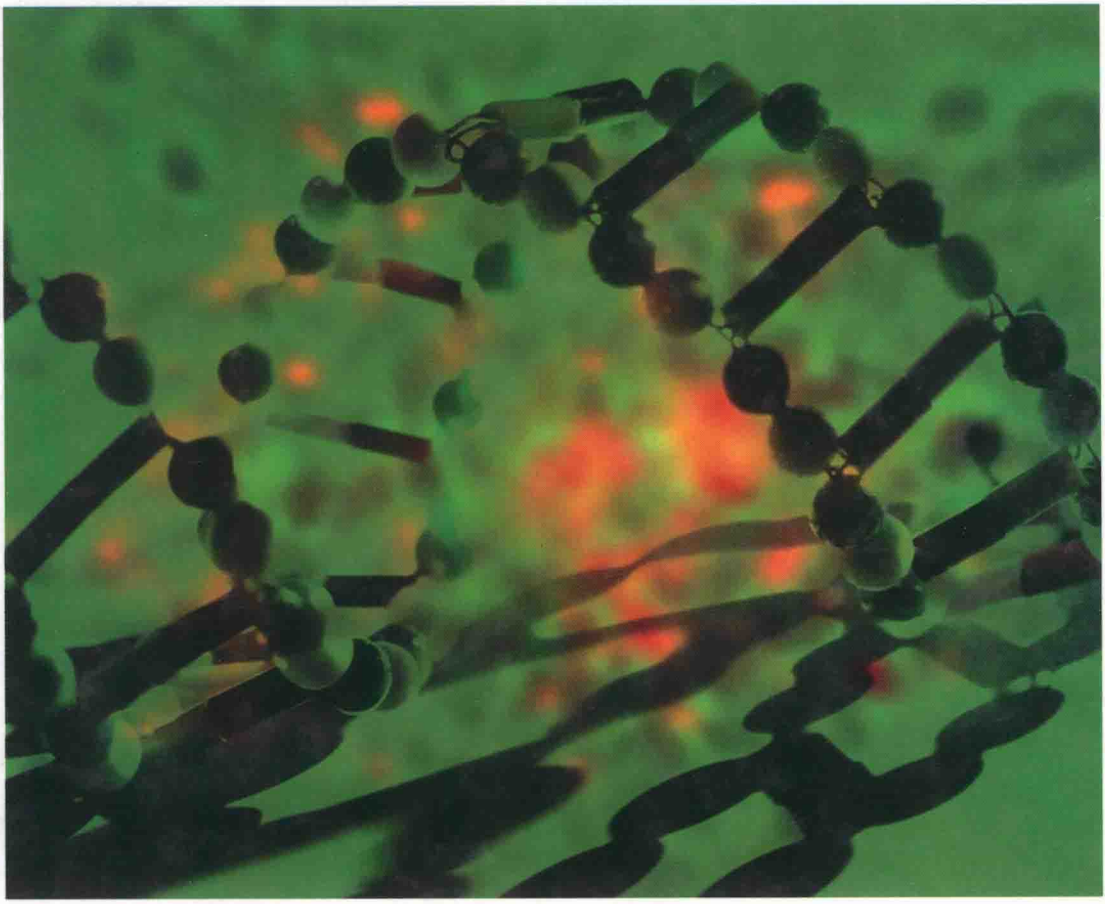
Markus Sauer, Johan Hofkens,
Jörg Enderlein

WILEY-VCH

Handbook of Fluorescence Spectroscopy and Imaging

From Single Molecules to Ensembles



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From Single Molecules to Ensembles

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Preface

Fluorescence spectroscopy and imaging have become powerful and widely used methods in almost any laboratory around the globe for the non-invasive study of polymers, inorganic materials, cells, and tissues. With the development of elaborate single-molecule fluorescence techniques, energy and electron transfer methods have experienced a renaissance and today they are used successfully to study protein folding, molecular interactions, and the motion and interaction of individual molecules, even in living cells with high temporal resolution. New methods that have been introduced to increase the optical resolution of microscopy beyond the diffraction barrier enable researchers to study cellular structures in fixed and living cells with unprecedented resolution, which seemed impossible to achieve only a few years ago. With the ongoing success of fluorescence spectroscopy and microscopy, fundamental knowledge about the chemical and photophysical properties of fluorophores lie at the heart of single-molecule sensitive experiments.

This book is intended to give interested readers the fundamental knowledge necessary for planning and designing successful fluorescence spectroscopy and imaging experiments with high spatiotemporal resolution. After a basic introduction to fluorescence spectroscopy, different fluorophores and their properties, we describe the details of specific fluorescence labelling of target molecules. A chapter that explains our current understanding of fluorophore photophysics and photobleaching pathways in single-molecule fluorescence experiments is followed by chapters giving introductions to fluorescence correlation spectroscopy and also to energy and electron transfer. Finally, the book introduces various super-resolution imaging methods and gives examples of how single-molecule spectroscopy can be used for the successful study of enzyme kinetics, conformational dynamics of biopolymers, protein folding, and for diagnostic applications.

The book is mainly aimed at graduate students and researchers who want to begin using the advanced fluorescence techniques of single-molecule fluorescence spectroscopy and imaging. However, we hope that even experts will find it a useful resource to consult for the planning and discussion of their experiments.

Würzburg, Leuven, Göttingen, November 2010

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Basic Principles of Fluorescence Spectroscopy

1.1
Absorption and Emission of Light

As fluorophores play the central role in fluorescence spectroscopy and imaging we will start with an investigation of their manifold interactions with light. A fluorophore is a component that causes a molecule to absorb energy of a specific wavelength and then re-remit energy at a different but equally specific wavelength. The amount and wavelength of the emitted energy depend on both the fluorophore and the chemical environment of the fluorophore. Fluorophores are also denoted as chromophores, historically speaking the part or moiety of a molecule responsible for its color. In addition, the denotation chromophore implies that the molecule absorbs light while fluorophore means that the molecule, likewise,emits light. The umbrella term used in light emission is luminescence, whereas fluorescence denotes allowed transitions with a lifetime in the nanosecond range from higher to lower excited singlet states of molecules.

In the following we will try to understand why some compounds are colored and others are not. Therefore, we will take a closer look at the relationship of conjugation to color with fluorescence emission, and investigate the absorption of light at different wavelengths in and near the visible part of the spectrum of various compounds. For example, organic compounds (i.e., hydrocarbons and derivatives) without double or triple bonds absorb light at wavelengths below 160 nm, corresponding to a photon energy of $>180\,\mathrm{kcal\,mol^{-1}}$ ($1\,\mathrm{cal} = 4.184\,\mathrm{J}$), or $>7.8\,\mathrm{eV}$ (Figure 1.1), that is, significantly higher than the dissociation energy of common carbon-to-carbon single bonds.

Below a wavelength of 200 nm the energy of a single photon is sufficient to ionize molecules. Therefore, photochemical decomposition is most likely to occur when unsaturated compounds, where all bonds are formed by σ-electrons, are irradiated with photon energies $>6.2\,\mathrm{eV}$. Double and triple bonds also use π-electrons in addition to a σ-bond for bonding. In contrast to σ-electrons, which are characterized by the rotational symmetry of their wavefunction with respect to the bond direction, π-electrons are characterized by a wavefunction having a node at the nucleus and rotational symmetry along a line through the nucleus. π-bonds

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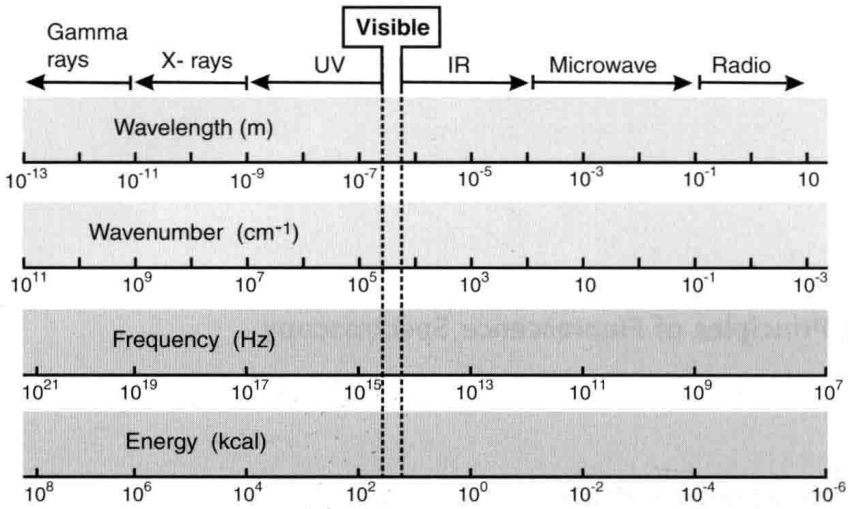

Figure 1.1 The electromagnetic spectrum.

are usually weaker than σ-bonds because their (negatively charged) electron density is further from the positive charge of the nucleus, which requires more energy. From the perspective of quantum mechanics, this bond weakness is explained by significantly less overlap between the component π-orbitals due to their parallel orientation. These less strongly bound electrons can be excited by photons with lower energy. If two double bonds are separated by a single bond, the double bonds are termed conjugated. Conjugation of double bonds further induces a red-shift in the absorption (a so-called bathochromic shift). All fluorophores that have a high absorption in the visible part of the spectrum possess several conjugated double bonds.

Above 200 nm only the two lowest energy transitions, that is, $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$, are achieved as a result of the energy available from the photons. When sample molecules are exposed to light having an energy that matches a possible electronic transition within the molecule, some of the light energy will be absorbed as the electron is promoted to a higher energy orbital. As a simple rule, energetically favored electron promotion will be from the highest occupied molecular orbital (HOMO), usually the singlet ground state, S_0, to the lowest unoccupied molecular orbital (LUMO), and the resulting species is called the singlet excited state S_1. Absorption bands in the visible region of the spectrum correspond to transitions from the ground state of a molecule to an excited state that is 40–80 kcal mol^{-1} above the ground state. As mentioned previously, in saturated hydrocarbons in particular, the lowest electronic states are more than 80 kcal mol^{-1} above the ground state, and therefore they do not absorb light in the visible region of spectrum. Such substances are not colored. Compounds that absorb in the visible region of the spectrum (these compounds have color) generally have some weakly bound or delocalized electrons. In these systems, the energy difference between the lowest LUMO and the HOMO corresponds to the energies of quanta in the visible region.

On the other side of the electromagnetic spectrum, there is a natural limit to long-wavelength absorption and emission of fluorophores, which is in the region of 1 μm [1]. A dye absorbing in the near-infrared (>700 nm) has a low-lying excited singlet state and even slightly lower than that, a metastable triplet state, that is, a state with two unpaired electrons that exhibits biradical character. Even though no generally valid rule can be formulated predicting the thermal and photochemical stability of fluorophores, the occupation of low-lying excited singlet and triplet states potentially increases the reactivity of fluorophores. Therefore, it is likely that fluorophores with long-wavelength absorption and emission will show less thermal and photochemical stability, due to reactions with solvent molecules such as dissolved oxygen, impurities, and other fluorophores. In addition, with increasing absorption, that is, with a decreasing energy difference between S_1 and S_0, the fluorescence intensity of fluorophores decreases owing to increased internal conversion. That is, with a decreasing energy difference between the excited and ground state, the number of options to get rid of the excited-state energy by radiationless deactivation increases. Hence, most known stable and bright fluorophores absorb and emit in the wavelength range between 300 and 700 nm.

Fluorophores with conjugated doubled bonds (polymethine dyes) are essentially planar, with all atoms of the conjugated chain lying in a common plane linked by σ-bonds. π-electrons, on the other hand, have a node in the plane of the molecule and form a charge cloud above and below this plane along the conjugated chain (Figure 1.2). The visible bands for polymethine dyes arise from electronic transitions involving the π-electrons along the polymethine chain. The wavelength of these bands depends on the spacing of the electronic levels. The absorption of light by fluorophores such as polymethine dyes can be understood semiquantitatively by applying the free-electron model proposed by Kuhn [2, 3]. The arrangement of alternating single–double bonds in an organic molecule usually implies that the π-electrons are delocalized over the framework of the "conjugated" system. As these π-electrons are mobile throughout the carbon atom skeleton containing the alternating double bonds, a very simple theoretical model can be applied to such a system in order to account for the energy of these electrons in the molecule. If one makes the

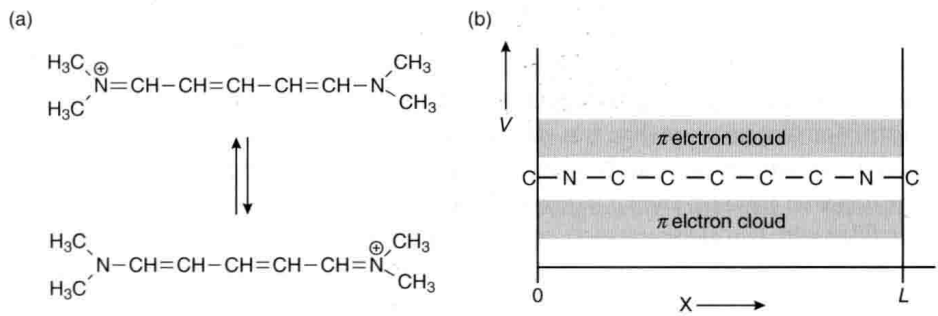

Figure 1.2 (a) Limiting structures of a resonance hybrid of a simple positively charged cyanine dye. (b) The π-electron cloud of the cyanine dye as seen from the side in a simplified potential energy (V) trough of length L.

seemingly drastic assumption that the several π-electrons that comprise the system are non-interacting (presumably, if the π-electrons are delocalized over the $-C=C-C=C-C=C-$ framework, they spread out, minimizing repulsion between them), then one can view the energetics of this system as arising from the simple quantum mechanical assembly of one-electron energy levels appropriate to the *particle in the box model*. In this case, one considers the potential energy of the electron as being constant throughout the length of the molecular box and then rising to infinity at each end of the conjugated portion of the molecule. As an example, consider a positively charged simple cyanine dye. The cation can "resonate" between the two limiting structures shown in Figure 1.2a, that is, the wavefunction for the ion has equal contributions from both states. Thus, all the bonds along this chain can be considered equivalent, with a bond order of 1.5, similar to the C–C bonds in benzene.

Assuming that the conjugated chain extends approximately one bond length to the left and right beyond the terminal nitrogen atoms, application of the Schroedinger equation to this problem results in the well known expressions for the wavefunctions and energies, namely:

$$\psi_n = \sqrt{\frac{2}{L}} \sin\left(\frac{n\pi x}{L}\right)$$

and

$$E_n = \frac{n^2 h^2}{8 mL^2}$$

where

n is the quantum number ($n = 1, 2, 3, \ldots$) giving the number of antinodes of the eigenfunction along the chain
L is the "length" of the (one dimensional) molecular box
m is the mass of the particle (electron)
h is Planck's constant
x is the spatial variable, which is the displacement along the molecular backbone.

Each wavefunction can be referred to as a molecular orbital, and its respective energy is the orbital energy. If the spin properties of the electron are taken into account along with the *ad hoc* invocation of Pauli's exclusion principle, the model is then refined to include spin quantum numbers for the electron (½) along with the restriction that no more than two electrons can occupy a given wavefunction or level, and the spin quantum numbers of the two electrons occupying a given energy level are opposite (spin up and spin down). Thus, if we have N electrons, the lower states are filled with two electrons each, while all higher states are empty provided that N is an even number (which is usually the case in stable molecules as only highly reactive radicals posses an unpaired electron). This allows the electronic structure for the π-electrons in a conjugated dye molecule to be constructed. For example, for the conjugated molecule $CH_2=CH-CH=CH-CH=CH_2$ 6 π-electrons have to be considered. The lowest energy configuration, termed the electronic ground state,

corresponds to the six electrons being in the lowest three orbitals. Higher energy configurations are constructed by promoting an electron from the HOMO with quantum number $n = 3$ to the LUMO with $n = 4$. This higher energy arrangement is called the electronically excited singlet state. The longest wavelength absorption band corresponds to the energy difference between these two states, which is then given by the following expression:

$$\Delta E = E_{\mathrm{LUMO}} - E_{\mathrm{HOMO}} = \frac{h^2}{8\,mL^2}\left(n^2_{\mathrm{LUMO}} - n^2_{\mathrm{HOMO}}\right)$$

The energy required for this electronic transition can be supplied by a photon of the appropriate frequency, given by the Planck relationship:

$$E = h\,\nu = hc/\lambda$$

where

h is Planck's constant
ν is the frequency
c is the speed of light
λ is the wavelength.

Because the ground state of a molecule with N π-electrons will have $N/2$ lowest levels filled and all higher levels empty, we can write $n_{\mathrm{LUMO}} = N/2 + 1$ and $n_{\mathrm{HOMO}} = N/2$:

$$\Delta E = \frac{h^2}{8\,mL^2}(N+1) \quad \text{or} \quad \lambda = \frac{8\,mc}{h}\frac{L^2}{N+1}$$

This indicates that to a first approximation the position of the absorption band is determined only by the chain length and the number of delocalized π-electrons. Good examples for this relationship are symmetrical cyanine dyes of the general formula shown in Figure 1.2a.

1.2 Spectroscopic Transition Strengths

The probability of a molecule changing its state by absorption or emission of a photon depends on the nature of the wavefunctions of the initial and final states, how strongly light can interact with them, and on the intensity of any incident light. The probability of a transition occurring is commonly described by the transition strength. To a first approximation, transition strengths are governed by selection rules that determine whether a transition is allowed or disallowed. In the classical theory of light absorption, matter consists of an array of charges that can be set into motion by the oscillating electromagnetic field of the light. Here, the electric dipole oscillators set in motion by the light field have specific natural characteristics, that is, frequencies, ν_i, that depend on the material. When the frequency of the radiation is near the oscillator frequency, absorption occurs, and the intensity of the radiation

decreases on passing through the substance. The intensity of the interaction is known as the oscillator strength, f_i, and it can be thought of as characterizing the number of electrons per molecule that oscillate with the characteristic frequency, ν_i. Therefore, practical measurements of the transition strength are usually described in terms of f_i. The oscillator strength of a transition is a dimensionless number that is useful for comparing different transitions. For example, a transition that is fully allowed quantum mechanically is said to have an oscillator strength of 1.0. Experimentally, the oscillator strength, f, is related to the intensity of absorption, that is, to the area under an absorption band plotted versus the frequency:

$$f = \frac{2303\, mc}{\pi N_A e^2 n} \int \varepsilon(\nu) d\nu$$

where

ε is the molar absorptivity
c is the velocity of light
m is the mass of an electron
e is the electron charge
n is the refractive index of the medium,
N_A is Avogadro's number.

The integration is carried out over the frequency range associated with the absorption band.

The quantum mechanical description, which is the most satisfactory and complete description of the absorption of radiation by matter, is based on time-dependent wave mechanics. Here, a transition from one state to another occurs when the radiation field connects the two states. In wave mechanics, the connection is described by the transition dipole moment, μ_{GE}:

$$\mu_{GE} = \int \psi_G \mu \psi_E d\upsilon$$

where

ψ_G and ψ_E are the wavefunctions for the ground and excited state, respectively
$d\upsilon$ represents the volume element.

The transition dipole moment will be nonzero whenever the symmetry of the ground and excited states differ. For example, ethylene (CH_2=CH_2) has no permanent dipole moment, but if ψ_G is a π-molecular orbital and ψ_E is a π^*-molecular orbital, then $\mu_{\pi\pi^*}$ is not zero. The direction of the transition moment is characterized by the vector components: $\langle \mu_x \rangle_{GE}$, $\langle \mu_y \rangle_{GE}$, and $\langle \mu_z \rangle_{GE}$. It has to be pointed out that for most transitions the three vectors are not all equal, that is, the electronic transition is polarized. The ethylene $\pi \rightarrow \pi^*$ transition, for example, is polarized along the C=C double bond. The magnitude of the transition is characterized by its absolute-value squared, which is called the dipole strength, D_{GE}:

$$D_{GE} = |\mu_{GE}|^2$$

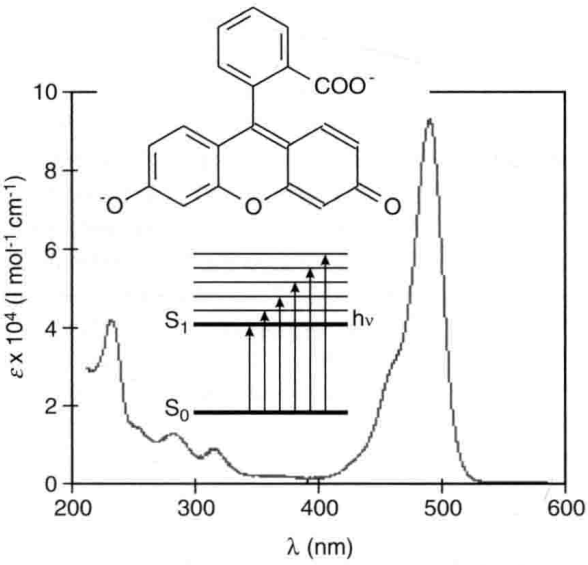

Figure 1.3 Molecular structure and absorption spectrum of the dianion fluorescein in ethanol, pH 9.0. The inset also shows the electronic ground and first excited singlet state level and possible absorptive transitions involving different vibronic states.

A peculiarity of the absorption spectra of organic dyes as opposed to atomic spectra is the width of the absorption band, which usually covers several tens of nanometers. This is easy to understand recalling that a typical dye molecule is composed of several tens of atoms, giving rise to manifold vibrations of the skeleton. These vibrations together with their overtones densely cover the spectrum between a few wave numbers and 3000 cm^{-1}. Furthermore, most of these vibrations are coupled to the electronic transitions through the change in electron densities over the bonds constituting the conjugated chain. That is, after electronic excitation the electron density changes, which is associated with a change in bond length. Quantum mechanically this means that transitions have occurred from the electronic and vibrational ground state S_0 of the molecule to an electronically and vibrationally excited state S_1. This results in broad absorption spectra like that shown for the fluorescein dianion in Figure 1.3 and depends on how many of the vibrational sublevels spaced at $h\nu$ $(n + ½)$, with $n = 0, 1, 2, 3, \ldots$, are reached and what the transitions moments of these sublevels are.

1.3
Lambert–Beer Law and Absorption Spectroscopy

Lambert–Beer Law is a mathematical means of expressing how light is absorbed by matter (liquid solution, solid, or gas). The law states that the amount of light emerging from a sample is diminished by three physical phenomena: (i) the amount of absorbing material (concentration c), (ii) the optical path length l, that is, the

distance the light must travel through the sample, and (iii) the probability that the photon of that particular energy will be absorbed by the sample (the extinction coefficient ε of the substance). Considering a sample of an absorbing substance placed between two parallel windows that transmit the light and supposing that the light of intensity I_0 is incident from the left, propagates along the x direction, then the intensity I decreases smoothly from left to right and exits with an intensity I_t. If the sample is homogeneous, the fractional decrease in light intensity is the same across a small interval dx, regardless of the value of x. As the fractional decrease for a solution depends linearly on the concentration of the absorbing molecule, the fractional change in light intensity dI/I can be written as:

$$-\frac{dI}{I} = \alpha c\, dx$$

where

α is a constant of proportionality.

Because neither α nor c depends on x, integration between limits I_0 at $x = 0$ and I_t at $x = l$, provides

$$\ln\frac{I_0}{I_t} = \alpha\, cl \quad \text{or} \quad I_t = I_0\, e^{-\alpha cl}$$

For measurements made with cuvettes of different path lengths, the transmitted intensity, I_t, decreases exponentially with increasing path length. Alternatively, the transmitted intensity decreases exponentially with increasing concentration of an absorbing solute. The absorbance or optical density, A, is defined as base 10 rather than natural logarithms,

$$A = \log\frac{I_0}{I_t} = \varepsilon\, c\, d$$

where

$\varepsilon = \alpha/2.303$ is the molar extinction coefficient (or molar absorptivity) with units M^{-1} cm^{-1}, when the concentration, c, and the path length, d, are given in molarity, M, and cm, respectively.

The Lambert–Beer Law shows that the absorbance is proportional to the concentration of the solute and path length with ε as the proportionality constant. The relationship between absorbance and transmission, $T = I_t/I_0$ is given by

$$A = -\log T$$

Because the absorption intensity depends strongly on wavelength, the wavelength at which the measurement was performed always has to be specified. The wavelength dependence of ε or of A is known as the absorption spectrum of the compound (Figure 1.3).

When measuring absorption spectra, several error sources have to be considered. Firstly, it should be known that a small but significant portion of light is lost by