the acceptor transition dipole is parallel or antiparallel (ω=0, π) to the donor electrical field. κ^2 assumes the minimum value of zero in more configurations, namely when these two vectors are orthogonal ($\omega=\pi/2$, $3\pi/2$) and at least one is perpendicular to the intermolecular distance vector. For this reason, the relative orientation of two fluorophores plays a crucial role in the yield of energy transfer.

A complete description of this aspect of ET is given by Lakowicz (1999) and Andres&Demidov (van der Meer B.W., 1999). The latter work shows that the probability to obtain a maximum value for κ^2 is almost negligible compared to other, lower, values. In any case, the probability on a particular value of κ^2 decreases with increasing values. The FRET efficiency between two rigidly connected chromophores will most probably be reduced by an unfavourable orientation of the two dipole moments. κ^2 is equal to 2/3 when complete rotational freedom between the two molecules can be assumed. This might hold true for small conjugated organic fluorophores, but is likely not the case for genetic fusions with the VFPs. In this case, the chromophore mobility is restricted inside the β-barrel of the VFP structure and the VFPs themselves are likely to bury into the surface of the fused protein. The orientation factor must therefore be optimised in the design of FRET-based biosensors by linker mutagenesis strategies to avoid the most probable but less optimal relative donor-acceptor orientation outcome. In fact, a colleague of the Cell Biophysics Group at the European Neuroscience Institute developed a standardized method for the optimization of the linker ("Linear Extensions for Good Orientation (LEGO)", M. Mitkovski *et al.*, unpublished work).

Another important parameter is the refractive index of the medium. The energy transfer efficiency depends on the inverse of the fourth power of the refractive index. Some problems can arise when changing the mounting medium or buffer solution. Mounting cells in mowiol or glycerol, for example, causes an increase of the refractive index up to 1.48-1.49, which is considerably higher than the refractive index of the cytoplasm which is estimated to be 1.38. At very low FRET efficiency, this can cause a significant reduction of the energy transfer. The possible effect of the refractive index should be considered critically under experimental conditions where the refractive index is varied over a broader range.

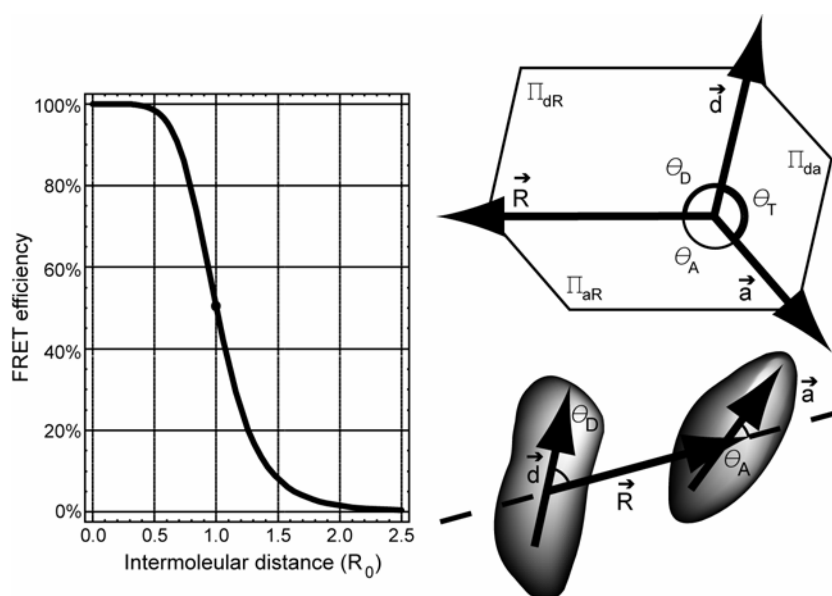


Figure 3. Spatial degrees of freedom in a FRET pair. The energy transfer phenomenon is a dipole-dipole interaction that depends on the intermolecular distance (R) and on the relative orientation of the two molecular dipole moments. The FRET efficiency increases with the sixth power of the intermolecular distance (left graph). The Förster radius, the distance at which the donor transfers energy to the acceptor with 50% efficiency, is a characteristic of the pair. Two interacting molecules are depicted (bottom-right) with their electrical dipole moment and the intermolecular distance vectors. The angle definitions of the three vectors are drawn in the upper-right scheme. Π indicates the plane defined by the different vectors and θ represents the angles (defined in the text) that are most commonly used as coordinates for the orientational factors.

Theory: Energy transfer, energy migration and lifetimes

Energy migration (EM) is the radiative migration of energy between molecules, *i.e.* the re-absorption of emitted photons. This phenomenon could dominate over longer distances where EM is relevant because it only falls with the second power of the intermolecular distance. Although we can consider EM as a distinct phenomenon, there are theoretical frameworks in which the two processes are explained as different manifestations of the