Chapter 1

Fluorescence

Cells are small and complex in their structure and even more complex in their function. It is difficult to see their structure, even more so to understand their molecular composition, and arbitrarily complex to understand the molecular interactions. Cell biology started with microscopy, and microscopy stayed an essential tool for biology and medicine.

Typical animal cells are 50–200μm in diameter, but the naked eye can resolve structures of about 100μm only. These cells are not only too small to be seen without aid but also almost colorless and translucent. Consequently, the discovery of their internal structures relied on a variety of stains developed for providing sufficient contrast to make cell structures visible. Because of their high visibility and advantageous features, fluorophores form a major and widely applied class of stains for visible light microscopy. For these reasons and up to date, microscopy in biology depends as much on techniques for preparing the specimen as on the performance of the microscopes themselves.

This chapter describes the energy levels of fluorophores based on the Jablonski diagram, as well as their molecular states and the events causing the molecules to undergo transitions between these states. Basic concepts of quantum mechanics are applied for understanding the interactions of molecules with light and for describing the fundamental behavior of fluorophores exposed to light. The chapter concludes with considerations regarding the suitability of various types of fluorophores for visible light microscopy.

1.1 Molecular states and energy levels

Molecules possess a large number of electronic energy levels and, moreover, an even larger number of kinetic energy sublevels due to the availability of mechanical vibrations on the molecules' atom–atom bonds as well as rotations of the molecule. Fortunately, the number of electronic energy levels that interplay in interactions of the molecule with visible light is quite limited. In general, only electrons with delocalized orbitals interact strongly with visible light, because their oscillation frequencies match the light frequencies and because they can transiently polarize the molecule.

A **Jablonski diagram** 1.1, named after the Polish physicist Aleksander Jabłoński, illustrates the energy states of a molecule and the relevant transitions between them. The molecular states are arranged vertically by energy and grouped horizontally by spin multiplicity. Small diagonal displacements indicate variations in the structural configuration (conformation) of the molecule. The horizontal axis is called **general coordinate** because it groups the spin multiplicity and multiple dimensions of conformation changes.

Electronic states are characterized by discrete energy levels with large energy differences well above the thermal energy. Electronic states are classified as singlet or triplet states based upon their angular momentum of the electron spin. The electrons in most organic molecules are paired with opposite spins ($\pm 1/2$) in the bonding and non-bonding orbitals, resulting in a net zero spin for the state. Such states have a single energy level (0) in an applied magnetic field and are therefore called **singlet states**. Electronic states in which two electrons with identical spin occupy different orbitals (Pauli exclusion principle) have a net spin of one ($2 \cdot 1/2$). In a magnetic field such states have three energy levels ($-1, 0, +1$) and are called

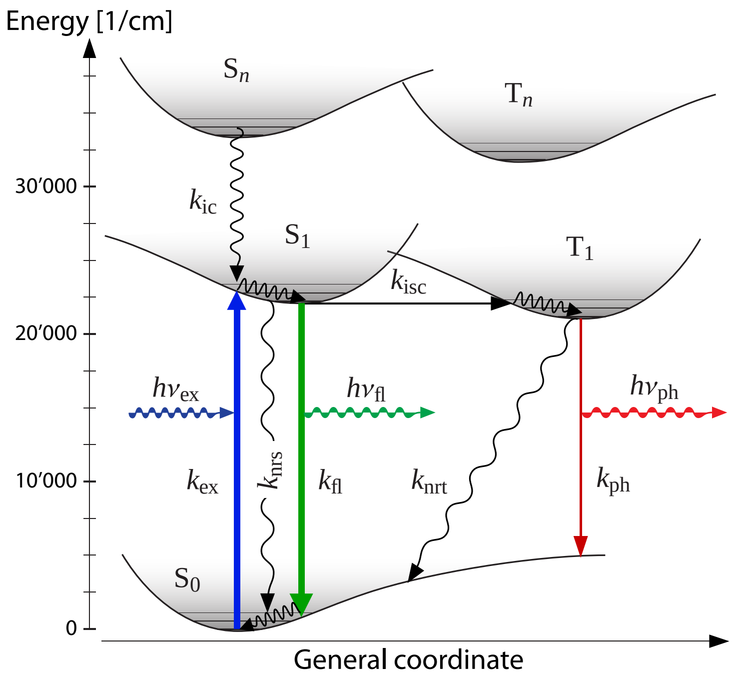

Figure 1.1: Jablonski diagram of molecular states, energy levels and transitions. The energy is scaled in waves per centimeter as $1/\lambda$.

triplet states. The distinction between singlet and triplet states is important because photon induced transitions always lead to a state of the same **spin multiplicity** (singlet→singlet or triplet→triplet).[1]

The **ground state** is the electronic state with the lowest energy that is occupied by a molecule in thermal equilibrium and without perturbation. For most molecules, the ground state is a singlet state denoted S_0. One notable exception is molecular oxygen O_2, which is found in its lowest triplet state T_0.[2] All other electronic states are **excited states** because they possess a higher energy than the ground state. S_1 to S_n denote excited singlet states and T_1 to T_n excited triplet states, respectively.

Each electronic state has associated **vibration** and **rotation substates**. The corresponding associated kinetic energy sublevels are discrete and quantized alike the electronic energy levels, but with a much smaller energy quantum. In thermal equilibrium, the molecule occupies these sublevels with a probability according to the Boltzmann distribution.

[1] In quantum mechanics, the spin multiplicity indicates the number of possible quantum states of a system with given principal spin quantum number S. The different states are distinguished by the spin projection quantum number S_z, which can take the values $-S, -S+1, ..., S-1, S$. Therefore, the spin multiplicity is $2S+1$, where S is the number of singly occupied orbitals multiplied by the electron spin quantum number m_s. A system with $S = 0$ has exactly one possible state. It is therefore in a singlet state. A system with $S = 1/2$ is a doublet; $S = 1$ is a triplet, and so on.
The most important application is to electrons in a molecular system. A single electron (as in a free radical) has $S = 1/2$ and is therefore always in a doublet state. Two electrons (as in a diradical) can pair up in a singlet or in a triplet state. Normally the singlet is the ground state.

[2] Oxygen has two singly occupied orbitals with electron spins that can be ↑↑, ↑↓ or ↓↓. Therefore, oxygen has a spin multiplicity of $2S + 1 = 2(2 \cdot 1/2) + 1 = 3$, where the term in brackets stands for the two singly occupied orbitals multiplied by the electron spin $m_s = 1/2$.

1.2 Light–molecule interactions

When it comes to study the interaction of light with matter, it is worth referring Albert Einstein's seminal finding for which he got the Nobel price in physics. He showed that the description of light as a continuous electromagnetic wave could not explain the threshold frequency required for extracting electrons out of a metallic material. Prior to this finding, Max Planck, physics Nobel price laureate himself, spent years in verifying experimental results on the electromagnetic radiation of black bodies. He finally succeeded in describing the full radiation spectrum with one well-defined equation by suggesting that the radiation is emitted in discrete energy quanta with an energy proportional to the light frequency [1]. A few years later, Albert Einstein reinterpreted the photoelectric effect and proved that light indeed consists of many energy quanta, called **photons** [2].

1.2.1 Quantum properties of light

In the classical picture, a light beam is described as an electromagnetic wave traveling at the speed of light c [m/s] and oscillating with a frequency ν [Hz], i.e. an angular frequency $\omega = 2\pi\nu$ [rad/s]. In vacuum, the speed of light is $c_0 = 2.998 \cdot 10^8$m/s. The speed of light slows down to $c = c_0/n$ in a material with refraction index n. The wavelength is given by $\lambda = c/\nu$ [m]. The propagation direction of the wave is described by a wave vector $\vec{k}$, which has a magnitude $|\vec{k}| = 2\pi/\lambda$ [rad/m]. The intensity I of the electromagnetic wave is obtained as the ratio P/A of the power P flowing through an area A and is linked to the amplitude $|\vec{U}|^2$ of the oscillating electromagnetic field. An associated plane wave is then given as

$$\vec{E}(\vec{r}, t) = \vec{U} \exp\left(i\vec{k} \cdot \vec{r} - i\omega t\right). \tag{1.1}$$

In the quantum-mechanical picture, a light beam is described by a stream of photons traveling at the speed of light c in the direction of the wave vector $\vec{k}$. Photons can be imagined as confined wave packets oscillating with a central frequency ν (figure 1.2). According to Max Planck and Albert Einstein, each **photon** carries an **energy** $E_{ph} = h\nu = \hbar\omega$, where the Planck constant is $h = 6.626 \cdot 10^{-34}$Js and the reduced Planck constant is $\hbar = h/2\pi = 1.055 \cdot 10^{-34}$Js. Therefore, an intensity I [W/m^2] of a light beam corresponds to a photon flux density $\Phi = I/h\nu$ [photons/m^{2}s].

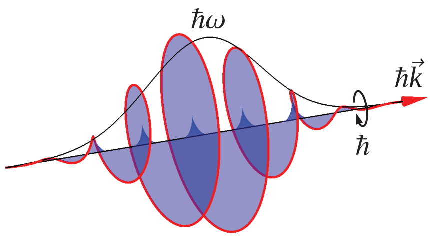

Figure 1.2: Graphical representation of a photon as a wave packet consisting of a few oscillations of the electromagnetic field with energy $\hbar\omega$, momentum $\hbar\vec{k}$ and angular momentum $\hbar$.

Photons carry a kinetic **momentum**, which is described by the momentum vector $\vec{p} = \hbar\vec{k}$. Its magnitude is also found by $|\vec{p}| = h/\lambda$. This momentum is responsible for the radiation pressure light exerts on matter.[3] Because photons only exist as long as they are traveling at the speed of light,[4] they do not possess mass at rest as one might conclude from Albert Einstein's famous relation $E = mc^2$.

[3] The radiation pressure may be used for satellite propulsion by solar sails, for instance.

[4] Photons are said to be ”stable” because they can travel for billions of years whilst crossing the universe. Applying the

Photons also possess **intrinsic angular momentum**, called **spin**. The magnitude of this spin is quantized to the two values $S = \pm\hbar$, often denoted as ± 1. The spin is responsible for the radiation torque light excerts on matter.[5]

1.2.2 Radiative transitions

Energy, momentum and angular momentum are conserved quantities. This imposes consequences for the interaction between a photon and a molecule. The energy of the photon determines the coupling strength of the photon's oscillating electromagnetic field with the electrons of the molecule. This coupling is strongest if a transition from the current molecular state to another electronic state offers an energy gap similar to the photon energy.

During a **radiative transit** a photon is created or destroyed. The conservation of energy implies that the photon energy equals the transit energy. Moreover, the conservation of momentum implies that the photon's momentum and its angular momentum are exchanged with the molecule as well. Therefore, the molecule not only transits to a different electronic state, but it also gets kinetic energy. In general and as a result thereof, the molecule finds itself in an energetic vibration and rotation substate of the new electronic state.

In the Jablonski diagram 1.1, radiative transitions are represented as straight vertical arrows between electronic states. The arrows are outlined vertically because the interaction time of a photon with a molecule is in the order of a femtosecond, which leaves no time for the molecule to relax from the experienced kinetic stress by finding a new thermal equilibrium. This is often referred to as the **Franck-Condon principle**, which states that during an electronic transit, a change from one vibration energy sublevel to another will be more likely to happen the better the two vibration wave functions overlap. The induced kinetic stress is then typically relaxed within picoseconds by dissipating heat. This **thermal relaxation** $ST^* \rightsquigarrow ST$ is outlined in a Jablonski diagram by a wavy diagonal arrow leading from an energetic substate ST^* to a more relaxed kinetic substate of the *same* electronic state ST.

1.2.3 Absorption of light

If a molecule is exposed to light, it may capture the energy of photons to transit into a different, more energetic electronic state. During this **absorption** event, the photon energy is transferred to the molecule and excites it. The momentum and the angular momentum of the absorbed photons are transferred as well, which puts the molecule in an energetic kinetic substate of the new electronic state. By heat dissipation, the molecule relaxes until it reaches a transient thermal equilibrium.

The better the photon energy matches with the transition energy of the molecule, the more likely the photon gets absorbed. A spectral measurement of the **transmissivity** $T(\lambda)$ of light traveling through a small cuvette containing the sample solution allows determining the absorption efficiency of the molecules with respect to the photon energy $E_{ph} = hc/\lambda$. This measurement is commonly performed with a dual-beam spectrometer, which measures the transmission through two sample arms simultaneously. After mutual calibration of the spectrometer arms with blanc samples, one arm measures the transmission $T_1(\lambda)$ through a cuvette containing only the solvent, whereas the other measures the transmission $T_2(\lambda)$ through an identical cuvette containing the dissolved analyte. The ratio $T_2(\lambda)/T_1(\lambda)$ yields then the net transmissivity $T(\lambda)$. This ratio measurement avoids perturbations by the reflectivity at the air–cuvette

relativity laws, the time a photon "experiences" between its creation and its destruction is exactly null, which renders obsolete any statement on stability from the viewpoint of a photon.

[5] Linearly polarized light does not induce torque because of evenly distributed spins, which sum up to zero in average.

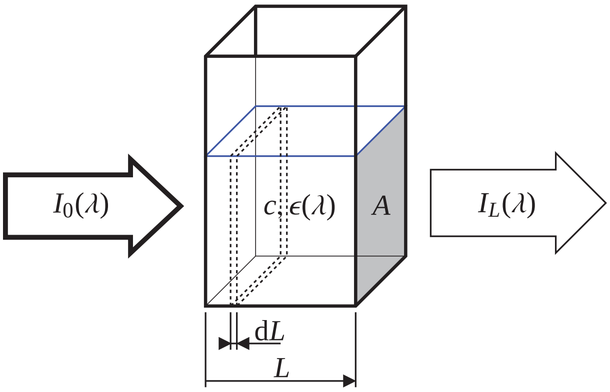

Figure 1.3: Absorption spectroscopy. An analyte solution in a cuvette is exposed to an incident light intensity $I_0(\lambda)$, which is reduced to the transmitted intensity $I_L(\lambda)$ after an absorption length L. The concentration of absorbing molecules is c and their extinction coefficient is $\epsilon(\lambda)$.

and the cuvette–sample interfaces or the absorption of light by the cuvette and the solvent. The ratio also rejects intensity fluctuations of the light source.

Figure 1.3 outlines the interpretation of the transmissivity $T(\lambda)$. L is the cuvette length and c is the analyte concentration. The incident light of intensity I_0 is partially absorbed by the analyte, which reduces the transmitted intensity $I_L \leq I_0$. Assuming a (macroscopically) homogeneous absorption in the cuvette, which is valid at low analyte concentration, a differential equation for the transmitted light intensity is obtained.

$$\frac{\mathrm{d}I(\lambda)}{I(\lambda)} = -\epsilon(\lambda)c\,\mathrm{d}L, \tag{1.2}$$

where $\epsilon(\lambda)$ [$\mathrm{M}^{-1}\mathrm{m}^{-1}$] is the **extinction coefficient**. Integration leads to the **law of Lambert-Beer**,

$$T(\lambda) = \frac{I_L(\lambda)}{I_0(\lambda)} = \exp\left(-\epsilon(\lambda)cL\right). \tag{1.3}$$

Of course, the absorption is not homogeneous at the molecular scale. Therefore, a different interpretation can be derived by attributing each absorbing molecule an **absorption cross-section** $\sigma(\lambda)$ [m^2]. Light falling on this cross-section shall get absorbed. Therefore, a number of molecules $\mathrm{d}N = cN_\mathrm{A}\,\mathrm{d}V$ in a small volume element $\mathrm{d}V = A\,\mathrm{d}L$ produce a shadow of $\sigma(\lambda)\,\mathrm{d}N$ area, where $N_\mathrm{A} = 6.022 \cdot 10^{23}/\mathrm{mol}$ is the Avogadro constant. The transmitted intensity becomes then proportional to the non-obstructed cross-section $A - \sigma(\lambda)\,\mathrm{d}N$, that is

$$T(\lambda) = \frac{I_L(\lambda)}{I_0(\lambda)} = 1 - \sigma(\lambda)cN_\mathrm{A}L. \tag{1.4}$$

Equating (1.3) and (1.4) results in the relation

$$\sigma(\lambda) = \frac{1 - \exp\left(-\epsilon(\lambda)cL\right)}{cN_\mathrm{A}L}, \tag{1.5}$$

which yields the equivalence $\sigma(\lambda) = \epsilon(\lambda)/N_\mathrm{A}$ in the limit of vanishing length $L \to 0$. The extinction coefficient ϵ and the absorption cross-section σ are both characteristic figures for the **polarizability** of a molecule, hence for its ability to get excited by light.

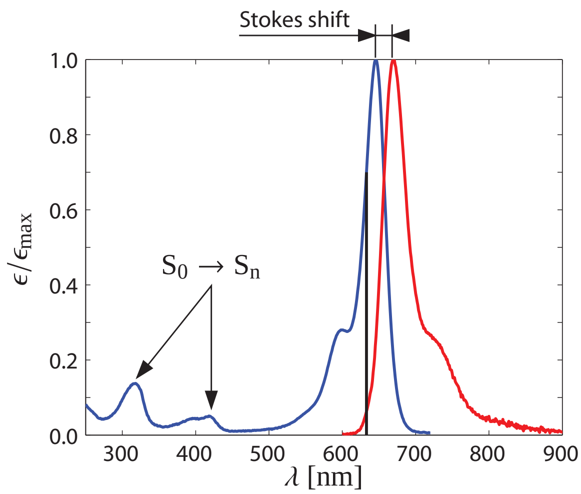

Figure 1.4: Absorption and emission spectra of the red fluorophore ATTO 647N in aqueous solution.

The absorption spectrum $\epsilon(\lambda)$ outlined in figure 1.4 shows a maximum at a wavelength $\lambda_{ex} = 644$nm. The maximum extinction coefficient $\epsilon_{max} = 1.5 \cdot 10^5 \mathrm{M}^{-1}\mathrm{cm}^{-1}$ corresponds to an absorption cross-section $\sigma_{max} = 2.5\text{Å}^2$, which is comparable to the size of a benzene ring in organic molecules. Secondary absorption peaks at $\lambda \approx 430$nm and $\lambda \approx 320$nm indicate excitations into higher singlet states S_n. The black vertical line shows a suitable excitation wavelength of 633nm (HeNe laser).

1.2.4 Emission of light

If a molecule is in a higher electronic state, it can give off its energy by emitting a photon for returning to a lower electronic state. The emitted photon will obtain the difference energy of this radiative transit. Because the photon also carries away momentum and angular momentum, the molecule will be again in an energetic kinetic substate after the transit, from which it relaxes by heat dissipation.

The conservation of the spin multiplicity favors emissive transitions between electronic states of the same kind – singlet or triplet. If a molecule stays in its lowest excited triplet state T_1, it can hardly return to its singlet ground state S_0 by emitting a photon. The probability of such a transition is small but non-null because of the overlap integral of the nuclear wave functions (heavy atom effect) and because of collisions with neighboring molecules, which can provide the necessary spin flip.

If the molecule is left in a higher electronic level, it tends to get rid of its excitation energy, for instance by **spontaneous emission** of a photon. If this emission stems from an allowed transition between states of identical spin multiplicity, it is termed **fluorescence**. Otherwise, it is called **phosphorescence**. Due to the much higher probability of performing an allowed transition, phosphorescence is in general much weaker than fluorescence. Fluorescence occurs typically within nanoseconds, whereas phosphorescence occurs on a microsecond to seconds time scale.

Because the molecule yet dissipated heat after absorbing a photon and will dissipate heat again after emitting a photon, the energy of the emitted photon is in general lower than the absorbed energy. This means that the emitted photons have a longer wavelength – the emission spectrum is red shifted. In a polar solvent, for instance water, the changes of the molecule's polarization induce a reorganization of the neighboring solvent molecules as well. This reorganization leads to a partial compensation of the polarization change, which also contributes to the red shift. The red shift of the peak emission wavelength versus the peak absorption wavelength is called **Stokes shift** [m]. Due to the stochastic nature of all these events, the emission spectrum is broadened. The spectral bandwidth of the emission is much larger than the homogeneous line width. For instance, the fluorescence spectrum of the red fluorophore ATTO 647N outlined in figure 1.4 peaks at a wavelength $\lambda_{\mathrm{fl}} = 669\mathrm{nm}$. The measured Stokes shift of this fluorophore is therefore 25nm.

If an excited molecule is exposed to light matching (part of) its *fluorescence* spectrum, a **stimulated emission** can be induced. The photon emitted by this stimulated transit carries the same energy, momentum and angular momentum as the stimulating photon. This process is exhaustively used in lasers because it provides a coherent amplification of the number of photons in an excited gain medium. The efficiency of the stimulation can be described with a cross-section much alike the absorption cross-section $\sigma(\lambda)$. However, the stimulation cross-section does not absorb the stimulating photons. Instead of producing a shadow, it duplicates the incident photons. Albert Einstein showed that the stimulation cross-section is identical to the emission cross-section, which means that the stimulated emission efficiency and the spontaneous emission spectrum match perfectly.

Molecules that are prone to emit a photon swiftly after excitation are called **fluorophores**. In general, fluorophores are small organic molecules with benzene rings, or fluorescent proteins. Many organic molecules absorb and emit light in the near ultraviolet range. The absorption and emission spectra of engineered fluorophores are usually in the visible to near infrared range. The **chromophore** is the photoactive core of a fluorophore or fluorescent protein.

Molecules that are good absorbers but bad emitters belong to the class of **colorants** or **dyes** (though dye became synonym to fluorophore). Colorants do hardly emit light but are very photostable, thereby providing permanent colors for textiles, paints and so on.

1.2.5 Non-radiative transitions

An excited molecule is not required to return to a lower electronic state by radiation. Indeed, most molecules tend to give off the excitation energy swiftly by dissipating heat or by transferring their polarization to neighboring molecules.

By **internal conversion**, that is by transforming electronic energy (polarization) into kinetic energy (vibrations), a molecule can transit from a higher to a lower electronic state. In this case, the kinetic energy is so high that the molecule is free to transit into an electronic state of different spin multiplicity whilst dissipating heat. A transition from a singlet to a triplet state or vice versa is called **intersystem crossing**.

The thermal relaxation can be considered as non-radiative too, although it does not bridge electronic energy levels and can therefore only dissipate heat (phonons) by definition, not photons.

1.2.6 Förster resonant energy transfer

A molecule can also get excited by receiving energy via direct resonant dipole–dipole energy transfer from a nearby fluorophore. In this **Förster resonant energy transfer** (FRET), the receiving molecule is the **acceptor**, whereas the sending fluorophore is the **donor**. FRET is a near field process and has a typical coupling range of about 10nm or less over which this process is likely to occur, provided that the acceptor and donor transitions have matching energies. Because of its strong dependence on the donor–acceptor distance, FRET can be used as a ruler of molecular distances, see for instance [4, 5].

Depolarization of a fluorophore from its excited triplet state T_1 to its singlet ground state S_0 is very much favored by a FRET of the fluorophore's triplet energy to molecular oxygen O_2. Because oxygen is normally in its triplet ground state T_0, this energy transfer is efficient and typically occurs within microseconds at room temperature. If oxygen is removed from a fluorophore solution, the fluorophores stay $10^2 - 10^4\times$ longer in their (dark) triplet state T_1.

Light is an electromagnetic wave with quantized energy. The light quanta are called **photons** and carry an energy $h\nu$, a momentum $\hbar\vec{k}$ and an angular momentum (spin) $\pm\hbar$; but no rest mass.

Molecules possess energy states of different kind. **Electronic states** reflect the electron energy levels and the (transient) polarizability of the molecule. **Vibration** and **rotation substates** reflect kinetic energy sublevels of the electronic states. These kinetic substates are due to vibrations of the molecule's atoms (bond stretches) and due to rotations of the molecule as a whole.

Molecules can interact with light by **absorbing** or **emitting** photons. The absorbed photon transfers its energy to the molecule, which transits into a higher electronic state of identical spin multiplicity – **singlet** or **triplet**. The molecule can emit a photon be returning into a lower electronic state. Absorption and emission are **radiative transitions** bridging the large energy gaps between electronic states of the molecule.

Due to the exchange of momentum and angular momentum between the photon and the molecule, the molecule transits into an energetic kinetic substate of the new electronic state. After the transit, the molecule relaxes from this mechanical stress by dissipating heat, called **thermal relaxation**. Moreover, in a polar solution, a transient polarization of the molecule will cause a reorganization of the neighboring solvent molecules, which partially shields the polarization change.

Fluorophores are (organic) molecules that likely emit a **fluorescence** photon swiftly after absorption. **Intersystem crossing** to and emission from an electronic state with different spin multiplicity are unlikely to occur but can result in the emission of **phosphorescence** photons. The dissipated heat and the polarization shielding are responsible for the Stokes shift and the broad emission spectrum. The **Stokes shift** denotes the red shift of the emission as compared to the absorbed light.

1.3 Transition rates

Fluorophores are frequently used as specific stains for fluorescence microscopy of cells at ambient or physiological temperature. This means that their typical environment is an aqueous solution. Under these conditions, table 1.1 summarizes the time scales and defines the various transitions between the most relevant molecular states of a fluorophore. A fluorophore takes the characteristic **transition time** τ_{xy} before it undergoes a transit. The dwelling time before a transit is stochastic and can be well described

Process	**Transition**	**Time scale**
Internal conversion	$S_n \rightarrow S_1$	$\tau_{ic} \approx 10^{-14} - 10^{-11}$s
Thermal relaxation	$S_n^* \rightarrow S_n$	$\tau_{tr} \approx 10^{-12} - 10^{-10}$s
Intersystem crossing	$S_1 \rightarrow T_1$	$\tau_{isc} \approx 10^{-11} - 10^{-6}$s
Fluorescence	$S_1 \rightarrow S_0$	$\tau_{fl} \approx 10^{-9} - 10^{-6}$s
Phosphorescence	$T_1 \rightarrow S_0$	$\tau_{ph} \approx 10^{-6} - 10^{2}$s
Non-radiative decay	$S_1 \rightarrow S_0$ $T_1 \rightarrow S_0$	$\tau_{nrs} \approx 10^{-7} - 10^{-5}$s $\tau_{nrt} \approx 10^{-6} - 10^{2}$s

Table 1.1: Time scales of molecular state transitions.

by an exponential probability density with characteristic decay time τ_{xy}.[6] The characteristic **transition rate** k_{xy} is given by $1/\tau_{xy}$. If a number of N fluorophores "prepare" for the same transition xy, the observable **transit rate** K_{xy} equals to $k_{xy}N$.

With these definitions, it is now possible to draw conclusions about the characteristic response of a fluorophore if continuously exposed to light. This characteristic response includes the lifetime of the electronic states as well as the average emission rates.

How long does a fluorophore reside in a particular state ST? The Jablonski diagram should show all transitions xy that start from the state ST, as well as their transition rates k_{xy}. Therefore, the state can be left with a total transition rate equal to the total rate of all leaving transitions xy. The characteristic **residence time** in the state ST is therefore given by

$$\tau_{ST} = \left(\sum_{xy} k_{xy}\right)^{-1} = \left(\sum_{xy} \frac{1}{\tau_{xy}}\right)^{-1}. \tag{1.6}$$

This is the **lifetime** τ_{ST} of this molecular state ST.

Considering now the probability that a fluorophore undertakes a particular transition xy from the state ST, it becomes immediately clear that this probability is given by

$$q_{xy} = k_{xy}\tau_{ST}. \tag{1.7}$$

This is the **quantum efficiency** $q_{xy} \in (0, 1]$, also called **yield**. These terms imply that the fluorophore "selects" and undertakes one and exactly one transit for leaving the current molecular state.

How does a fluorophore respond if exposed to a constant photon flux? A fluorophore exposed to a constant light intensity I_{ex} will continuously perform absorption–emission cycles. Its average cycling behavior can be derived directly from the lifetime of its molecular states and the quantum efficiencies of its transitions. This average behavior is exemplified by considering the Jablonski diagram 1.1. For this example, the internal conversion k_{ic} can be neglected by assuming that the fluorophore never reaches the excited state S_n.

[6] In principle, the thermal relaxation would require a multi-exponential probability density for describing this multi-level multi-step process. In practice however, experimental results are adequately described by a single exponential decay, because the thermal relaxations are much faster than the other transitions.

Let assume that the fluorophore initially resides in its ground state S_0. The incident light provides the fluorophore excitation energy because the fluorophore captures photons at an absorption rate k_{ex}.

$$k_{ex} = \sigma_{ex}\Phi_{ex} = \sigma_{ex}\frac{I_{ex}\lambda_{ex}}{hc_0} = \frac{1}{\tau_{ex}} \tag{1.8}$$

The fluorophore will take an average time $\tau_{ex} + \tau_{tr}$ to reach the excited singlet state S_1. There, it will reside for the excited singlet state lifetime $\tau_{S1} = 1/(k_{fl} + k_{nrs} + k_{isc})$, the **fluorescence lifetime**, before it returns to S_0 or crosses to the triplet state T_1. With a probability $q_{isc} = k_{isc}\tau_{S1}$ it passes to the triplet state T_1 and will take an additional time of $\tau_{tr} + \tau_{T1}$ for returning to S_0, where $\tau_{T1} = 1/(k_{ph} + k_{nrt})$ is the **triplet state lifetime**.

The average time τ for the cycle $S_0 \to \ldots \to S_0$ is given by the sum of the residence times in the traversed states weighted by the probability of encountering these states.

$$\tau = \tau_{ex} + \tau_{S1} + 2\tau_{tr} + q_{isc}\left(\tau_{tr} + \tau_{T1}\right) \approx \tau_{ex} + \tau_{S1} + q_{isc}\tau_{T1} \tag{1.9}$$

The time-averaged probability that the fluorophore resides in the molecular state ST is obtained by normalizing the residence time in that state by the average cycle time. This probability is the **occupation probability** $P_{ST} = \tau_{ST}/\tau$, from which the emission rates per molecule are derived.

$$K_{fl} = k_{fl}P_{S1} = k_{fl}\frac{\tau_{S1}}{\tau} = \frac{q_{fl}}{\tau} \tag{1.10}$$

$$K_{ph} = k_{ph}P_{T1} = k_{ph}\frac{q_{isc}\tau_{T1}}{\tau} = q_{isc}\frac{q_{ph}}{\tau} \tag{1.11}$$

How does the fluorophore respond if the excitation intensity is increased? Equation (1.8) shows that the absorption rate k_{ex} is directly proportional to the photon flux Φ_{ex}. If the excitation intensity is increased more and more, the absorption time $\tau_{ex} \to 0$. In the limit, the cycle time (1.9) reduces to $\tau \to \tau_{S1} + q_{isc}\tau_{T1}$. The cycling rate is then limited by the time the fluorophore takes to return spontaneously to the ground state. This means that the emission rates saturate.

The **saturation intensity** I_{sat} is defined as the excitation intensity I_{ex} required for depleting the ground state population to 50% of the fluorophores, which corresponds to an S_0 occupation probability of 50%. Therefore, the saturation intensity is obtained by fulfilling the condition $\tau_{ex} = \tau_{S1} + q_{isc}\tau_{T1}$.

$$I_{sat} = \frac{hc_0}{\sigma_{ex}\lambda_{ex}}\left(\tau_{S1} + q_{isc}\tau_{T1}\right)^{-1} \tag{1.12}$$

1.4 Rate equations

The principal time-averaged response of a fluorophore to a continuous incident photon flux Φ_{ex} was just depicted by calculating the lifetimes of the molecular states and the yields of all transitions connecting them. In the following, this response is described by the rate equations linking the transit rates with the variations of the molecular state populations, which allows studying the fluorophore's transient behavior to varying excitation conditions $\Phi_{ex}(t)$ as well.

Let P_{ST} be the occupation probability of the molecular state as defined before. For a given total number N of identical fluorophores, the **populations** of state ST are then given by the number of fluorophores in that state, that is $N_{\mathrm{ST}} = P_{\mathrm{ST}} N$. In other words, the occupation probability is tantamount to the population if a single fluorophore is considered ($N = 1$).

The variation of the population of the state ST can be expressed by the transit rates from and to all connected molecular states. This relation is called **rate equation** and is of the general form

$$\frac{\partial P_{\mathrm{ST}}}{\partial t} = \sum_{\overleftarrow{\mathrm{xy}} \text{ from } \overleftarrow{\mathrm{ST}}} k_{\overleftarrow{\mathrm{xy}}}(t) P_{\overleftarrow{\mathrm{ST}}}(t) - \sum_{\overrightarrow{\mathrm{xy}} \text{ to } \overrightarrow{\mathrm{ST}}} k_{\overrightarrow{\mathrm{xy}}}(t) P_{\overrightarrow{\mathrm{ST}}}(t), \tag{1.13}$$

where all transits $\overleftarrow{\mathrm{xy}}$ from connected states $\overleftarrow{\mathrm{ST}}$ increase the population and all transits $\overrightarrow{\mathrm{xy}}$ to connected states $\overrightarrow{\mathrm{ST}}$ decrease the population of the state ST. Therefore, the complete set of rate equations (1.13) for all molecular states describes the response of the fluorophore in all detail. This system of rate equations can be expressed in matrix form as

$$\frac{\partial \vec{P}}{\partial t} = \overleftrightarrow{\mathbb{K}}(t) \vec{P}(t), \tag{1.14}$$

where $\vec{P}$ is a column vector describing the populations $P_{\mathrm{ST}}(t)$ of all molecular states ST, and where $\overleftrightarrow{\mathbb{K}}$ is the $m \times m$ connection matrix formed by all transition rates $\pm k_{\mathrm{xy}}(t)$ connecting these m states.

Solving the rate equations for the excited state populations $P_{\mathrm{S1}}(t)$ or $P_{\mathrm{T1}}(t)$ allows then to obtain the emission rates. Much like before, the fluorescence emission rate per molecule is finally given by $K_{\mathrm{fl}}(t) = k_{\mathrm{fl}}(t) P_{\mathrm{S1}}(t)$ and the phosphorescence rate by $K_{\mathrm{ph}}(t) = k_{\mathrm{ph}}(t) P_{\mathrm{T1}}(t)$, respectively.

> The **transition rate** k_{xy} describes the characteristic rate at which the molecule will undergo a transit if it resides in the departure state ST of the transition xy. The characteristic **transition time** τ_{xy} is given by $\tau_{\mathrm{xy}} = 1/k_{\mathrm{xy}}$. The **transit rate** $K_{\mathrm{xy}} = k_{\mathrm{xy}} N_{\mathrm{ST}}$ describes the observed rate at which the molecules undertake the transition, where N_{ST} is the population in the departure state ST.
>
> The **population** N_{ST} of a molecular state ST is the number of molecules residing in that state. Normalizing N_{ST} by the total number N of molecules yields the **occupation probability** P_{ST}. The transit rate per molecule is then given by $k_{\mathrm{xy}} P_{\mathrm{ST}}$.
>
> The **residence time** τ_{ST} describes the characteristic time a molecule stays in the state ST. It is the inverse of the total rate of all transitions xy that depart from ST: $\tau_{\mathrm{ST}} = 1/\sum k_{\mathrm{xy}}$. The **quantum efficiency** or **yield** $q_{\mathrm{xy}} = k_{\mathrm{xy}} \tau_{\mathrm{ST}}$ is the probability a molecule undergoes a particular transition xy if it resides in its departure state ST.
>
> The characteristic time τ for a complete cycle $\mathrm{S}_0 \to \ldots \to \mathrm{S}_0$ is obtained by summing the weighted residence times in the traversed states, where the weights are the probabilities of encountering these states. The occupation probabilities are then obtained by normalizing the weighted residence times by τ, from which the emission rates at the dynamic equilibrium are immediately obtained.

It is worth noting that the solution of the rate equations can become arbitrarily complex, in particular if the transition rates are time-dependent. Fortunately, the complexity can be reduced significantly by noting that the thermal relaxation can often be neglected or attributed to the lifetime of the relaxed state.

Moreover, the excitation rate $k_{ex}(t)$ is typically the only relevant time-varying transition rate. For (piecewise) constant k_{ex}, the eigenvalues of the transition matrix $\overleftrightarrow{\mathbb{K}}$ are the equilibration rates of the states, whereas the eigenvectors are the corresponding populations. The column-wise sums of the transition rates in $\overleftrightarrow{\mathbb{K}}$ are always zero if the number of fluorophores stays constant. In this case, one eigenvalue is zero and its eigenvector yields the steady state populations.

The following sections exemplify the rate equations and their solutions for two frequently applied models. These models provide much simplified but sufficiently accurate descriptions of the fluorophore's photophysical behavior. The first model uses just two states, the ground state S_0 and the excited state S_1. The second model uses three states for taking into account the triplet state T_1 too.

1.5 Two-level model

The simplified Jablonski diagram 1.5 shows the most essential electronic states and transitions of a fluorophore. This two-level model forms the simplest possible description of fluorescence. Here, the ground state S_0 and the excited state S_1 are both singlet states.[7] The rate equations are readily obtained by applying the general form (1.13).

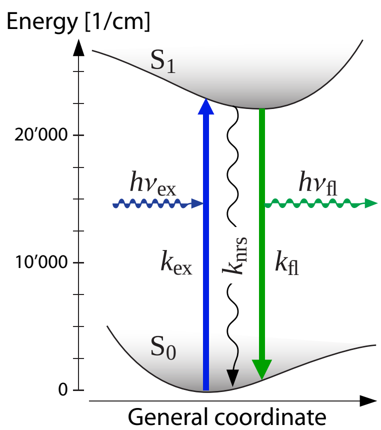

Figure 1.5: Two-level Jablonski diagram showing the singlet states S_0 and S_1 as well as the transition rates of the excitation k_{ex}, the fluorescence k_{fl} and the non-radiative decay k_{nrs}.

$$\begin{cases} \dfrac{\partial P_{S0}}{\partial t} = -k_{ex} P_{S0} + (k_{fl} + k_{nrs})\, P_{S1} \\ \dfrac{\partial P_{S1}}{\partial t} = k_{ex} P_{S0} - (k_{fl} + k_{nrs})\, P_{S1} \end{cases} \tag{1.15}$$

We note that $k_{fl} + k_{nrs} = k_{S1}$ is the total transition rate $S_1 \to S_0$, which yields the excited state lifetime $\tau_{S1} = 1/k_{S1}$. Alternatively, the rate equation system (1.15) can be written in the matrix form $\dot{\vec{P}} = \overleftrightarrow{\mathbb{K}}\vec{P}$, where

$$\vec{P}(t) = \begin{pmatrix} P_{S0}(t) \\ P_{S1}(t) \end{pmatrix} \quad \text{and} \quad \overleftrightarrow{\mathbb{K}}(t) = \begin{pmatrix} -k_{ex}(t) & k_{S1} \\ k_{ex}(t) & -k_{S1} \end{pmatrix}. \tag{1.16}$$

[7] The two-level model neglects the thermal relaxations but assumes a Stokes shift. In fact, the Stokes shift is essential for an efficient absorption because it largely prevents stimulated emission by the excitation beam. Implicitly, any two-level model of a fluorophore is a three- or four-level model.

With the condition $P_{S0}+P_{S1} = 1$ and the abbreviation $k(t) = k_{ex}(t)+k_{S1}$, two independent inhomogeneous differential equations are obtained.

$$\begin{aligned} \dot{P}_{S0}(t) + k(t)P_{S0}(t) &= k_{S1} \\ \dot{P}_{S1}(t) + k(t)P_{S1}(t) &= k_{ex}(t) \end{aligned} \tag{1.17}$$

Because the fluorescence emission per molecule is given by equation (1.10) as $K_{fl}(t) = k_{fl}P_{S1}(t)$, it is sufficient to solve for the excited state population $P_{S1}(t)$. Unfortunately, there is no general solution for the behavior of a fluorophore in response to a varying excitation rate $k_{ex}(t)$, not even for this simple two-level model. However, we can easily draw the main characteristics of the fluorophore's response to piece-wise constant conditions.

What happens if the excitation stays constant? If the excitation is kept continuously at the rate k_{ex}, the populations will reach a **dynamic equilibrium** characterized by $\vec{P}(t) = \text{constant}$. This is the **steady state** solution that we find by letting $\partial\vec{P}/\partial t = 0$, which immediately leads to $P_{S1} = k_{ex}/k$.

It is quite instructive to check whether this steady state solution is equal to the solution found in section 1.3. In this two-level model, the characteristic cycle time is $\tau = \tau_{ex} + \tau_{S1}$. Therefore, the average population of the excited state is $P_{S1} = \tau_{S1}/\tau$, which is indeed identical to k_{ex}/k.

What happens if the excitation is switched on? Let assume no excitation for $t < 0$ and a constant excitation rate k_{ex} for $t > 0$. The fluorophore will be initially in the ground state, that is $P_{S1}(t \leq 0) = 0$. For $t > 0$, the steady state solution is approached and will be reached for $t \gg 0$. The transient response of the fluorophore is found to be

$$P_{S1}(t \geq 0) = \frac{k_{ex}}{k}\left(1 - \exp\left(-kt\right)\right), \tag{1.18}$$

which can be verified easily by putting it back into the differential equation (1.17). We observe that $k(t)$ is the decay rate at which the singlet state populations track transients in the excitation rate $k_{ex}(t)$.

What happens if the excitation is switched off? Let assume the excitation is switched off at $t = 0$. For obtaining the response at $t > 0$, we solve the homogeneous differential equation $\dot{P}_{S1} + k_{S1}P_{S1} = 0$ or we reuse the result (1.18) for a negative transient. We find

$$P_{S1}(t \geq 0) = P_{S1}(0)\exp\left(-k_{S1}t\right), \tag{1.19}$$

where the initial condition is the excited state population $P_{S1}(t = 0)$. After switching the excitation off, the excited state population decays exponentially. As we could expect, the characteristic decay time is the fluorescence lifetime of the fluorophore.

What happens if the excitation rate changes? Let assume that the excitation rate changes to k_{ex} at the time $t = 0$. The initial condition is again the excited state population $P_{S1}(t = 0)$ due to prior excitations. From the transient response (1.18), we obtain directly the response to the change in the excitation rate if we note that the transient only applies to the change of the excited state population.

$$P_{S1}(t \geq 0) = P_{S1}(0) + \left(\frac{k_{ex}}{k} - P_{S1}(0)\right)\left(1 - \exp\left(-kt\right)\right) \tag{1.20}$$

Letting $P_{S1}(0) = 0$ reproduces the result (1.18) and $k_{ex} = 0$ yields the solution (1.19), respectively.

What happens if the excitation rate varies continuously? In principle, the differential equation (1.17) has no general solution for a continuously changing excitation rate $k_{ex}(t)$ because the excitation rate also changes the equilibration rate $k(t)$. However, if this influence is small compared to k_{S1} (no saturation) or if the excitation rate variations are small (small perturbation), the fluorophore can be modeled like a linear shift invariant (LSI) system. Assuming a LSI system, the response of the fluorophore can be approximated by an exponential impulse response convolved with the excitation rate.

Let $\overline{k}_{ex} = \langle k_{ex}(t) \rangle$ be the average excitation rate. The average equilibration rate for the singlet populations is then given by $\overline{k} = \overline{k}_{ex} + k_{S1}$. If the variations $|\delta k_{ex}(t)| \ll \overline{k}$, the differential equation (1.17) can be approximated by

$$\dot{P}_{S1}(t) + \overline{k} P_{S1}(t) \approx k_{ex}(t). \tag{1.21}$$

The homogeneous solution is identical to the result (1.19) but with a characteristic decay time $\overline{k}$. If the fluorophore is brought in the excited state at $t = 0$ by a short excitation pulse (Dirac), it will respond by

$$P_{S1}(t > 0) \approx \exp\left(-\overline{k}t\right), \tag{1.22}$$

which is the **impulse response** or the **Green's function** of the fluorophore. Therefore, the response to a variable excitation rate is given by the convolution of $k_{ex}(t)$ with this impulse response.

$$P_{S1}(t) \approx \int_0^\infty k_{ex}(t - \tau) \exp\left(-\overline{k}\tau\right) \, d\tau \tag{1.23}$$

1.5.1 Exercise

Using the Jablonski diagram 1.5, calculate the fluorescence emission rate $K_{fl}(t)$ for a sinusoidal excitation intensity $I_{ex}(t) = 2I_0 \cos^2(\omega t/2)$, where $I_0 \ll I_{sat}$. What happens if the modulation frequency ω is increased from zero to infinity?

Hint Characterize the response in the frequency domain.

1.6 Three-level model

The simplified Jablonski diagram 1.6 shows the essential electronic states and transitions of a fluorophore for modeling fluorescence and phosphorescence. This three-level model extends the previous two-level model by including the lowest triplet state T_1. Although this model is still fairly simple, it is well appreciated because it allows modeling most features of fluorophores with sufficient fidelity.

Once more, the rate equations are obtained by applying the general form (1.13).

$$\begin{cases} \dfrac{\partial P_{S0}}{\partial t} = -k_{ex} P_{S0} + (k_{fl} + k_{nrs}) P_{S1} + \left(k_{ph} + k_{nrt}\right) P_{T1} \\ \dfrac{\partial P_{S1}}{\partial t} = k_{ex} P_{S0} - (k_{fl} + k_{nrs} + k_{isc}) P_{S1} \\ \dfrac{\partial P_{T1}}{\partial t} = k_{isc} P_{S1} - \left(k_{ph} + k_{nrt}\right) P_{T1} \end{cases} \tag{1.24}$$

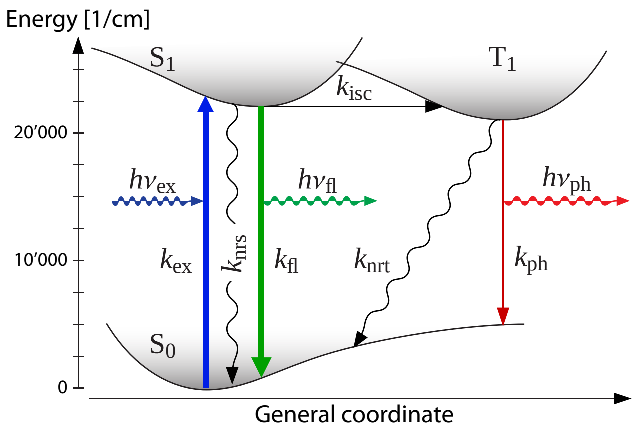

Figure 1.6: Three-level Jablonski diagram showing the singlet states S_0 and S_1 as well as the triplet state T_1 and the relevant transition rates.

The second equation shows the fluorescence lifetime, that is the total S_1 depopulation rate $k_{S1} = k_{fl} + k_{nrs} + k_{isc}$. The third equation does the same for the triplet state lifetime, that is $k_{T1} = k_{ph} + k_{nrt}$. Using these abbreviations, the rate equations (1.24) can be rewritten in the matrix form $\dot{\vec{P}} = \overleftrightarrow{\mathbb{K}}\vec{P}$, where

$$\vec{P}(t) = \begin{pmatrix} P_{S0}(t) \\ P_{S1}(t) \\ P_{T1}(t) \end{pmatrix} \quad \text{and} \quad \overleftrightarrow{\mathbb{K}}(t) = \begin{pmatrix} -k_{ex}(t) & k_{fl} + k_{nrs} & k_{T1} \\ k_{ex}(t) & -k_{S1} & 0 \\ 0 & k_{isc} & -k_{T1} \end{pmatrix}. \tag{1.25}$$

Recalling and observing that the column-wise sums of the transition rates in $\overleftrightarrow{\mathbb{K}}$ are zero, one rate equation can be determined by the others. For the sake of completeness, all rates are outlined here. However, the response to an excitation $k_{ex}(t)$ will be derived from the excited states only. Applying the condition $P_{S0} + P_{S1} + P_{T1} = 1$ yields then the following differential equation system.

$$\begin{cases} \dot{P}_{S1}(t) = k_{ex}(t)\left(1 - P_{T1}(t)\right) - \left(k_{ex}(t) + k_{S1}\right)P_{S1}(t) \\ \dot{P}_{T1}(t) = k_{isc}P_{S1}(t) - k_{T1}P_{T1}(t) \end{cases} \tag{1.26}$$

At this point, we could solve for either excited state population. We decide to solve for the triplet state population first because it keeps a record of the excited singlet state population, which itself tracks the history of the excitation rate. Regardless of possibly abrupt changes in the excitation rate, the triplet state population evolves strictly continuously, and so does its first derivative.

Solving the triplet state differential equation for the excited singlet state yields

$$P_{S1}(t) = \frac{\dot{P}_{T1}(t) + k_{T1}P_{T1}(t)}{k_{isc}}. \tag{1.27}$$

Insertion into the excited singlet state differential equation (1.26) results then in an inhomogeneous second order differential equation.

$$\frac{\partial^2 P_{T1}}{\partial t^2} + \left(k_{ex} + k_{S1} + k_{T1}\right)\frac{\partial P_{T1}}{\partial t} + \left(k_{ex}k_{isc} + \left(k_{ex} + k_{S1}\right)k_{T1}\right)P_{T1} = k_{ex}k_{isc} \tag{1.28}$$

For a piece-wise constant excitation rate k_{ex}, we try an ansatz of the form $P_{T1}(t) = a\exp\left(-bt\right) + c$ and obtain by identification of constant terms

$$c = \frac{k_{ex}k_{isc}}{k_{ex}k_{isc} + \left(k_{ex} + k_{S1}\right)k_{T1}} = \frac{q_{isc}\tau_{T1}}{\tau_{ex} + \tau_{S1} + q_{isc}\tau_{T1}}, \tag{1.29}$$

which is the steady state solution calculated in section 1.3. The $\exp(-bt)$ terms yield the characteristic polynomial of second order for the rate b at which the triplet state population approaches its dynamic equilibrium.

$$b^2 - (k_{\text{ex}} + k_{\text{S1}} + k_{\text{T1}})\, b + k_{\text{ex}} k_{\text{isc}} + (k_{\text{ex}} + k_{\text{S1}})\, k_{\text{T1}} = 0 \tag{1.30}$$

Solving for the two possible rates b leads to

$$b_\pm = \frac{1}{2}\left(k_{\text{ex}} + k_{\text{S1}} + k_{\text{T1}} \pm \sqrt{(k_{\text{ex}} + k_{\text{S1}} - k_{\text{T1}})^2 - 4k_{\text{ex}} k_{\text{isc}}}\right). \tag{1.31}$$

Therefore, the triplet population is given by the general form

$$P_{\text{T1}}(t) = a_+ \exp(-b_+ t) + a_- \exp(-b_- t) + c, \tag{1.32}$$

where the amplitudes $a_\pm$ are obtained with the initial conditions given by the continuity of the triplet state population $P_{\text{T1}}(t)$ and its derivative $\dot{P}_{\text{T1}}(t)$. The results (1.29) and (1.31) show that solving for the triplet state population first has the advantage of requiring neither the derivative nor the integral of the excitation rate $k_{\text{ex}}(t)$. Therefore, all parameters $a_\pm$, $b_\pm$ and c are well defined irrespective of potential discontinuities of the excitation rate such as steps or Dirac pulses.

What happens if the excitation is switched off? Let assume the excitation is switched off at $t = 0$. For obtaining the response at $t > 0$, we look up the equilibration rates (1.31) for $k_{\text{ex}} = 0$ and find $b_+ = k_{\text{S1}}$ and $b_- = k_{\text{T1}}$, respectively. Obviously, these are the decay rates of the excited states without excitation. The steady state triplet population (1.29) is of course zero, that is $c = 0$. Finally, we obtain the amplitudes $a_\pm$ with the initial conditions.

$$\begin{cases} P_{\text{T1}}(0) = a_+ + a_- \\ \dot{P}_{\text{T1}}(0) = -a_+ k_{\text{S1}} - a_- k_{\text{T1}} \end{cases} \tag{1.33}$$

The transient response of the triplet state population is then given by

$$P_{\text{T1}}(t \geq 0) = \frac{\dot{P}_{\text{T1}}(0) + k_{\text{T1}} P_{\text{T1}}(0)}{k_{\text{T1}} - k_{\text{S1}}} \exp(-k_{\text{S1}} t) + \frac{\dot{P}_{\text{T1}}(0) + k_{\text{S1}} P_{\text{T1}}(0)}{k_{\text{S1}} - k_{\text{T1}}} \exp(-k_{\text{T1}} t). \tag{1.34}$$

What happens if the excitation is switched on? Let assume no excitation for $t < 0$ and a constant excitation rate k_{ex} for $t > 0$. The fluorophore will be initially in the ground state, that is $P_{\text{T1}}(t \leq 0) = 0$ and $\dot{P}_{\text{T1}}(t \leq 0) = 0$. For $t > 0$, the steady state solution (1.29) is approached and will be reached for $t \gg 0$. The transient response of the fluorophore is found by solving the initial conditions for the amplitudes $a_\pm$ as before.

$$\begin{cases} P_{\text{T1}}(0) = 0 = a_+ + a_- + c \\ \dot{P}_{\text{T1}}(0) = 0 = -a_+ b_+ - a_- b_- \end{cases} \tag{1.35}$$

The transient response of the triplet state population is then given by

$$P_{\text{T1}}(t \geq 0) = \frac{b_- c}{b_+ - b_-} \exp(-b_+ t) + \frac{b_+ c}{b_- - b_+} \exp(-b_- t) + c. \tag{1.36}$$

Interpretation

For weak excitation $I_{ex} \ll I_{sat}$, the rate constants $b_\pm$ are approximately given by the decay rates k_{S1} and k_{T1} of the excited states as if no excitation were present. If this approximation seems too coarse, the excitation rate k_{ex} has to be taken into account. Nevertheless, the expressions in equation (1.31) can be further simplified by noting that typically $k_{isc} \ll k_{S1} - k_{T1}$. Therefore, the square root can be well approximated by

$$\begin{aligned}\sqrt{(k_{ex}+k_{S1}-k_{T1})^2 - 4k_{ex}k_{isc}} &= (k_{ex}+k_{S1}-k_{T1})\sqrt{1-4k_{ex}k_{isc}\,(k_{ex}+k_{S1}-k_{T1})^{-2}} \\ &\approx k_{ex}+k_{S1}-k_{T1} - \frac{2k_{ex}k_{isc}}{k_{ex}+k_{S1}-k_{T1}}.\end{aligned} \tag{1.37}$$

The rate constants (1.31) are then simplified by the approximate expressions

$$\begin{aligned} b_+ &\approx k_{ex}+k_{S1} - \frac{k_{ex}k_{isc}}{k_{ex}+k_{S1}-k_{T1}} \approx k_{ex}+k_{S1} \quad \text{and} \\ b_- &\approx k_{T1} + \frac{k_{ex}k_{isc}}{k_{ex}+k_{S1}-k_{T1}} \in [k_{T1}, k_{T1}+k_{isc}) \,. \end{aligned} \tag{1.38}$$

We observe that b_+ corresponds to the singlet state equilibration rate k found with the two-level model in section 1.5 and we note that b_- is the triplet state equilibration rate, respectively.

We recall that the singlet state population $P_{S1}(t)$ is readily obtained by applying the relation (1.27).

1.6.1 Exercise

Based on the Jablonski diagram 1.6, calculate the fluorescence emission rate in response to a rectangular excitation pulse providing an excitation rate $k_{ex} = 5 \cdot 10^6/\text{s}$ during a time interval of $t_{ex} = 20\mu\text{s}$. Plot the excited state populations for a fluorescence lifetime $\tau_{S1} = 12\text{ns}$, a triplet state lifetime $\tau_{T1} = 5\mu\text{s}$ and an intersystem crossing yield $q_{isc} = 5\%$.

The **steady state** is the **dynamic equilibrium** achieved for constant excitation when the variations of all molecular states vanish, that is $\partial\vec{P}/\partial t = 0$. For the three-level model, the steady state excited singlet state population is given by

$$P_{S1} = \frac{k_{ex}k_{T1}}{k_{ex}k_{isc} + (k_{ex}+k_{S1})\,k_{T1}} = \frac{\tau_{S1}}{\tau_{ex}+\tau_{S1}+q_{isc}\tau_{T1}}.$$

For the two-level model the intersystem crossing rate k_{isc}, respectively its yield q_{isc}, is set to zero. For large excitation rates $k_{ex} \to \infty$, the **emission saturates** and is limited by the spontaneous relaxation of the excited states.

The transient response of a fluorophore due to a change of the excitation rate $k_{ex}(t)$ is in general exponentially approaching the steady state with characteristic **equilibration rates** of the populations $P_{ST}(t)$ of the states ST. These equilibration rates are approximately given by the absolute sums of the rates of all transitions connected with each state ST.

1.7 Fluorescence quenching

Any extrinsic process that decreases the emission of a fluorophore is a **quenching process**. The characteristic parameters for the fluorescence emission such as quantum yield and lifetime are influenced in the presence of a **quencher** Q. This quencher Q interacts with excited molecules in various ways to inhibit fluorescence. These fluorophore–quencher interactions compete with the intrinsic de-excitation of the fluorophore. In the Jablonski diagram 1.1, the quenching rate can be outlined as an additional non-radiative transition rate $S_1, T_1 \rightarrow S_0$ or simply added to the existing non-radiative transition rates k_{nrs} and/or k_{nrt}. Therefore, the general forms of the rate equations and their solution do not change except that the fluorescence and/or phosphorescence quantum yields and lifetimes decrease with increasing quenching rate.

There are a manifold of molecular energy transfer processes causing quenching, see for example B. Valeur [6]. Next, two important quenching processes and their influence on the fluorophore's response are exemplified.

1.7.1 Dynamic quenching

If the energy is transferred to the quencher molecule upon collision of the fluorophore with the quencher molecule, this fluorophore–quencher interaction is called **collisional quenching** or **dynamic quenching**. The collisional quenching process increases the rate of non-radiative transitions by the quenching rate $k_{dq}[Q]$, which is proportional to the concentration [Q] of the non-excited quencher.[8] Therefore, the fluorescence quantum yield decreases with the quencher concentration.

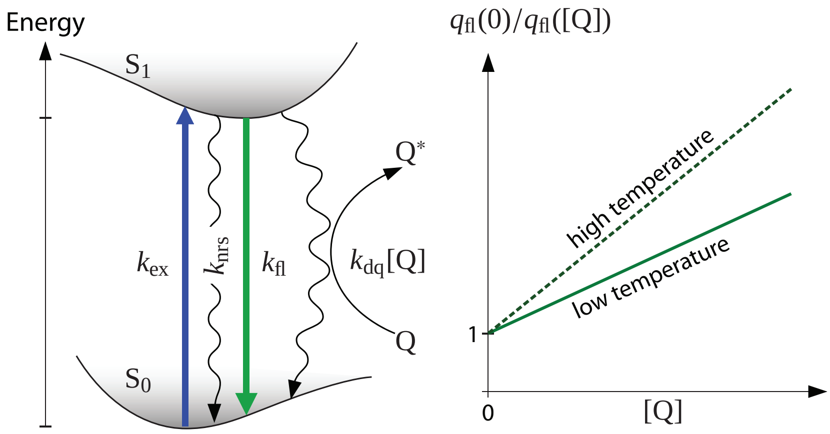

Figure 1.7: Collisional quenching process (left) and Stern-Vollmer plot (right). The Stern-Vollmer plot shows the impact of collisional quenching at different temperatures.

$$q_{fl}([Q]) = \frac{k_{fl}}{k_{fl} + k_{nrs} + k_{isc} + k_{dq}[Q]} = q_{fl}(0)\frac{k_{S1}}{k_{S1} + k_{dq}[Q]} \tag{1.39}$$

The **Stern-Vollmer equation** expresses the quenching efficiency by the ratio of the fluorescence quantum yield $q_{fl}(0)$ without quencher versus the quantum yield $q_{fl}([Q])$ in the presence of the quencher.

$$\frac{q_{fl}(0)}{q_{fl}([Q])} = 1 + \frac{k_{dq}[Q]}{k_{S1}} = 1 + \tau_{S1} k_{dq}[Q] \tag{1.40}$$

[8] Quencher molecules are mostly inactive if they are still excited due to a prior quenching interaction.

This ratio is represented as a function of the quencher concentration in the **Stern-Vollmer plot** 1.7. In most solvents, the collision rate and therefore the quenching rate k_{dq} increases with the temperature because the diffusion coefficient increases and because the solvent viscosity decreases. This dependence shows up in the Stern-Vollmer plot as an increased slope of the quenching efficiency with temperature.

In general, dynamic quenching already occurs with the solvent molecules acting as quenchers, which can become particularly significant at high temperature. On the other hand, at cryogenic temperature, many molecules become fluorescent as their non-radiative decay is sufficiently slowed down.

Molecular oxygen Oxygen O_2 is well known as a quencher for many fluorophores. The oxygen ground state is a triplet state. The collisional quenching of singlet states but in particular of triplet states is possible through an energy transfer process. The triplet state quenching is fairly efficient because of its long lifetime and the triplet ground state of oxygen, which allows to transfer the change in spin multiplicity as well. The oxygen does the transit $T_0 \rightarrow S_1$ whilst the fluorophore returns to the ground state $T_1 \rightarrow S_0$. The collisional quenching rate is proportional to the mean square distance $\left\langle \Delta r^2 \right\rangle = 6\,(D_F + D_Q)\,\Delta t$ the fluorophore and the quencher diffuse during Δt, that is the lifetime τ_{S1} or τ_{T1} of the excited state S_1 or T_1, respectively.

An oxygen molecule in water at a temperature of 25°C has a diffusion constant $D_Q = 2.4 \cdot 10^{-5}\mathrm{cm}^2/\mathrm{s}$. During the singlet lifetime of nanoseconds, the oxygen molecule diffuses over a distance of nanometers only. The probability to collide with a fluorophore in the triplet state is much higher because the triplet state lifetime is in the range of micro- to milliseconds.

1.7.2 Static quenching

The reversible formation of a non-fluorescent fluorophore–quencher complex FQ is called **static quenching**. The complex FQ often keeps absorbing photons but returns to the ground state S_0 without emission. The fluorescence of the free fluorophore F is not affected. If the lifetime of the complex FQ becomes shorter, the static quenching becomes gradually time-dependent like the dynamic quenching.

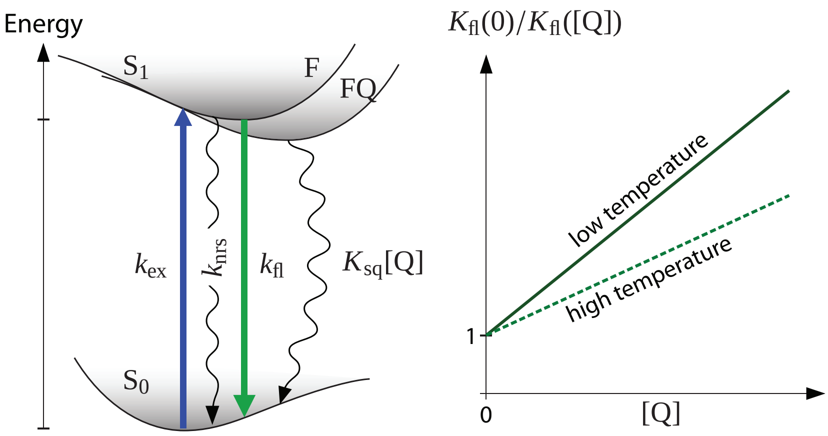

Figure 1.8: Static quenching and the complex formation process (left) and Stern-Vollmer plot (right). The Stern-Vollmer plot shows the impact of static quenching at different temperatures.

In equilibrium, the quencher–fluorophore complex formation is described as $F + Q \leftrightharpoons FQ$. The fluorescence intensity is related to the quencher concentration [Q] by the association constant K_{sq} for the complex formation, which is given as

$$K_{\mathrm{sq}} = \frac{[\mathrm{FQ}]}{[\mathrm{F}][\mathrm{Q}]}, \tag{1.41}$$

where [F] is the concentration of free fluorophores and [FQ] the concentration of complexes, respectively. Because of the mass conservation, the total fluorophore concentration $[\mathrm{F}]_0 = [\mathrm{F}] + [\mathrm{FQ}]$ is constant. The fraction of free fluorophores becomes then

$$\frac{[\mathrm{F}]}{[\mathrm{F}]_0} = \frac{1}{1 + K_{\mathrm{sq}}[\mathrm{Q}]}. \tag{1.42}$$

As the fluorescence is proportional to the concentration [F], the Stern-Vollmer relation leads to

$$\frac{K_{\mathrm{fl}}(0)}{K_{\mathrm{fl}}([\mathrm{Q}])} = 1 + K_{\mathrm{sq}}[\mathrm{Q}]. \tag{1.43}$$

There are remarkable similarities between the Stern-Vollmer equation (1.40) for collisional quenching and this result (1.43) for complex formation, respectively. An increase in temperature leads to a slightly faster association rate, because the complex formation is favored by fast diffusion, hence frequent collisions. However, the dissociation rate becomes significantly faster, because the dissociation energy is much more frequently achieved by thermal excitation. Overall, the association constant K_{sq} usually shows a pronounced negative temperature dependency. The Stern-Vollmer plot 1.8 shows therefore a decreasing slope of the static quenching efficiency with increasing temperature.

Quenching is an extrinsic inhibition of the fluorophore's cabability to fluoresce. A **quencher** is a molecule or compound that can deplete the fluorophore's excited states by non-radiative energy transfers.

Dynamic quenching is caused by fluorophore–quencher collisions, at which occasions the fluorophore's energy is taken up by the quencher molecule(s). Dynamic quenching becomes more efficient with increasing quencher concentration and with increasing diffusion speed of the quencher molecules and the fluorophores. In general, the diffusion speed increases with temperature and so does the dynamic quenching efficiency.

Static quenching is caused by the reversible formation of non-fluorescent fluorophore–quencher complexes. Static quenching becomes more efficient with increasing quencher concentration and with increasing association constant of the complex formation. In general, the association constant decreases with temperature and so does the static quenching efficiency.

A **Stern-Vollmer plot** outlines the quenching efficiency versus the quencher concentration. The **quenching efficiency** is the ratio of the non-quenched versus the quenched fluorescence emission rate or quantum yield.

A notable difference is the influence on the measured lifetime and quantum yield of the fluorophores. The collisional quenching reduces both characteristics of the fluorophore, because this dynamic quenching affects all fluorophores. The complex formation heavily affects the complexed fraction of fluorophores but not the free. If the complex FQ is completely non-fluorescent, the measurable lifetime of the free fluorophores is not affected at all.

1.8 Photobleaching and -ionization

An irreversible, permanent loss of the fluorophore's capability to absorb and emit at its characteristic wavelengths is called **photobleaching** or fading. Photobleaching is due to photoinduced chemical damage and/or covalent modification,[9] which either inhibits fluorescence completely or shifts the absorption and/or emission spectra to non-addressed wavelengths. Recently, it turned out that many fluorophores that were believed to bleach are instead undergoing reversible but long-lived **photoionization**, which renders them temporarily non-fluorescent [7].

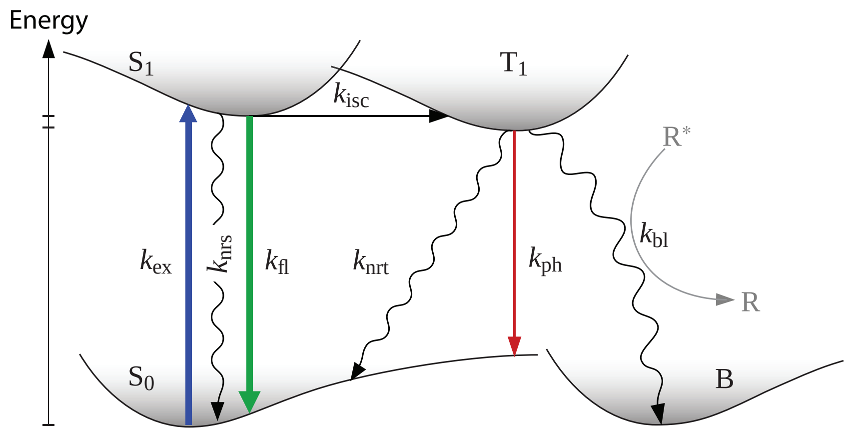

Figure 1.9: Photobleaching from the excited triplet state T_1 to an irreversible bleached state B. The bleaching rate k_{bl} may be increased by the presence of free radicals R^*.

A fluorophore in an excited state may interact with another molecule to produce irreversible chemical modifications. As the triplet state is relatively long-lived with respect to the singlet state, fluorophores in the excited triplet state have a significant probability of undergoing chemical reactions with components in their neighborhood. The Jablonski diagram 1.9 exemplifies photobleaching from the excited triplet state T_1 with or without additional activation energy by a free radical R^*. The average number of excitation and emission cycles before photobleaching depends on the molecular structure of the fluorophore and its local environment. Some fluorophores bleach quickly after emitting only a few photons, whereas others are more robust and undergo many thousands or even millions of excitation cycles before bleaching.

Photobleaching degrades the fluorescence emission in many specimens. Therefore, controlling this adverse artifact is critical for achieving high quality fluorescence microscopy. As an example, the quantum yield for photobleaching of fluorescein at medium to high illumination intensity dictates that a molecule will only emit $3 \cdot 10^4$ to $4 \cdot 10^4$ photons in average before bleaching. Provided that no excitation to higher excited states S_n or T_n occur, the number of excitation and emission cycles is nearly constant for a given fluorophore and almost independent of the temporal shape of the excitation light. On the other hand, highly energetic states S_n or T_n have typically bleaching rates that are orders of magnitude larger than for the first excited states S_1 and T_1, because they can directly provide the energy required for breaking up bonds of the fluorophore [8]. Photobleaching accross these states becomes particularly important with picosecond or femtosecond pulsed excitation.

An important bleaching agent is molecular oxygen. As outlined in subsection 1.7.1, oxygen plays an important role as triplet quencher. In principle, quenching the triplet state is an advantageous reaction

[9] Mainly dissociation of the molecule (breaking covalent bonds), isomerization of the molecule (reforming covalent bonds) or reaction with another molecule.

because it reduces the chance that the fluorophore bleaches by removing the excitation energy more quickly. However, the oxygen molecule becomes activated during this process and may turn into free radical singlet oxygen. If such a radical encounters a fluorophore it may oxidize and thereby bleach it, in particular if the fluorophore is yet in an excited state. Hence, the presence of oxygen in the fluorophore's environment has a two-fold effect. It increases the brightness of the fluorophore by quenching its triplet population at the price of simultaneously increasing the bleaching rate of the fluorophore.

The amount of photobleaching due to this **photodynamic reaction** is depending on the molecular oxygen concentration and the diffusion between the fluorophores, the oxygen molecules and other components. Photobleaching can be reduced efficiently by lowering the excitation energy, which also reduces the measurable fluorescence signal. Fortunately, suitable combinations of reducing and oxidizing agents help avoiding photobleaching by preventing the formation of oxygen radicals whilst keeping the triplet state quenched [7]. Such anti-bleaching agents are commercially available. However, they introduce additional substances, which asks for a case-per-case investigation of their compatibility with the biological sample.

1.9 Comparing fluorophores

A simple parameter for comparing different fluorescent molecules is the product of the extinction coefficient $\epsilon(\lambda)$ and the fluorescence quantum yield q_{fl}. This term is directly proportional to the brightness of the dye, accounting for both the amount of light absorbed and the quantum efficiency of the fluorophore. Meaningful comparisons between fluorophores should include both parameters and keep an eye on the photostability as well. Figure 1.10 plots the fluorophore brightness $\epsilon_{\text{max}}q_{\text{fl}}$ versus the wavelength λ_{ex} of peak absorption for major classes of biologically significant fluorophores [9].

Further reading

[1] Max Planck, "Über das Gesetz der Energieverteilung im Normalspectrum," Ann. Phys. **309,** 553–563 (1901).

[2] Albert Einstein, "Über einen die Erzeugung und Verwandlung des Lichtes betreffenden heuristischen Gesichtspunkt," Ann. Phys. **322,** 132–148 (1905).

[3] Joseph R. Lakowicz, *Principles of Fluorescence Spectroscopy,* Plenum Press (1999). ISBN: 0-306-41285-3.

[4] I. Majoul, M. Straub, S. W. Hell, R. Duden and H.D. Söling, "KDEL-Cargo Regulates Interactions between Proteins Involved in COPI Vesicle Traffic: Measurements in Living Cells Using FRET," Develop. Cell **1,** 139–153 (2001). DOI: 10.1016/S1534-5807(01)00004-1

[5] P. J. Rothwell, S. Berger, O. Kensch, S. Felekyan, M. Antonik, B. M. Wöhrl, T. Restle, R. S. Goody and C. A. M. Seidel, "Multiparameter single-molecule fluorescence spectroscopy reveals heterogeneity of HIV-1 reverse transcriptase: primer/template complexes," Proc. Nat. Acad. Sci. USA **100,** 1655–1660 (2003). DOI: 10.1073/pnas.0434003100

[6] Bernard Valeur, *Molecular Fluorescence: Principles and Applications,* Wiley-VCH Verlag GmbH (2001). ISBN: 3-527-29919-X (hardcover); 3-527-60024-8 (electronic).

[7] J. Vogelsang, T. Cordes, C. Forthmann, C. Steinhauer and P. Tinnefeld, "Controlling the fluorescence of ordinary oxazine dyes for single-molecule switching and superresolution microscopy," Proc. Nat. Acad. Sci. USA **106,** 8108–8112 (2009). DOI: 10.1073/pnas.0811875106

[8] C. Eggeling, J. Widengren, L. Brand, J. Schaffer, S. Felekyan and C. A. M. Seidel, "Analysis of Photobleaching in Single-Molecule Multicolor Excitation and Förster Resonance Energy Transfer Measurements," J. Phys. Chem. A **110,** 2979–2995 (2006). DOI: 10.1021/jp054581w

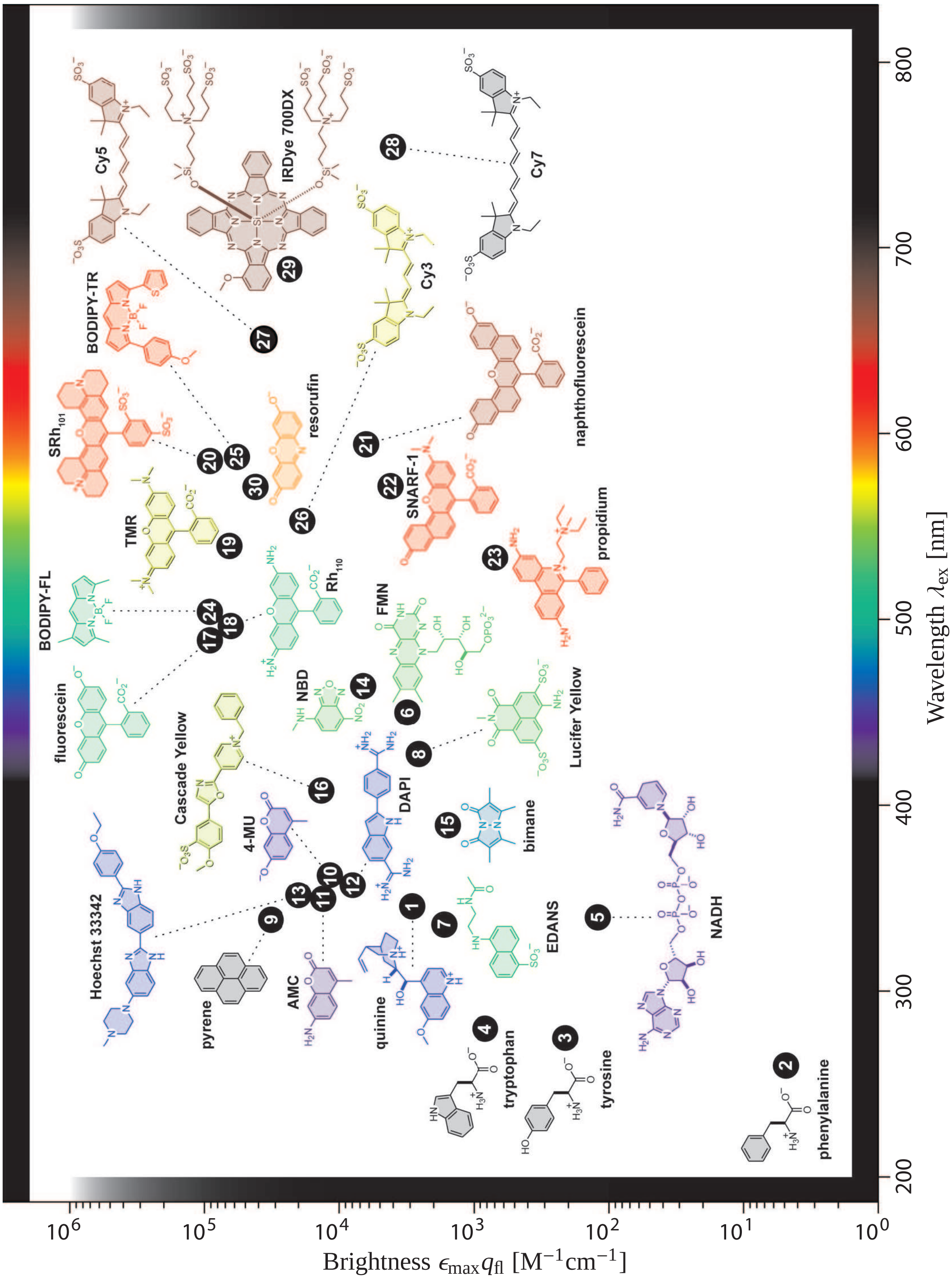

Figure 1.10: Fluorophore brightness $\epsilon_{max}q_{fl}$ versus wavelength of maximum absorption λ_{ex} for major fluorophore classes. The colors of the structures indicate their wavelengths of maximum emission. For some molecules, only the chromophores are shown. Reprinted with courtesy by Lavis and Raines [9].

[9] L. D. Lavis and R. T. Raines, ”Bright Ideas for Chemical Biology,” ACS Chem. Biol. **3,** 142–155 (2008). DOI: 10.1021/cb700248m