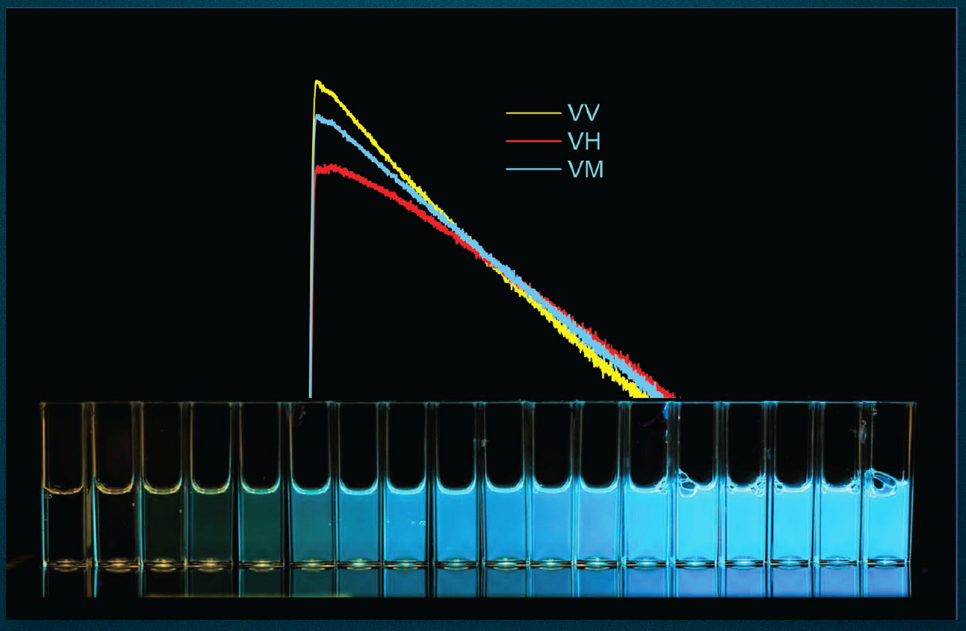
PRACTICAL FLUORESCENCE SPECTROSCOPY



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Practical Fluorescence Spectroscopy

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Preface

Fluorescence spectroscopy has become an indispensable tool in many scientific disciplines from biology and ecology to chemistry and physics. This book is meant to be a practical reference and guide for those who plan to use fluorescence in their respective field, or to those who are new to using fluorescence. The main focus is to describe how to perform experiments, from initial design and set up to data analysis and presentation. Advantages and disadvantages of various techniques are presented with an emphasis on the design of experiment (DOE) to suit the reader's specific needs. This book is designed to include sufficient theory and background to bridge the gap between introductory classroom textbooks and current journal articles as related to particular experiments. It is intended for students starting lab work and professionals from different fields venturing into the fluorescence spectroscopy. This is not a theoretical textbook with problem sets, but rather a tutorial for practical applications. Starting from basic experiments on transmission and absorption of light, simple design and interpretation of steady-state fluorescence experiments, to advanced experiments involving oriented systems, time-resolved fluorescence, fluorescence quenching, and Förster Resonance Energy Transfer (FRET).

Design and discussions of specific experiments are meant to teach less known nuances of experimental strategy, data fitting, and analyses, as well as potential problems and pitfalls. It is important to remember that a single fluorescence technique/experiment usually is applicable across many fields and sample types. Data from an absorption measurement can be used to determine how many fluorophores have labeled a protein (i.e., after using a commercial labeling kit) or the percent of incorporation of magnesium into a ZnMgO thin film on sapphire (i.e., after semiconductor growth). Thus, it is important to consider that while the exact experiment one wishes to perform may not be described within this text, an identical or at least similar experimental design is likely to be presented but applied to a different type of problem and/or sample.

The first two chapters introduce necessary theoretical concepts, experimental designs, and the basics of data analysis. Chapters 3 through 8 present sets of different experiments. Experiments are divided into two parts. Chapters 3 through 5 describe basic steady-state experiments starting from absorption/transmission measurements, basic concepts of steady-state fluorescence measurements, and simple examples of basic applications of steady-state fluorescence like fluorescence quenching. This part is meant to introduce the new experimentalist/user to the principals of proper fluorescence measurement. Chapters 6 through 8 cover more advanced fluorescence concepts from fluorescence anisotropy and

time-resolved fluorescence to examples of more advanced experiments that will typically involve both steady-state and time-resolved approaches. This part demonstrates more powerful applications of fluorescence technology and potential experimental problems that an experimentalist may encounter.

Each chapter in the experimental section is preceded by an abbreviated introduction that serves as a refresher of basic principles applicable to a particular group of experiments. This acknowledges that the nomenclature and certain formalisms differ across disciplines. It aims to show that these are different ways of writing the same thing and that they facilitate cross-disciplinary collaboration. Each introduction presents and discusses experimental basics and instrumentation used in presented experiments. Guidelines are presented, as well as, a general rule of thumb for conducting experiments and selecting the most appropriate types of tools with some practical limitations. In addition, each experiment is preceded by a short description of a problem and the theoretical and experimental basis needed for this particular experiment. Each experiment ends with brief "*Conclusions*." Also, to address some potential problems in the experiment description, a "*Note*" is indicated in the text addressing/emphasizing certain specific issues.

Chapter 3 presents a series of introductory experiments on how to properly measure sample transmittance/absorption and discuss problems and limitations of such experiments. Chapter 4 includes a series of basic fluorescence experiments that are independent of time (steady-state measurements), as well as interpretations applicable to those experiments. Chapter 5 follows the format of Chapter 4 but expands to some practical steady-state applications and examples. In Chapter 6, steady-state anisotropy experiments are discussed, Chapter 7 describes time-resolved experiments, and Chapter 8 is dedicated to examples of more advanced fluorescence experiments.

Readers may be somewhat surprised that this book contains only a few original references. This is because all experiments were done by us and our students from the beginning to the end.

For a broader perspective, we recommend these textbooks on fluorescence:

- Joseph R. Lakowicz: *Principles of Fluorescence Spectroscopy*, 2006, Springer Science + Business Media, LLC.
- Bernard Valeur and Mario N. Berberan-Santos: *Molecular Fluorescence. Principles and Applications*, 2012, Wiley-VCH Verlag GmbH & Co. KGaA.
- David M. Jameson: *Introduction to Fluorescence*, 2014, Taylor & Francis Group/CRC Press.

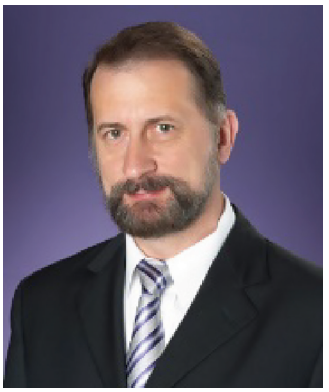
This book has been a lengthy project that took over 5 years to complete. During this time, many of our colleagues and friends became involved and contributed numerous valuable suggestions and insights. Initially, we were unsure of how to approach the problem of writing a book on the experimental aspects of fluorescence and Dr. Karol Gryczynski (son of Dr. Zygmunt (Karol) Gryczynski) was instrumental in keeping us running and patiently writing/rewriting initial chapters. We also need to thank many

of our students who were involved in testing and reading the numerous experiments in this book. We would like to acknowledge Dr. Sangram Raut, Dr. Sunil Shah, M.Sc. Zhangatay Nurekeyev, Dr. Hung Doan, Dr. Joe Kimball, Dr. Rahul Chib, Dr. Sebastian Requena, Jose Chavez, Luca Ceresa, and Bobby Pendry. We also want to thank our long-term collaborators and friends for reading and correcting parts of the experiments—Dr. Anna Synak, Dr. Rafal Fudala, Dr. Rafal Luchowski, and Jack Gryczynski (son of Dr. Ignacy Gryczynski) for help with proofreading. We also thank the University of North Texas Health Science Center and Texas Christian University for providing the intellectual environment that supported this project.

We also would like to acknowledge the publishers, Taylor and Francis. With particular thanks to Mr. Lou Chosen for originally supporting the idea for writing the experimental handbook and Mr. Aoife McGrath, in the final phase, for his patience and understanding.

Finally, we have to sincerely acknowledge the patience of our families, especially our wives, for their long and tireless support over this time, as well as over 40 years supporting our adventure with fluorescence.

Authors



Dr. Zygmunt (Karol) Gryczynski, PhD, is "Tex" Moncrief Jr. chair and professor of Physics, Department of Physics and Astronomy, Texas Christian University, and director and professor, Center for Fluorescence Technologies and Nanomedicine (CFTN), Department of Microbiology, Immunology, and Genetics, University of North Texas, Health Science Center. Dr. Gryczynski received his MS in experimental physics in 1982 from the University of Gdansk, Poland, and PhD in spectroscopy in 1987, working on the basic spectroscopic studies of isotropic and oriented systems of organic molecules. In 1991 he became a research assistant professor in the Department of Biochemistry and Molecular Biology, University of Maryland and 1998–2004 he was an assistant director in the Center for Fluorescence Spectroscopy at the University of Maryland. From 2005 he is a professor of Molecular Biology and Immunology at the University of North Texas Health Science Center at Fort Worth, Texas. In 2006 with the support from the Emerging Technology Funds (ETF) of Texas together with his colleagues he established a Center for Commercialization of Fluorescence Technologies (CCFT) that in 2013 has been transformed to the Center for Fluorescence Technologies and Nanomedicine (CFTN). In 2010 he became the "Tex" Moncrief Jr. Chair and professor of Physic in the Department of Physics and Astronomy, Texas Christian University at Fort Worth.

He has authored over 300 peer-review publications, 12 book chapters, 10 patents, and 15 edited books. He is also a member of editorial boards of *Journal of Experimental Biology and Medicine* and *Methods and Applications in Fluorescence*.

Dr. Ignacy Gryczynski, PhD, is professor in the Department of Microbiology, Immunology and Genetics, University of North Texas Health Science Center. Dr. Gryczynski received his MS (1973) and PhD (1977) in Physics from the University of Gdansk, Poland. For 20 years, since 1985, he was working in the Center for Fluorescence Spectroscopy (at the University of Maryland School of Medicine in Baltimore) directed by Dr. Joseph R. Lakowicz. In 2005 he moved to UNTHSC in Fort Worth, Texas, where he directed Microscopy Core Facility and co-directed Center for Commercialization of Fluorescence Technologies at UNTHSC. His research interests cover basics of fluorescence spectroscopy and its applications in biochemistry and biology.

He has authored over 470 peer-reviewed publications and is a member of editorial boards of *Journal of Experimental Biology and Medicine* and *Journal of Photochemistry and Photobiology B: Biology*.

CHAPTER 1

Theory of Light and Light Interaction with Matter

Conventionally, we call "light" a small range of a broad spectrum of electromagnetic radiation that corresponds to the visible spectral range (400–700 nm) as shown in Figure 1.1. Light is electromagnetic radiation which presents properties of both a wave and a particle. A "particle" of light is called a photon. The dual nature of light is often a source of confusion, but experiments confirming both interpretations exist. For example, experiments involving single-photon double-slit interference and the photoelectric effect have shown both the wave and particle nature of light, respectively. A photon is a quanta or the smallest possible amount/part of an electromagnetic energy/wave. Since we almost always refer to multiple photons, a photon can be referred to as a quantum of electromagnetic radiation. Both definitions will be used throughout this text. For example, a single chromophore (molecule) absorbs or emits a photon of a certain energy. It is important to remember that when discussing photons or electromagnetic waves, we are talking about light; both representations are equivalent, but one is often much easier applied to a particular scenario. For example, it is more natural to discuss a metal interacting with an oscillating electric wave rather than a stream of "particles" (photons) that never physically collide with the metal surface.

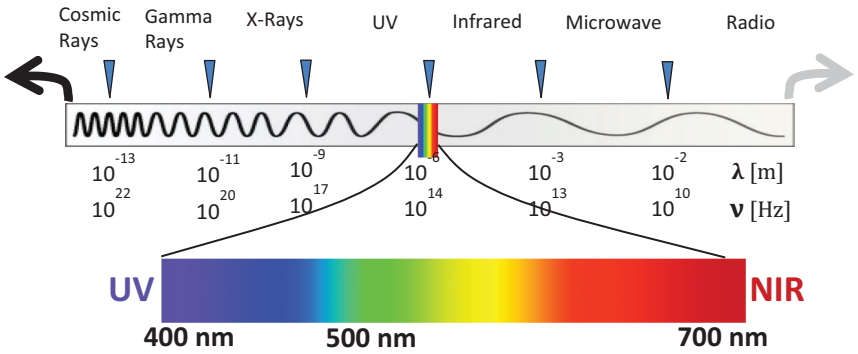


FIGURE 1.1 Electromagnetic radiation. The expanded range of 400–700 nm represents visible light.

1-1 BASICS OF LIGHT

This section is meant as a review of the basics of light as electromagnetic waves or photons. More importantly, it should demonstrate that there are many different ways of looking at light from a mathematical perspective, but they all lead to the same interpretation. Various uses of light are presented across many different fields, especially biology, chemistry, physics, and engineering. Within each field, and indeed specializations within each individual field, the nomenclature and properties of interest vary. For example, in biology, spectra are frequently given in a wavelength scale (nanometers) but in the semiconductor field, they are typically presented on a scale in terms of energy (electron volts). However, any spectrum may be presented in wavelength or energy without a loss of information. By the end of this chapter, the reader should have a fluid understanding of the relationships between different notations.

When looking at the light, one most readily thinks about its brightness and color. The brightness of the light is determined by the intensity (energy), passing through a certain surface area orthogonal to the direction of light propagation per unit of time (we also call it photon flux). The intensity of light is typically denoted as I. A simple way of quantifying the brightness of the light is to think about how many photons hit a detector (e.g., human retina, charge-coupled device, photodiode) per unit time. The more photons that hit a detector, the brighter (i.e., more intense) the light source is. From the wave point of view, the intensity of the electromagnetic radiation is proportional to the square of the electric field amplitude. Color is related to the period (duration of time of one cycle of a wave) of the electromagnetic radiation; equivalently, color (wavelength) is related to the frequency or energy of the light wave.

Figure 1-1.1 shows a typical schematic of a light wave. The electromagnetic field is depicted as orthogonal electric and magnetic fields $\boldsymbol{E}$ and $\boldsymbol{B}$, respectively. It should be noted that $\boldsymbol{E}$ and $\boldsymbol{B}$ are rarely drawn to scale as the amplitude of $\boldsymbol{B}$ is roughly $1/c$ the size of that of $\boldsymbol{E}$. In Figure 1-1.1, the wave is traveling in the $\hat{z}$-direction, the electric field points in the $\hat{x}$-direction, and the magnetic field points in the $\hat{y}$-direction. It is important to notice

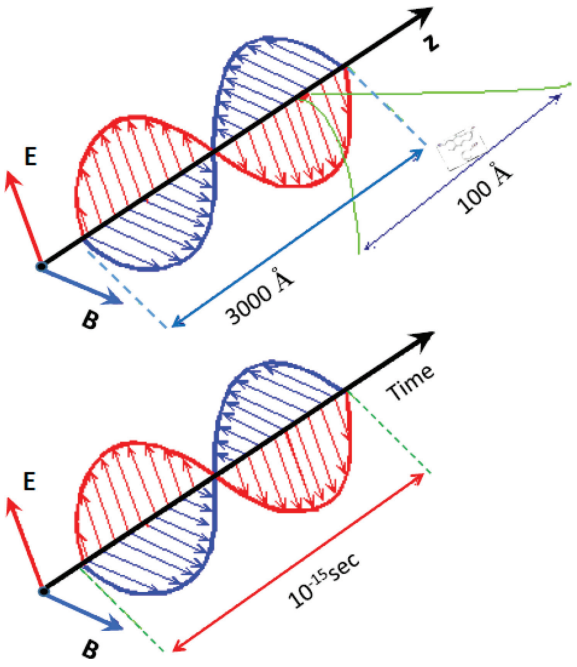


FIGURE 1-1.1 A light wave and its comparison with a typical chromophore.

that the electric field, magnetic field, and the direction of propagation are all orthogonal (akin to the "right-hand rule"). The electric field of light is given as:

$$\boldsymbol{E}(x,t) = E_0 \hat{\boldsymbol{e}} \sin(kx + \omega t + \varphi) \tag{1-1.1}$$

E_0 is the maximum intensity of the electric field or the amplitude of the electric vector and $\hat{e}$ is the directional unit vector. This directional unit vector indicated allows for the direction of the electric field to point in any arbitrary direction. In the example in Figure 1-1.1, $\hat{e} = \hat{x}$; one may also see $\boldsymbol{E}_0$, a constant vector, in place of $E_0 \hat{x}$. In this case, $E_0 = |\boldsymbol{E}_0|$, and $\hat{e} = \frac{E_0}{|E_0|}$. Thus, Equation 1-1.1 becomes:

$$\boldsymbol{E}(x,t) = \boldsymbol{E}_0 \sin(kx + \omega t + \varphi) \tag{1-1.2}$$

Thus, the intensity of this light ray is:

$$I = \frac{cn\varepsilon_0}{2} |\boldsymbol{E}|^2 = \frac{cn\varepsilon_0}{2} \boldsymbol{E}_0^2 \tag{1-1.3}$$

where c is the speed of light, n is the refractive index of the medium the light is traveling in, and ε_0 is the permittivity of vacuum. Light sources such as lamps and lasers are often described by the power, P of the light they emit:

$$P = \int I dS \tag{1-1.4}$$

where S is the surface area of the light used, usually the focused area of the lamp or laser is called the spot size. Equation (1-1.4) often simplifies to the product of an intensity and an area $P = I \times S$. The area of the spot size is usually a circle ($S = 4\pi r^2$) or a rectangle (width × height). Power is given as a time average, for pulsed light sources this includes the time intervals when there is no light emitted. Thus, the instantaneous intensity of a pulsed laser, while hitting a sample, is greater than that of a non-pulsed (steady state) light source for the same given average power. This is an important consideration for sample damage. Power relates well to the number of photons emitted per second, $N_\gamma\ s^{-1}$:

$$\frac{N_\gamma}{s} = P\frac{\lambda}{h\,c} \tag{1-1.5}$$

where λ is the wavelength, a parameter corresponding to the color. Equation (1-1.5) relates the particle and wave natures of light. It gives the number of light particles (photons) as a function of the power of a light wave.

The electric field, in the example of Figure 1-1.1, is a sine wave on the XZ plane depicted temporally in Figure 1-1.2.

E_0 is the amplitude of the wave. The angular frequency ω (rad/s) is $\omega = 2\pi\nu$ where ν is the frequency of light in cycles per second (Hz). The distance from crest to crest of the wave is the wavelength, $\lambda = c/\nu$. An increase in ω decreases the distance from crest to crest or increases the frequency with which the wave repeats. The phase, φ, relates to a translation of the wave on the t-axis. In the example of Figure 1.1-2, the wave is a sine wave and φ is depicted as the distance from the origin to the first zero. The phase shift (translation on t-axis) is the same at any point of the wave.

Light is characterized in space by a wave vector $\boldsymbol{K}$:

$$\boldsymbol{K} = \frac{2\pi}{\lambda}\hat{r} = \frac{2\pi\nu}{c}\hat{r} = \frac{\omega}{c}\hat{r} \tag{1-1.6}$$

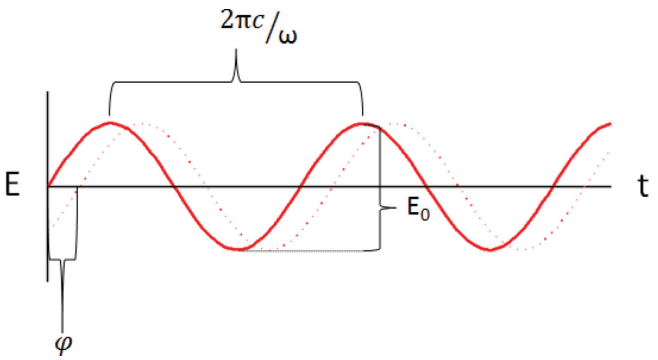


FIGURE 1-1.2 Electric field wave (solid) and electric field with a non-zero phase shift (dotted).

where $\hat{r}$ is the propagation direction of the wave in Figure 1-1.1, $\hat{r} = \hat{z}$ since the wave propagates in the $\hat{z}$-direction. The wavenumber k is defined as:

$$k = |\boldsymbol{K}| = \frac{2\pi}{\lambda} \tag{1-1.7}$$

It is important to note that not all disciplines include the factor of 2π in the definition of $\boldsymbol{K}$ and k, and thus other texts may use:

$$k = |\boldsymbol{K}| = \frac{1}{\lambda} \tag{1-1.8}$$

The factor of 2π will be somewhere else in the equations. The momentum of a photon is:

$$p = \boldsymbol{K}\hbar \tag{1-1.9}$$

where $\hbar$ is the Plank's constant, h, divided by 2π.

The color of light is determined by the period of the sine wave of the electromagnetic field or in other words frequency or wavelength. The electric and magnetic fields are always in phase. Depending on the application, the periodicity of the light wave may be given in wavelength, frequency, wavenumber, or energy. All of these properties of waves are related to each other in a one-to-one fashion. Table 1-1.1 is meant as a review on how to convert among these representations. These conversions are important because these will be the units of the independent axis in most absorption and fluorescence data. Any of these properties are equally applicable to waves and photons (particles), and it is correct to say that a photon has a wavelength.

TABLE 1-1.1 Different Units Used to Describe a Property of Electromagnetic Radiation (Light)

	Wavelength (λ)	**Wavenumber (k)**	**Frequency (ω)**	**Energy ($\mathcal{E}$)**
Commonly Encountered Units	Distance (nm, μm, or Å)	Inverse distance (nm^{-1} or cm^{-1} or kK)	Inverse time (Hz or MHz or GHz or s^{-1})	Energy (eV or J)
Conversion to Wavelength		$\lambda = \frac{1}{k}$	$\lambda = \frac{2\pi c}{\omega}$	$\lambda = \frac{hc}{\mathcal{E}}$
Conversion to Wavenumber	$k = \frac{1}{\lambda}$		$k = \frac{\omega}{2\pi c}$	$k = \frac{\mathcal{E}}{hc}$
Conversion to Frequency	$\omega = \frac{2\pi c}{\lambda}$	$\omega = 2\pi ck$		$\omega = \frac{2\pi\mathcal{E}}{h}$
Conversion to Energy	$\mathcal{E} = \frac{hc}{\lambda}$	$\mathcal{E} = hck$	$\mathcal{E} = \frac{h\omega}{2\pi}\left(=\hbar\omega\right)$	
Why we use it	Many instruments are set up to output wavelength. Easier to draw in the diagram.	Often used in equations. Relates well to the wave vector.	Often used with plasmonics. In natural units is same as energy.	Study transition/ vibration energies.
Visible Region	400–700 nm	25,000–14,286 cm^{-1}	4.7×10^{15} Hz–2.7×10^{15} Hz	3.1–1.77 eV

It is important to consider that the relationship between energy and wavelength is an inverse one with energy and frequency being directly proportional. The inverse relationship between energy and wavelength means that the number of electron volt (eV) in a 50 nm window will differ for different wavelength ranges; in fact, over a large range of energies, the difference can be quite large. For example, the difference between 200 and 250 nm is 50 nm or 1.24 eV, while the difference between 800 and 850 nm is also 50 nm, but 0.9 eV. This is the reason why one may see a graph with an axis labeled in both wavelength and energy with different scales. This also means that spectral fitting in a broad range may differ depending on if a wavelength or energy scale is used and should be done with the proper conversion.

To gain some familiarity with the scales of light waves, we can consider that the "size" of the Bohr Atom is roughly 1 Å. For 1 nm = 10 Å, the wavelength of 500 nm (green) light is 5000 Å = 0.5 µm which is roughly 5×10^3 atoms long. Considering that a typical molecule (fluorescence dye) is only tens of atoms in size, the wavelength of typical light is much larger than the molecule. Even quantum dots are on the scale of a few to tens of nm in size, making their physical structure non-resolvable to visible light. The frequency of current microprocessors is about 3 GHz, with some liquid helium microprocessors that can operate up to about 8.5 GHz, a time scale of 10^9 Hz. In contrast, the frequency for 500 nm of light is about 3.8×10^{15} Hz or 3.8×10^6 GHz or 3.8 PHz (Petahertz). An electron volt is defined as the energy needed to move an electron through a potential difference of 1 V. A 500 nm wavelength of light has an energy about 2.5 eV that is roughly 100 times greater than the average thermal energy of a molecule at room temperature, and about 10 times greater than the energy of an average non-covalent bond. For comparison, it takes 13.6 eV (Rydberg constant) to ionize a single hydrogen atom with its electron in the ground state.

1-2 LIGHT POLARIZATION

Polarization of light occurs when the electric field vibrates in one direction (in one plane). The polarization of light is given by the direction of its electric field $\hat{e}$ from Equation (1-1.1). Figure 1-2.1 shows the single wave traveling in the direction z that has vertical polarization. Most light in everyday life is a composition of many single waves that have no constraints on the $\hat{e}$ direction in the plane orthogonal to the traveling direction. For example, the direction of the electric field of any given light wave from the Sun has an arbitrary random direction in the plane orthogonal to the direction of light propagation. Any two or more photons emanating from the Sun are very likely to have different electric field directions, and thus the light coming from the Sun has randomly distributed electric field vectors around the direction of light propagation. This type of light is non-polarized, and we call it isotropic light. A single wave (Figure 1-2.1) has only one direction for the electric field $\hat{e}$, and any discussion of linear polarization therein is trivial.

This section is mostly concerned with linearly polarized light. Light is polarized in a direction, $\hat{\boldsymbol{r}}$, when its electric field vector points in that direction, $\hat{r}$. When light passes through a polarizer, a device that only allows the light of a certain polarization to be transmitted (say $\hat{r}$), it may lose some or all of its intensity. If $\boldsymbol{E}$ points along $\hat{r}$ no intensity is lost as the light is already polarized. If $\boldsymbol{E}$ and $\hat{r}$ are orthogonal, all intensity is lost (no light travels

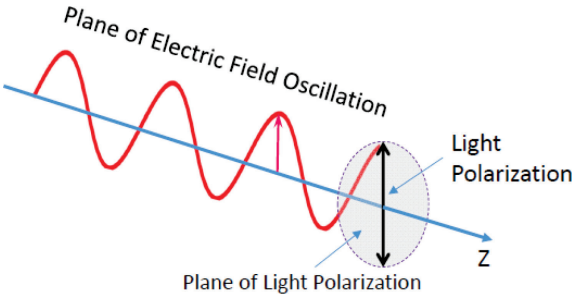


FIGURE 1-2.1 A single electromagnetic wave traveling in the direction z.

through the polarizer). If $\boldsymbol{E}$ and $\hat{r}$ are neither parallel nor orthogonal, the amount of light transmitted is determined by Malus' Law:

$$I = I_0 \cos^2\left(\frac{\boldsymbol{E} \bullet \hat{r}}{|\boldsymbol{E}||\hat{r}|}\right) = I_0 \cos^2\left(\hat{r} \bullet \hat{e}\right) = I_0 \cos^2(\alpha) \tag{1-2.1}$$

where α is the angle between the polarization direction of the polarizer and the direction of $\boldsymbol{E}$ of the incident light (Figure 1-2.2a). Isotropic light loses half of its intensity traveling through a polarizer. It is important to remember that light has no memory, for lack of a better word, of its previous states, including information about its polarization. A common example of this is light passing through two orthogonal polarizers ($\theta = 90°$) where there is no light transmitted (Figure 1-2.2b). However, when there is a third polarizer inserted in-between the two orthonormal polarizers light is indeed transmitted (Figure 1-2.2c). To illustrate this mathematically, consider the light that has passed through the first polarizer and now enters the orthonormal one:

$$I_{\text{final}} = I_0 \cos^2(90) = 0 \tag{1-2.2}$$

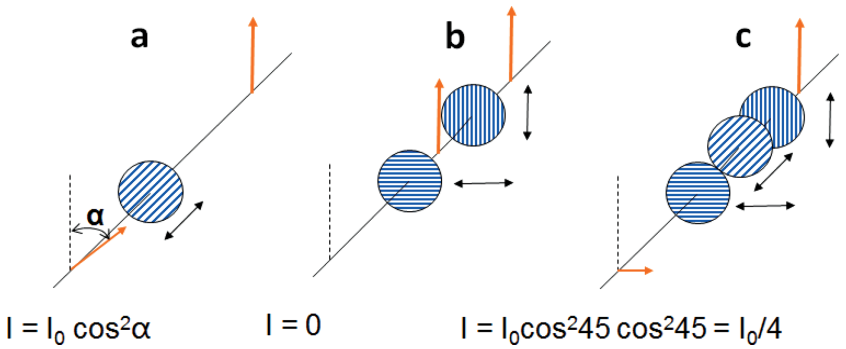


FIGURE 1-2.2 Light being transmitted through polarizers. Light is traveling out of the page and downward. a) Single polarizer; b) Two crossed polarizers; c) Inserted third polarizer enables transmission of the light through a crossed polarizers.

As expected, no light is transmitted. However, in the presence of a third intermediate, polarizer offset to 45° to both original polarizers, the final intensity becomes:

$$I_{\text{intermediate}} = I_0 \cos^2(45) = \tfrac{1}{2} I_0 \tag{1-2.3}$$

$$I_{\text{final}} = \tfrac{1}{2} I_0 \cos^2(45) = \tfrac{1}{4} I_0 \tag{1-2.4}$$

Light is indeed transmitted for any angle in-between 0° and 90°. Thus, light entered both systems in the same state and left through the same final polarizer, but its final intensity varied greatly due to the presence of a third polarizer in one of the systems. The reason for this is that after the light passes through the intermediate polarizer in the second example (Figure 1-2.2b); all information about its original state was completely lost.

Linearly polarized light is a collection (ensemble) of light waves (photons) that all have the same direction of the electric field, thus $\hat{e}_i = \hat{e}_j$. Here i and j are arbitrary iterators overall photons in the ensemble. If we have the light that is an ensemble of randomly oriented electric field vectors (isotropic light) entering an arbitrary linear polarizer, we consider the average angle of all vectors on the $\hat{e}$ direction in the ensemble, which turns out to be 45°. In other words, the isotropic light passing through a linear polarizer loses half its intensity.

Polarizers

The equations in the previous section deal with ideal polarizers and media. A polarizer which transmits through 50% of isotropic light is called a perfect polarizer since 50% is the theoretical maximum of its transmission. In reality, polarizers will reflect and absorb light due to material imperfections so transmitted light is typically no greater than 90–95% of the theoretical limit. Surfaces of many optical elements (polarizers, mirrors, etc.) are specially coated and optimized to work in a specific spectral range achieving efficiencies greater than 95%, but their efficiencies will significantly decrease with light outside the specified range. It is also important to remember that light does not have "memory" due to what process its intensity was lost or how it was polarized other than an apparent intensity decrease or polarization change.

The first modern study of polarizers was conducted by the Danish scientist Bartholin in 1667. He studied a rare transparent mineral called Icelandic spar. Light propagating through this stone is separated into two orthogonally polarized components via refraction. This results in light passing through the stone, creating two side-by-side images. This double image only hinted at polarization since the human eye is not capable of resolving such a phenomenon (this is the principle by which 3D movies operate, only they use circularly polarized light, and each eye sees the differently polarized light through the special polarizing glasses). Polarizers made from these types of minerals are still used in Glan-Thompson polarizers that also split incident light into two beams of different polarizations. In 1929, the Polaroid Corporation filed for a patent of a plastic sheet polarizer. The common sheet polarizers, we see

today, were invented in 1938 by Edwin Land who worked for Polaroid Corporation. Polarizers consist of free moving charges on parallel conducting wires. Light with an electric field parallel to these wires will be absorbed; whereas light with a perpendicular electric field will pass through. Current polarizer sheets polarize visible light using stretched hydrocarbon polymer chains covered in conducting iodide ions. The spacing of these conduction wires must be less than the wavelength of light they polarize. The polymers are stretched to be as thin as possible, ideally to the width of a monomer. For visible light, the spacing of these wires is to be less than 400 nm. Today, we can also use wire grid polarizers where very thin metallic (aluminum) wires are layered (embedded) between fused silica or quartz. Such polarizers may have the working range from UV to NIR. Longer wavelength electromagnetic radiation such as radio waves may be polarized using conductors that are much sparser such as those found on the surface of a metal grill.

1-3 INTERACTION OF LIGHT AND CHROMOPHORES

A *chromophore* is a chemical molecule or group responsible for giving color to a substance through absorption of certain wavelengths of light. If the chromophore is capable of the emission of light (fluorescence) is called a *fluorophore*. Chromophores may also be referred to as "color radicals." For the purposes of this book, a chromophore will usually be an organic molecule or dye.

For organic molecules, the loosely bound electrons in the "outer shell" orbital(s) are shared between atoms forming "molecular orbitals" that are responsible for interacting with light. The energy of molecular orbitals in a molecule is quantized similar to an atom's energy. When the molecule does not have extra energy, the electrons are in a relaxed state called the *ground state*. When light is shone at such a molecule it may be absorbed and the energy from the light (photon) is transferred to the outer electrons leading to a transition (excitation) to a higher energy state. Such a molecule is said to be in an *excited state*. The excitation to the first excited state requires the smallest discrete amount of energy the molecule can absorb and each consequent excitation state requires larger discrete amounts of energy. It is important to remember that electronic states, including the ground state, are discrete, meaning the molecule is in one of these states and nowhere in-between. This means that molecules take on only a discrete amount of extra energy of the light they have absorbed. The energy of the electrons is also quantized corresponding to an eigenstate of a wave function ψ. The electronic energy of a molecule is $\mathcal{E}^n$ where the superscript n denotes the excited state of the molecule; $n = 0$ in the ground state. At higher excited states, there will be higher values of n, and the molecule will possess more energy.

The total energy of a real molecule is determined by its electronic energy state, vibrational energy state, and rotational energy state. When a molecule transition (decays/relaxes) from an excited state to a lower state (usually ground state) it may emit a photon. The total energy of a molecule at given state i, $\mathcal{E}_i$, is:

$$\mathcal{E}_i = \mathcal{E}_i^n + \mathcal{E}_i^v + \mathcal{E}_i^r \tag{1-3.1}$$

where $\mathcal{E}_i^n$, $\mathcal{E}_i^v$, and $\mathcal{E}_i^r$ are electronic, vibrational, and rotational energies for the state i, respectively. The wavelength of the emitted photon is thus proportional to the energy difference $\Delta\mathcal{E} = \mathcal{E}_i \rightarrow \mathcal{E}_0$:

$$\lambda = \frac{hc}{\Delta\mathcal{E}} \tag{1-3.2}$$

The wavelength of the emitted photon depends on the change of the total energy, not just the electronic transition. Experimentally, the number of photons with wavelengths λ emitted by the chromophore is counted for all wavelengths of interest. The vibrational energy of molecules is typically in a range of 0.004–0.4 eV. Since molecules are made of many bonded atoms they can vibrate in many different ways called modes. Each mode of vibration has its own set of energy levels, and combined energy levels are also observed. For example, benzene has 30 vibrational modes while water has 3. However, individual molecular species have a smaller range and only the change in vibrational energies, $\Delta\mathcal{E}_v$, not $\mathcal{E}_v$, contributes to the wavelength of emitted light. For most liquid and solid samples, rotational energy levels are broad and overlap, so that no rotational structure is distinguishable. The rotational energy is typically an order of magnitude smaller than the vibrational energy.

A single chromophore may absorb and emit light multiple times. However, every time a chromophore is in the excited state, the probability for degradation is significantly higher than in the ground state. The excited-state energy is significantly higher than typical thermal energy, and after the excitation cycle has been repeated many times, the chromophore will no longer emit light due to photochemical degradation. This process is called photobleaching. The probability for photobleaching of an individual fluorophore is relatively low and an average fluorophore can be excited hundreds of thousands to millions of times before it undergoes photochemical degradation. For a perfect emitter, millions of excitations will lead to millions of emitted photons. This may sound like a very big number, but one needs to remember that a typical laser pointer (5 mW) emits about 10^{16} photons per second. In a real case, it may take a single fluorophore a fraction of a second to photobleach. The photobleaching of dyes in solution, where we have a very large number of dye molecules, depends on the excitation light intensity and the volume of the sample; typically, photobleaching is not a fast process. However, under powerful laser excitation conditions, in many cases, a significant decrease of intensity for common fluorophore solutions can be easily detected in minutes. This can be a significant problem in microscopy, where the intensity of light in the focal point is very high and the number of dyes available in excitation volume is much smaller.

Absorption of Light

The three primary factors affecting light absorption by molecules are:

1. The energy of the incident photon corresponds to the energy difference of two states, $h\nu = \mathcal{E}_i - \mathcal{E}_0$.
2. The intensity of the excitation light source.
3. The relative orientation between the polarization of the excitation light and the molecular transition moment.

Liquid samples are typically placed in a transparent rectangular container called a cuvette. The molecules of interest are dispersed in a solvent. Different wavelengths of light are passed through the liquid and the light coming out on the other side of the cuvette is observed. The intensity of light is decreased (attenuated), as it passes through the sample. The attenuation of the light as it passes through a cuvette containing a sample (chromophore molecules) depends on:

- The absorption cross-section, σ, of the used molecule (chromophore); parameter that reflects the probability of the photon passing in the molecule proximity to be absorbed. The cross-section of a molecule depends on the wavelength/frequency of light.
- The concentration of molecules in the solution, C.
- The path, l, the light travels through the sample and cuvette.

The concentration of molecules in a solution, C, is given in moles per liter (mol/L). Sometimes, the symbol (M) is used and it means the same as mol/L. The number of molecules per cubic centimeter (number of molecules/cm^{-3}) is often used, in which case, the conversion factor from mol/L is $C \times N_A/1000$, where N_A is Avogadro's number (6.0225×10^{23} mol^{-1}). The absorption cross-section of the molecule at a given wavelength is σ and has units of (cm^2/mol). Absorption cross-section reflects probability for a photon of a given wavelength (energy) to be absorbed as it passes in chromophore proximity. It is instructive to calculate the number of chromophores in a typical sample. Most often concentrations of measured samples will be in a range of micromole per liter (μM).

How many chromophores are in a typical 2 mL sample at 1 μM concentration? 1M means that there are about 6×10^{23} molecule in 1 L of the solution. In 2 mL of this solution (1 μM), it will be about $6 \times 10^{23} \times 10^{-6} \times 2 \times 10^{-3} = 1.2 \times 10^{15}$ chromophores. One needs to remember that a typical molecule is much smaller than the wavelength of a given light and incoming/passing electromagnetic radiation produces a local field perturbation at the chromophore location. The chromophore in such a field has a certain probability to absorb energy that depends on the wavelength (frequency of the field). As light travels through the solution, it will get absorbed by individual chromophores and the number of absorbed photons will depend on the number of chromophores in the light path. In typical conditions, one chromophore may absorb one photon and the number of absorbed photons will be proportional to the length light travels through the solution, Δl. Thus, the change in intensity, ΔI of the light as it travels through the solution layer is:

$$\Delta I = I_0 n \Delta l \sigma \tag{1-3.3}$$

where I_0 is the intensity of the incoming light wave (number of photons per surface unit per second), Δl is the path length, and n is the number of chromophores per unit of volume. The intensity of the transmitted light, I for a sample of thickness l is:

$$I = I_0 e^{-\sigma n l} \tag{1-3.4}$$

Equation (1-3.4) represents the Beer–Lambert law. It is useful to rewrite the Beer–Lambert law to be a function of wavelength since the absorption cross-section is wavelength dependent:

$$I(\lambda) = I_0(\lambda)e^{-\sigma(\lambda)nl} \tag{1-3.5}$$

Absorption may be calculated using log base 10 or log base *e*, called decadic or natural (Napierian), respectively. In photochemistry and photobiology, the extinction coefficient ($\varepsilon(\lambda)$) is more frequently used. The extinction coefficient is a measure of how much a chromophore at a concentration of 1 mole in a 1 cm layer absorbs at a particular wavelength. The units for the molar extinction coefficient are (L mol^{-1} cm^{-1}), although the units used may vary by field (i.e., [m^2 mol^{-1}]). In this text, the decadic molar extinction coefficient, $\varepsilon(\lambda)$, is used. It relates to the absorption cross-section as.

$$\sigma(\lambda) = \frac{2.303\ \varepsilon(\lambda)}{N_A} = 3.823\times10^{-21}\varepsilon(\lambda) \tag{1-3.6}$$

Thus, as a general rule, when the absorption cross-section σ is used then the natural logarithm will also be used. When the extinction coefficient ε is used, the logarithm will be taken with base 10. The Beer–Lambert law then becomes:

$$I(\lambda) = I_0(\lambda)10^{-\varepsilon(\lambda)Cl} \tag{1-3.7}$$

where *C* is the molar concentration of the molecule and the exponent's base is 10 instead of *e* (this convention is not adopted by all texts). The absorbance or optical density, *Ab* (*OD*), is defined as:

$$Ab(\lambda) = \varepsilon(\lambda)Cl \tag{1-3.8}$$

The absorbance is a unitless quantity which is the argument of the exponent in the Beer–Lambert law. It is a quantity that is measured experimentally. The fraction of light absorbed by the sample can then be expressed:

$$\frac{I_0 - I}{I_0} = 1 - 10^{-\varepsilon(\lambda)Cl} = 1 - 10^{-Ab(\lambda)} \tag{1-3.9}$$

Samples with low absorbance ($Ab(\lambda) < 0.1$), we called optically thin and the fraction of light absorbed by the molecules is approximated by $(I_0 - I)/I_0 = 2.303Ab(\lambda)$. As discussed later, optically thin samples will be used for fluorescence measurements.

The extinction coefficient, $\varepsilon(\lambda)$, is the principle parameter characterizing spectroscopic properties of a chromophore. In order to experimentally evaluate $\varepsilon(\lambda)$, a sample of known chromophore concentration is placed in a cuvette of a well-calibrated thickness (typically 1 cm), and $\varepsilon(\lambda)$ is then evaluated from the measured absorbance according to Equation (1-3.8).

It is important to clarify the terminology we are using. By ***absorption***, we will understand the physical process of converting the wave energy to the matter. ***Absorbance*** is a measure of absorption expressed in the common (decadic) logarithm of the ratio of incident to transmitted light. We will use alternatively both terms for the description of our absorption measurements. ***Extinction*** is a total attenuation of the light due to absorption, scattering, and reflections expressed in the common logarithm of the ratio of incident to transmitted light. ***Optical density*** (***OD***) is the measure of the transmission of an optical medium for a given wavelength, also expressed in the common logarithmic scale. The higher the OD, the lower the transmittance and vice versa, e.g., optical density of 1 means 90% of incident light is extinct and 10% is transmitted. We will sometimes use this term to characterize our samples.

Emission of Light

Similar to atoms, in a molecular system the character of a given state depends on the spin of the excitable electron(s) (molecular orbital), and typically we are dealing with singlet states or much more rarely with triplet states. Both are physically allowed states, however, transitions between singlet and triplet states are forbidden. The ground state is typically a singlet state, and by absorbing a photon (energy), a molecule can be excited to any of its allowed singlet states. Even if all allowed states are quantum mechanically real states, the molecule in solution will very quickly relax to the lowest energy excited state, which is typically the most stable state. In fluorescence, the emissive state is typically the lowest excited state, a principle also called Kasha's rule. A molecule in an excited state $\mathcal{E}_i$ stays for a specific (finite) amount of time and then it transitions back to the ground state (or decays or relaxes to the ground state). If this transition involves emission of a photon it is a radiative transition and the wavelength of the emitted photon is determined by the energy difference between the two states. It is possible for the chromophore to lose the extra energy via a non-radiative process, one not involving the emission of light. Such non-radiative decay may be the result of a collision and heat dissipation. Table 1-1.1 may be used to determine other properties of this emitted photon. While the extra energy absorbed from a photon is in the molecule, portions of this extra energy may be used to alter rotational and vibrational states. This is why the energy of the photon absorbed by the molecule is not necessarily the same as the energy of the photon being emitted by the molecule. The energy difference between the absorbed and emitted photons is called the Stokes' shift. When the molecule transitions in one step from the excited state to the ground state, it releases the excess energy in the form of a photon (light). This photon is emitted in an arbitrary/random direction. It is conceptually wrong to think of the emitted photon in some way being related to the photon absorbed, it is merely the result of the molecule releasing the excess energy. In fact, the excess energy may not even come from absorbing a photon; it can come from a chemical reaction (chemiluminescence), electric (current/electroluminescence), or heat (thermoluminescence) interactions. The molecule has no recollection of how it got into the excited state. There are no phase dependences between absorption and emission processes.

Most fluorescence experiments are done on large collections or ensembles of molecules and involve many photons. However, even a single fluorophore when excited many times

will stay in the excited state for a different time after every consecutive excitations. In other words the transition to the ground state is a statistical process and we can only talk about the probability for a given transition per unit of time. This is why we talk about averages, such as the average lifetime or the center of the emission peak. An individual molecule stays in an excited state for a random time t_i after each excitation. Another molecule in the experiment will stay in the excited state for a random time t_j. In general, t_i does not equal t_j but an average time for a large number of events for each molecule will be the same. So, to characterize the ensemble of relaxing molecules the average lifetime, τ, is used. The average lifetime, τ represents the time after which the population of excited molecules decreases $1/e$ of the initial number of excited molecules. In fact, only a few molecules may decay at $t = \tau$ but most excited molecules in the ensemble (~63%) will decay during the period of time from $t = 0$ to $t = \tau$. In general, the number of molecules in the excited state, $N(t)$ can be described:

$$N(t) = N_0 e^{-t/\tau} \tag{1-3.10}$$

At time $t = \tau$, the number of molecules remaining in the excited state will be:

$$N = N_0 e^{-1} = 0.368\, N_0 \text{ or } 36.8\% \text{ of } N_0 \tag{1-3.11}$$

It is important to notice that in this discussion once N_0 molecules are excited, no more molecules in the ensemble become excited. Since the decay process is a statistical process and because there is no relationship between a molecule's excitation source and how long it is in the excited state, after the next time interval:

$$N' = Ne^{-1} = 0.368\, N \text{ or } 0.135\, N_0 \tag{1-3.12}$$

The number of molecules in the excited state decreases exponentially.

The decay of excited molecules depends on both radiative relaxation (radiative decay) and non-radiative process occurring in the excited state. Oscillations, rotations, and collisions of molecules may induce transitions to the ground state (without emission of the photons) with the dissipation of the excitation energy into heat. The photo-processes are described with the rates, which are related to probabilities. The deactivation of the excited state can be described with the decay rate k, which is the sum of the radiative rate (Γ) and non-radiative rate k_{nr}:

$$k = \Gamma + k_{nr} \tag{1-3.13}$$

A cumulative rate k reflects how long in average the molecule stays in the excited state before it relaxes via one of the specific channels. The number of molecules in the excited state as a function of time is described as follows:

$$N(t) = N_0 e^{-kt} = N_0 e^{-(\Gamma + k_{nr})t} \tag{1-3.14}$$

This process may be generalized to account for more decay channels.

The average time fluorophores spend in the excited state, called *lifetime* is:

$$\tau = 1/\left(\Gamma + k_{\mathrm{nr}}\right) \tag{1-3.15}$$

The probability that a molecule will emit a photon is the ratio between the radiative rate and the sum of all rates, and describes the efficiency of the radiative process:

$$Q_Y = \frac{\Gamma}{\Gamma + k_{\mathrm{nr}}} \tag{1-3.16}$$

The efficiency of the radiative process, Q_Y, is called quantum efficiency (quantum yield) and reflects the number of emitted photons as compared to the total number of absorbed photons. Importantly, in fluorescence we do not use energy efficiency since the wavelength of emission is typically longer than the wavelength of excitation light (Stoke's shift), a fact that already reflects energy being lost. A $Q_Y = 1$ typically does not mean energy efficiency is equal to 1, it only means that each absorbed photon leads to the emission of a photon typically of lower energy.

If the non-radiative rates are equal to zero the fluorescence lifetime $\tau_n = 1/\Gamma$ is called *natural* fluorescence lifetime.

Jablonski diagram: Figure 1-3.1 shows a classic energetic representation of molecular energy levels called a Perrin–Jablonski diagram (historically called a Jablonski diagram). Each horizontal line represents an energy state, $\mathcal{E}_i$. The solid lines are electronic states and the dashed lines are sums of electronic and vibrational states. Under normal conditions,

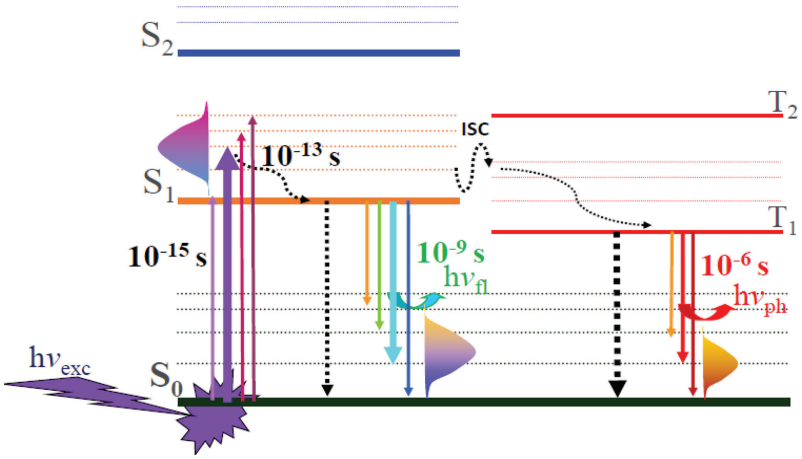


FIGURE 1-3.1 Jablonski diagram describing molecular photo processes. Solid horizontal lines indicate electronic levels and dotted horizontal lines indicate vibrational levels. The left side shows the singlet states and the right side shows the triplet states. Arrows depict transitions labeled with a typical time of that transition (1 ns = 10^{-9} s).

molecules are in the lowest energetic state (ground state, S_0 that has energy $\mathcal{E}_0$ or the black line on the bottom of the diagram). Incoming excitation light causes mixing of the ground state with the excited states, leading to the transition to the excited state (light absorption). The absorbed energy of light ($E = h\nu$) brings the molecule from the ground state energy $\mathcal{E}_0$ (typically at room temperature the lowest vibrational state of the state $\mathcal{E}_0$) to a new energy state for which quantized total energy is $\mathcal{E}_i^j$. Absorption is a very fast process happening on a 10^{-15} s time scale. Depending on the energy of the light (wavelength), the transition is to one of a number of vibrational energy levels, *j*:

$$\Delta\,\mathcal{E} = \mathcal{E}_i^j - \mathcal{E}_0 \tag{1-3.17}$$

After absorption, the molecule quickly relaxes to the lowest vibrational level of the first excited state $\mathcal{E}_1$. This relaxation process depends on the molecule and molecular environment and is in the order of 10^{-14} to 10^{-12}. Emission is typically a transition from the lowest vibrational level of the first excited state, $\mathcal{E}_1$ to one of the vibrational levels of the ground state, $\mathcal{E}_0^j$. The energy difference $\Delta\mathcal{E}'$ can be expressed as:

$$\Delta\mathcal{E}' = \mathcal{E}_i - \mathcal{E}_0^j \tag{1-3.18}$$

$\Delta\mathcal{E}'$ is not the same as the energy difference for absorption, $\Delta\mathcal{E} \geq \Delta\mathcal{E}'$. This difference is due to molecular relaxation in the excited state and the fact that the emission transition could be to higher vibrational levels of the ground state. In real absorption and emission measurements where many molecules contribute to the measured spectrum, we will observe a distribution of all $\Delta\mathcal{E}$ due to the distribution of vibrational and rotational states. Typically, the distribution of vibrational energy levels in combination with the probability for the transition has a so-called normal distribution (also called a Gaussian or bell curve) centered at specific transition energies. Such a normal distribution in a spectrum is referred to as a *peak*. The peak is centered at the mean value of the normal distribution called the maximum. The wavelength value of the maximum of the peak is normally given as absorption and emission positions of the dye. For absorption transitions that have more than one electronic state involved in the same experiment, their energies are typically far enough apart to result in multiple distinguishable peaks in the spectrum. Contrary to this, the emission is typically one electronic transition with vibrational and rotational states contributing to the width of the distribution, resulting in a bell-shaped emission. For some rigid molecules like anthracene, the emission spectrum is structured and vibrational states can be clearly seen as separated peaks in the spectrum.

The schematic representation of the bell-shaped absorption and emission spectra are shown in Figure 1-3.1 drawn horizontally. Correctly, the intensity should be drawn as a function of wavenumber that directly corresponds to energy. As we discussed above, the distribution in the spectrum corresponds to the energy levels of vibrational and rotational states. Drawing the intensity as a function of energy (wavenumber or eV) is a proper way of doing so for many applications that we will discuss later. However, for many historical reasons, absorption and emission spectra are frequently represented on

a wavelength scale. The apparent shapes, for wavenumber and wavelength representations, are different and any data operation involving surface area calculations under the emission curve should be done carefully.

The Jablonski diagram is often called an energy diagram because the horizontal lines represent energy levels. Compare transitions in absorption $S_0 \rightarrow S_1$ with transitions in fluorescence $S_1 \rightarrow S_0$. The energy differences for absorption transitions are larger than for fluorescence transitions (lengths of the arrows in Figure 1-3.1 pointing up (absorption) are longer than arrows pointing down (fluorescence)). So, the energy of the emitted photon will typically be lower than the energy of the absorbed one. In effect, the wavelengths for absorption spectrum are shorter than for emission spectrum. It could be rephrased that the fluorescence emission is shifted toward longer wavelengths as compared to the absorption. First, who realized this was Sir Stokes and this phenomenon is now called a *Stoke's shift*.

1-4 INTERACTIONS AMONG CHROMOPHORES

One of the most important properties of fluorescence molecules is the fact that the absorption and emission process are separated in time. Typically excited molecules will stay in the excited state for a short time in the range of a nanosecond. But this time is comparable to the timescale of molecular processes and interactions. So, such excited molecules may interact during their fluorescence lifetime with other molecules. Due to such interactions, the fluorescence signal may significantly change bringing fundamental information about interacting entities. Such interactions can occur by direct contact (collisions due to the diffusion of molecules during the finite time when they are in the excited state). Or the interaction can be through space (due to physical distances larger than the molecule size) when there is no physical contact between interacting molecules.

We need to realize that in typical fluorescence experiments we will deal with many chromophores. Depending on the fluorophore concentration, the number of fluorophores in 1 cm^3 is very large (for very dilute 1 nM solution it is about 6×10^{11}). As a consequence of this high number, the separations between chromophores are small, comparable to the average distance molecules may diffuse during their fluorescence lifetime. Also, even if in most fluorescence experiments we excite only a small percentage of molecules, a chromophore in the excited state may easily find a molecule of solvent, some other charged atom/molecule, an unexcited chromophore, or even another excited chromophore. In many cases, interactions with atoms or molecules like iodide, oxygen, or acrylamide lead to a non-radiative deactivation of the fluorophore's excited state. Such collisions with other molecules or among chromophores itself result in a non-radiative deactivation with an extra non-radiative deactivation rate, leading to a decrease in fluorescence intensity and fluorescence lifetime. A different type of quenching is fluorescence (Förster) resonance energy transfer (FRET). In this case, molecules that have suitable spectroscopic properties can interact through space without direct contact. Excitation energy from one molecule (called donor) can be transferred to another suitable molecule (called acceptor) through a significant distance (up to 10 nm). In this case, the donor fluorescence is quenched and fluorescence lifetime of the donor is shortened.

Fluorescence Quenching

In molecular fluorescence, the number of emitted photons is usually lower than the number of absorbed photons, which is reflected in quantum yields lower than one. This is because of intrinsic non-radiative processes such as non-radiative transition or intersystem crossing to a triplet state. Atoms and functional groups incorporated in molecular structures of fluorophores can accelerate these processes. In this case, we observe an *intramolecular quenching*. Examples of such quenchings include heavy atoms within molecular structures or covalently attached quenchers, like in molecular beacons. If the fluorescence emission is decreased by species not linked to fluorophores, we refer to this as *external quenching*.

It should be noted that fluorescence depends on temperature (higher temperatures result usually in lower quantum yields); often this is a dramatic dependence, especially in the case of indoles.

Therefore, quenching experiments should be conducted with rigorous temperature control.

External quenching falls into two categories: static and dynamic (the last is also called collisional).

***Static quenching*:** If quenching species (atoms or molecules) form non-fluorescent complexes with fluorophore molecules in a solution, then the observed fluorescence will be decreased. For example, when half of the fluorophore molecules form non-fluorescent complexes with quenchers, the observed fluorescence will be reduced to 50% compared to a non-quenched solution. Static quenching can be described with the association constant K_a.

Assuming that the solution has a concentration of $[N_0]$ fluorophores and part of these fluorophores, $[N_c]$, formed complexes with quenchers, the concentration of unquenched fluorophores will be reduced to $[N]$. The concentration of complexes, N_c, is proportional to the number of free fluorophores and to the number of quenchers, $[Q]$, and the complex formation depends on the product $[N] \times [Q]$:

$$\boldsymbol{N_c = N_0 - N = K_a \cdot N \cdot [Q]} \tag{1-4.1}$$

This leads to:

$$\frac{N_0}{N} = 1 + K_a \cdot [Q] \tag{1-4.2}$$

The fluorescence intensity is proportional to the number (concentration) of free fluorophores, therefore:

$$\frac{I_0}{I} = 1 + K_a \cdot [Q] \tag{1-4.3}$$

The ratio I_0/I depends linearly on the quencher concentration.

In the case of static quenching, unaffected (free) fluorophores behave exactly as they would in the absence of the quencher, and other fluorescence properties such as lifetime and polarization are not changed. The solution behaves as if it has a lower concentration of fluorophores.

The complex formation depends on the temperature; at higher temperatures, static quenching is less effective because there are fewer complexes in the solution.

If external species do not perturb fluorophores in the ground state (do not form non-fluorescent complexes) they can still affect fluorophores while they are in the excited state. However, this requires direct contact of the excited fluorophores and quenchers (as in static quenching). In solutions, diffusion of quenchers and excited fluorophores allow this direct contact. Such quenching is called *dynamic or collisional.*

***Dynamic quenching*:** Dynamic (collisional) quenching depends on the effectiveness of the quenching process described by a bimolecular constant k_q and on the quencher concentration $[Q]$ which result in a quenching rate equal $k_q \times [Q]$.

It is convenient to illustrate molecular processes on the Jablonski diagram. In particular, the dynamic quenching is represented in Figure 1-4.1 by a non-radiative transition $S_1 \rightarrow S_0$ with the constant rate $k_q \times [Q]$. Other transitions are: absorption, Abs; fluorescence (radiative transition) with the constant rate Γ (k_f is sometimes used instead of Γ); non-radiative deactivation of the excited state (independent on the quencher), k_{nr}; and non-radiative transition to the triplet state (intersystem crossing), k_{isc}.

For consistency, we will use Γ in the following equations.

The constant rates describe the probability of deactivation processes and are expressed in units of 1/s. The time needed for the given process k_i is equal to $1/k_i$. In the case of fluorescence, $\tau_n = 1/\Gamma$ is called a natural lifetime. The average time of fluorophores in the excited state will depend on all deactivation processes and is given by:

$$\tau = \frac{1}{\Gamma + \sum k_i} \tag{1-4.4}$$

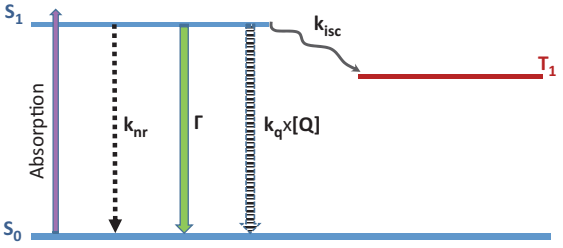


FIGURE 1-4.1 Jablonski diagram describing fluorescence processes in the presence of the quencher.

In the absence of the quencher:

$$\tau_0 = \frac{1}{\Gamma + k_{\mathrm{nr}} + k_{\mathrm{isc}}} \tag{1-4.5}$$

And in the presence of the quencher:

$$\tau = \frac{1}{\Gamma + k_{\mathrm{nr}} + k_{\mathrm{isc}} + k_q \cdot [Q]} \tag{1-4.6}$$

The efficiency of the given process is equal to the ratio of a given rate constant to the sum of all rates in the processes:

$$E = \frac{k_i}{\Gamma + \sum k_i} \tag{1-4.7}$$

The efficiency of the fluorescence (fluorescence intensity) in the absence of the quencher is:

$$E_0 = I_0 = \frac{\Gamma}{\Gamma + k_{\mathrm{nr}} + k_{\mathrm{isc}}} \tag{1-4.8}$$

And in the presence of the quencher:

$$E_f = I_f = \frac{\Gamma}{\Gamma + k_{\mathrm{nr}} + k_{\mathrm{isc}} + k_q \times [Q]} \tag{1-4.9}$$

Equations 1-4.5 through 1-4.9 give:

$$\frac{\tau_0}{\tau} = \frac{I_0}{I_f} = \frac{\Gamma + k_{\mathrm{nr}} + k_{\mathrm{isc}} + k_q \times [Q]}{\Gamma + k_{\mathrm{nr}} + k_{\mathrm{isc}}} = 1 + \tau_0 \times k_q \cdot [Q] = 1 + K_{sv} \cdot [Q] \tag{1-4.10}$$

This relationship is known as the *Stern–Volmer* equation, and K_{sv} is a Stern–Volmer constant. The graphical illustration of this dependence is called a Stern–Volmer plot.

In the case of dynamic quenching, a simultaneous decrease of the lifetime and fluorescence intensity is expected, whereas in the case of static quenching only intensity is decreased. Another difference between static and dynamic quenching is the temperature dependence—dynamic quenching intensifies with temperature because diffusion increases, whereas static quenching decreases with increased temperatures.

Often, the quencher acts in a dual role as a static and dynamic quencher, simultaneously. In such a case, the change in the fluorescence intensity will be given by the product of both processes:

$$\frac{I_0}{I} = \left(1 + K_a \cdot [Q]\right) \cdot \left(1 + K_{sv} \cdot [Q]\right) \tag{1-4.11}$$

The lifetime is dependent only on the dynamic process. The time-resolved (lifetime) measurements will directly reveal K_{sv} for dynamic quenching. The expression (1-4.11) is square dependent on the quencher concentration which results in an upper-curvature of the Stern–Volmer plots.

There are a number of approaches to the theoretical description of quenching mechanisms. These include a model of a sphere of efficient quenching, diffusion dependence with a transient effect, and Collins–Kimball's theory with distance-dependent quenching. Detailed descriptions of these models are provided in the recommended books (see Preface). In practice, most quenching experiments can be interpreted with the Stern–Volmer approach.

What information is provided by fluorescence quenching measurements?

There are two major applications of fluorescence quenching. First is the accessibility of quenchers to fluorophores—quenching experiments can directly provide information on the location of fluorophores. For example, a tryptophan moiety located on the surface of a protein will be quenched more effectively than if it was buried deep in the protein. Fluorescence quenching is applicable to macromolecules, proteins, membranes, and DNA. Second, the dependence of the intensity and lifetime of fluorescence on the quencher can be used for fluorescence-based sensing. For example, oxygen is an efficient dynamic quencher and can be detected with lifetime measurements of many fluorophores; of course, the quenching effect will be stronger with long-lived fluorophores which allow sufficient diffusion of oxygen.

Fluorescence (Förster) Resonance Energy Transfer (FRET)

Resonance energy transfer (FRET) is a process in which one chromophore transfers its excitation energy to another chromophore in a non-radiative way (without emission of the photon). The concept of radiationless resonance energy transfer originated almost 100 years ago with reports on fluorescence self-depolarization in solutions in the 1920s. The first approximation for the radiationless interaction of two oscillating dipoles based on classical physics was developed by Perrins in 1925. However, a classical model or its subsequent quantum mechanical expansion by Perrins in the early 1930s still was unable to give a quantitative account of the experimental data measured for the fluorophores at high concentrations. The exact theory of fluorescence resonance energy transfer (FRET) was correctly explained by Förster almost 20 years later. Recently, the radiationless energy transfer is also called Förster resonance energy transfer (FRET) to recognize Förster's contribution.

FRET is the most widely applied fluorescence-based technology today that allows researchers to study molecular processes with sub-nanometer resolution. The enormous power of FRET lies in the fact that it brings not only static information but also may give insight into the internal mobility/flexibility of a biomolecular system.

The concept of FRET for two interacting chromophores is presented in Figure 1-4.2. The chromophore which gives up excitation energy is called the donor and the chromophore which takes the energy is called the acceptor. In Figure 1-4.2, we also presented the schematic representation for electronic levels for acceptor and donor molecules. The energy level for an excited state of an acceptor is typically lower than the energy of the excited state of the donor. In practice, this means that the donor emission energetically overlaps with the acceptor absorption. When the donor and the acceptor are two different molecular species (typically the case), we call it hetero-FRET to distinguish from the FRET between the same molecular species that we call homo-FRET or energy migration. Often the ratio of donors to acceptors is not one to one especially in free solutions where typically the acceptor concentration is much higher than that of the donor. The efficiency of FRET is determined by multiple factors:

1. The separation between the donor and acceptor, *r*.
2. The overlap integral of the donor's emission and acceptors absorption.
3. The relative orientation of the donor and acceptor transition moments.
4. Donor fluorescence lifetime and quantum yield in the absence of acceptor.
5. Refractive index of the medium between the donor and acceptor.

The exact theory for RET is rather complex and we will present only the final results. Readers interested in the physical basis and mathematical derivation of FRET are referred to the original papers. For a weak coupling between two dipole moments, where the energy of interaction between the donor and acceptor is small compared to the vibrational splitting of the donor energy levels, for a single donor and a single acceptor separated by the

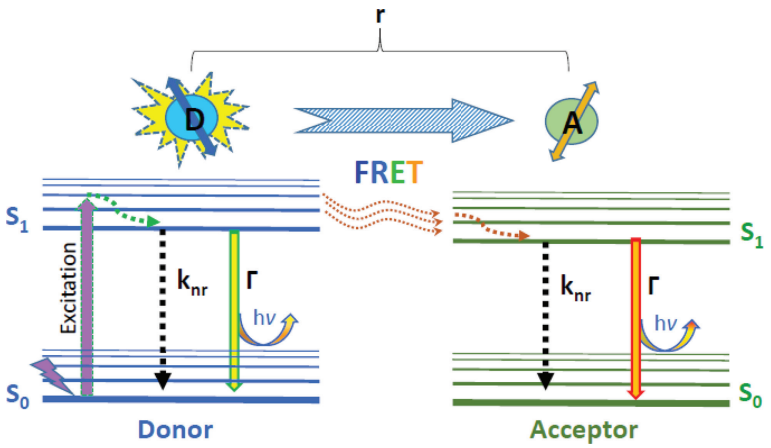


FIGURE 1-4.2 Schematics for electronic levels of an interacting donor-acceptor system.

distance, r one may calculate the rate of transfer (probability of transfer of energy quantum from donor to acceptor per unit time):

$$k_T = \frac{Q_D \kappa^2}{\tau_D r^6}\left(\frac{9000 ln10}{128\pi N n^4}\right)\int_0^\infty F_D(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda \tag{1-4.12}$$

where Q_D is the quantum yield of the donor in the absence of the acceptor; τ_D is the lifetime of the donor in the absence of the acceptor; n is the refractive index of the medium; N is Avogadro's number; F_D is the normalized fluorescence intensity of the donor (emission spectrum normalized to unity of surface area); $\varepsilon_A(\lambda)$ is the extinction coefficient of the acceptor at wavelength, λ; and κ^2 is the orientational factor describing the relative orientation of the transition moment of the donor and acceptor. The integral in Equation (1-4.12) is referred to as an overlap integral, $J(\lambda)$ expresses the spectral overlap between the donor emission and acceptor absorption and is given by:

$$J(\lambda) = \frac{\int_0^\infty F_D(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda}{\int_0^\infty F_D(\lambda) d\lambda} \tag{1-4.13}$$

$F_D(\lambda)$ is dimensionless. If the extinction coefficient $\varepsilon(\lambda)$ is expressed in units of M^{-1} cm^{-1} and λ in nanometers, then $J(\lambda)$ is in units of M^{-1} cm^{-1} nm^4. The overlap integral has been defined in several ways with different units. This sometimes causes confusion if one tries to calculate the so-called R_0 value for a specific donor-acceptor (D-A) system. We usually recommend the units of nanometers or centimeters for the wavelength and M^{-1} cm^{-1} for the extinction coefficient.

R_0 is a characteristic Förster distance and its meaning can be understood from the frequently used form of Equation (1-4.12) where the right-hand side can be written in the form:

$$k_T = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6 \tag{1-4.14}$$

where $R_0 = 8.79 \times \left[Q_D \kappa^2 n^{-4} J(\lambda)\right]^{1/6}$

In this expression, R_0 is given in Å and the overlap integral is given in $M^{-1}\,cm^{-1}$. It is important to realize that the energy transfer process competes with the spontaneous decay of the donor, which proceeds with the rate constant $1/\tau_D$. The probability p that the donor will not lose its energy at a time t after excitation is given by:

$$-\frac{1}{p}\frac{dp}{dt} = \frac{1}{\tau_D} + \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6 \tag{1-4.15}$$

From that relationship, one may understand the phenomenological meaning of R_0 that it is the distance between the donor and acceptor at which a probability for the excited donor to transfer the excitation energy to the acceptor is equal to the probability of losing excitation energy by all other processes. In other words, half of the excitation energy of the donor is transferred to the acceptor, while the other half is dissipated by all other processes, including emission.

If the transfer rate is much faster than the decay rate ($1/\tau_D$), then the energy transfer process will be efficient. If the transfer rate is slower than the decay rate, then the probability for a transfer to occur during the excited-state lifetime is low.

The efficiency of energy transfer (E) can be defined by rate constants for all processes involved in excitation energy losses. E represents the fraction of photons absorbed by the donor that is transferred to the acceptor and is given by:

$$E = \frac{k_T}{k_T + k_{nr} + \Gamma} = \frac{k_T}{k_T + 1/\tau_D} \tag{1-4.16}$$

which is the ratio of the transfer rate to the total decay rate (sum of all deactivation rates) of the donor. Taking into account Equation 1-4.14, we can describe the transfer efficiency as:

$$E = \frac{R_0^6}{R_0^6 + r^6} = \frac{1}{1 + \left(r / R_0\right)^6} \tag{1-4.17}$$

This equation shows that the energy transfer efficiency is strongly dependent on distance when the D-A separation is near R_0 as shown in Figure 1-4.3. The efficiency quickly increases to 1.0 as the D-A separation decreases below R_0. For instance, if $r = 0.5$, R_0 the energy transfer

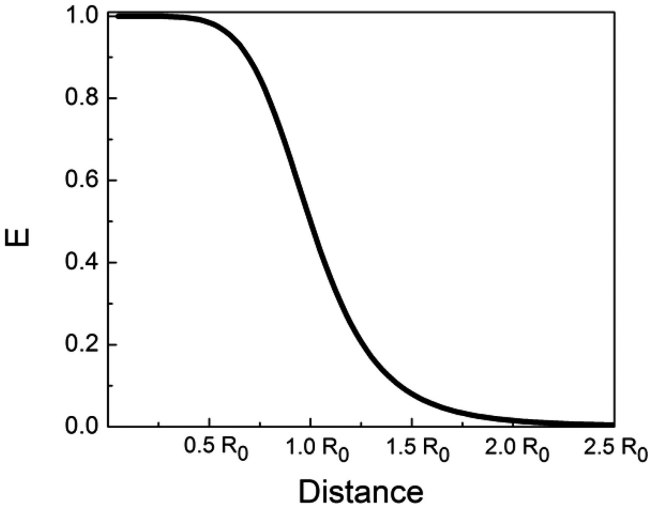


FIGURE 1-4.3 Dependence of energy transfer efficiency (E) as a function of donor-acceptor distance.

efficiency is 98.5% and if $r = 2R_0$ the energy transfer efficiency is only 1.5%. This outstanding dependence on $1/r^6$ of FRET has been extensively tested experimentally and leads to very precise distance estimation between donor and acceptor molecules. Because of the FRET capabilities to measure separation very precisely, it is frequently called a molecular ruler.

1-5 SCATTERING

When measuring absorption and emission, we may have an additional process affecting our result. In absorption and emission, an incident photon is absorbed by a chromophore which enters an excited state as a result of the interaction. When the chromophore relaxes from the excited state, it may release a photon. Interaction of electromagnetic radiation with the matter also leads to scattering in an outward direction of field energy at various directions. This process is much more effective for non-continuous media, such as solutions of biomolecules (proteins or DNA) and especially suspensions of large objects like cells. Scattering is an interaction between light and matter in which the light is not completely absorbed. The matter which scatters light may be referred to as a scatterer. During scattering, the traveling direction of a photon is altered and sometimes also is its energy. When the scattered photon does not change its energy (color), the scattering is called *elastic*; when the photon changes in energy, the scattering is called *inelastic*. Elastic and inelastic often refer to collisions, and in a way scattering of photons can be described as collisions between photons and matter particles. This description is not accurate, especially when the scatterer is small compared to the wavelength of light. A photon incident on a molecule will alter its direction and possibly its energy as a result of passing near a molecule, the energy of the molecule will also be altered (equal and opposite). The details of this mechanism are quite complex. The common way in which scattering is described is that a molecule absorbs a photon into a "forbidden" energy level during which time the molecule is in a "virtual excited" state. The energy, the molecule has in a virtual state is forbidden by quantum mechanics (the value of the energy lays inside the band-gap). This leads to a messy situation wherein it becomes acceptable to violate the laws of physics, but only if it is for a short enough time, something that should typically be avoided. All virtual states are extremely short-lived. A virtual state is not a real state but its effects are observable through scattering. Similarly, the centrifugal force is not a real force, but its effects may be observed by driving a car around a curve very quickly. Figure 1-5.1 shows a Jablonski diagram where the solid lines are electronic states and dotted lines are vibrational states (rotational states not shown). In elastic scattering, the molecule goes back to its initial state (left) and in inelastic scattering, it alters its rotational state (right). Thus the energy lost/gained by the photon results in the gain/loss of vibrations. The vibrations of molecules are quantum oscillators which have non-zero ground energy. This sometimes results in Jablonski diagrams being drawn with parabolic shapes with discrete lines in them where one parabola corresponds to an electronic state and the horizontal lines within it correspond to quantized vibrational states in the electronic state. However, it is often the case that the excited energy of a vibrational state is given as the difference between its energy and the vibrational ground energy. This is the quantity which is experimentally observed, $\Delta \mathcal{E}_v$.

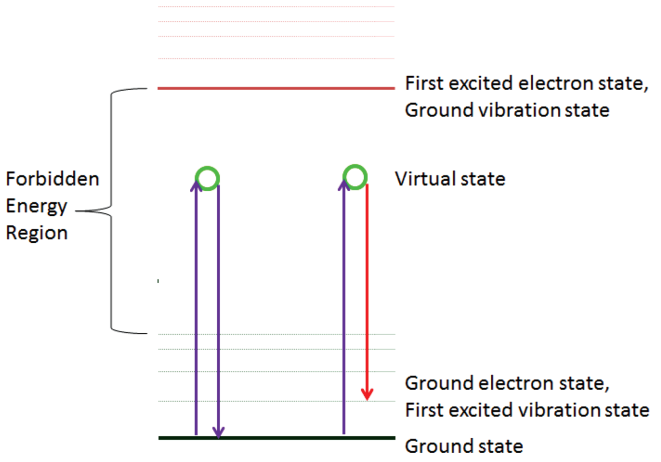


FIGURE 1-5.1 Jablonski diagram with virtual states depicting elastic (left) and inelastic (right) scatterings.

Elastic Scattering

Rayleigh scattering is a type of elastic scattering which appears in many experiments. It only alters the path of a photon and does not result in a change of the vibrational state of the scatterer. Rayleigh scattering is commonly observed when powerful lasers are shone through an air medium. For example, consider a green laser pointer, which would appear as a line that streaks through the air. If all photons emitted from the laser traveled in a straight line, they would not reach the observer's eye (unless the laser is shined directly into the point of view of the observer). All photons emitted from a laser travel in a straight line, but they also interact with air via Rayleigh scattering, causing some of the photons to change direction and be visible to an observer not looking directly into the laser (about 0.0001% of a laser beam's intensity is lost per meter while traveling in air under 1 atmosphere of pressure). In a 90° fluorescence measurement set up, the excitation light is scattered and reaches the detector which is perpendicular to the light's emission direction. Usually, it is scattered by aqueous samples. Rayleigh scattering is proportional to λ^{-4}, thus shorter wavelengths are scattered much more. The cross-section of Rayleigh scattering is given by:

$$\sigma_{\text{Rayleigh}} = \frac{1}{\lambda^4}\frac{2\pi^5 d^6}{3}\left(\frac{n^2-1}{n^2+1}\right)^2 \tag{1-5.1}$$

where d is the diameter of the particles. The λ^{-4} dependence is important because it is observed in the spectra. Rayleigh scattering is applicable to scattering particles that are much smaller than the wavelength of light they scatter. A strong dependence on wavelength results in a much higher scattering at shorter wavelengths making it frequently a concern for measuring protein absorption in the UV spectral range. This phenomenon is also responsible for the blue color of the sky where tiny particles scatter much more

blue/UV light than other colors. Mie scattering theory is a full solution to Maxwell's equations with a scattering sphere, whereas Rayleigh scattering is an approximation for Mie scattering for small scatterers.

Raman Scattering

Raman scattering is an inelastic form of scattering, also referred to as the *Raman Effect*. In Raman scattering, some of the energy of the incident photon is absorbed by the molecule and the scattered photon loses energy or the molecule absorbs some of the photons energy into its vibrational state. When the photon loses energy (increased observed wavelength), the change is called a Stokes shift; when it gains energy (decreased observed wavelength) this is called an anti-Stokes shift. The shift refers to the wavelength shift of the photon. In Raman scattering, the photon interacts with the vibrational energy states of the molecule, $\mathcal{E}_v$. Thus, Raman spectroscopy can be used to probe the vibrational states of molecules, proteins, crystal lattices, etc. Vibrations are quantized, thus it is possible to identify a species by its rotational energy transitions. A quantum of vibration is called a phonon; this term is often used when talking about vibration in solid-state lattices. The change in incident photon's energy $\Delta\mathcal{E}$ is equal and opposite to the change of the molecule's vibrational energy. The quantity which is experimentally observed is a light with a wavelength $\lambda + \Delta\lambda$ where $\Delta\lambda$ corresponds to $\Delta\mathcal{E}_v$, the molecule's vibrational energy where λ is the excitation wavelength. If Raman scattered light redshifts, the vibrational energy of the molecule increases, and if the scattered light blueshifts, the vibrational energy of the molecule decreases. Many instruments set up to collect Raman show $\Delta\lambda$ in their output window. Raman scattering may also be observed while performing fluorescence emission measurements. In this case, the Raman signal will look like an emission peak. Since $\Delta\lambda$ depends on the excitation wavelength while $\Delta\mathcal{E}_v$ is constant, a Raman peak will move with an inverse dependence to the excitation energy. An emission peak will stay in the same place or vanish, with varying excitation energy. Normally, a Raman signal is much weaker (many orders of magnitude) than an emission signal. Typically, the Raman signal of a solvent becomes a concern for fluorescence measurements made with very low fluorophore concentrations as an abundance of solvent compared to molecules of dye may result in a significant Raman contribution. For example, a nanomolar concentration of a UV fluorophore in water will give an easily measurable signal, but the signal can be weaker than the water Raman signal due to an overwhelmingly higher concentration of water (over 55.5 moles).

Water (similar to other transparent media) scatters light mainly via two processes, Rayleigh scattering and Raman scattering. Rayleigh scattering is due to any small particles (impurity or very small air bubbles) present in the water and Raman scattering is the scattering process on water molecules. Raman scattering is an inelastic scattering meaning that the scattered photon changes its energy (wavelength). In Raman scattering, a photon is "absorbed" by the water molecule but to a very short-lived virtual state. A virtual state is a forbidden energy state and a distinct form of an excited state. A molecule's decay forms the virtual state practically immediately but as it relaxes some of the energy of the photon is transferred into vibrational energy of the water molecule. Thus, the vibration energy, $\mathcal{E}_v$, of the molecule is increased and the energy of the photon is decreased. The photon

transfers part of its energy to the molecule. Transitions of vibrational states of water do not emit light, what is observed in this experiment is the excitation light which lost some of its energy due to the Raman interaction.

The vibrations of water can be modeled as a collection of harmonic quantum oscillators. The ground (not excited) state of water, $\mathcal{E}_0$, is not 0 but 0.574 eV, no quantum harmonic oscillator has a zero ground energy. Everyday liquid water is in the vibrational ground state. Since H_2O is a compound molecule it can undergo several types of vibrations, specifically, it has three vibrational modes. These modes are illustrated in Table 1-5.1. The vibrations of a water molecule may be symmetric, anti-symmetric, or bend. These types of vibrations are called modes and have distinct frequencies. Each mode has its own first excited state vibrational energy of 1.03, 0.772, and 1.104 eV respectively. What is observed in the spectra is the energy differences between the ground state energy and the first excited state energy of each of these vibrations subtracted from the energy of the excitation beam.

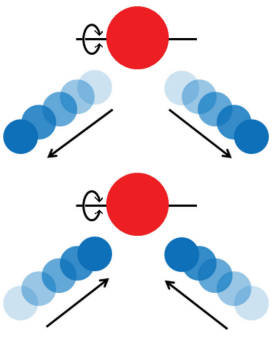
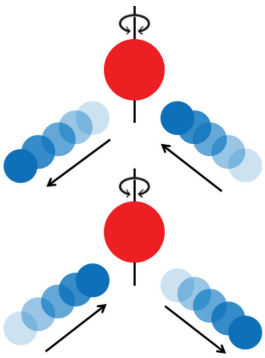
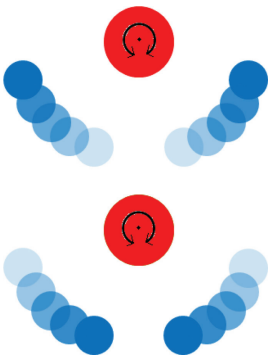
$$\mathcal{E}_{\text{observed}} = \mathcal{E}_{\text{excitation}} - \mathcal{E}_{\text{vibration}}$$

$$\lambda_{\text{observed}} = \lambda_{\text{excitation}} - \lambda_{\text{vibration}}$$

The electronic state does not change in Raman scattering. It is important to remember that in the above equation in wavelength $\lambda_{\text{vibration}}$ varies as a function of excitation wavelength but $\mathcal{E}_{\text{vibration}}$ does not. Table 1-5.1 lists the vibrational energy states and other useful information for the Raman effect in the water.

Each vibrational state of water has higher excited states and linear combinations of different modes, not listed in Table 1-5.1. What is observed in the water Raman spectra are

TABLE 1-5.1 Vibrational Modes of Water

	Symmetric	Anti-Symmetric	Bend
Motion Red: oxygen Blue: hydrogen			
$\mathcal{E}_0$ (eV)	0.574	0.574	0.574
$\mathcal{E}_1$ (eV)	$\mathcal{E}_1^{\text{symmetric}} = 1.04$	$\mathcal{E}_1^{\text{anti-symmetric}} = 1.03$	$\mathcal{E}_1^{\text{bend}} = 0.772$
$\mathcal{E}_2$ (eV)	$\mathcal{E}_2^{\text{symmetric}} = 1.5$	$\mathcal{E}_2^{\text{anti-symmetric}} = 1.47$	$\mathcal{E}_2^{\text{bend}} = 0.965$
$\Delta\mathcal{E}_{0-1}$ (eV)	0.466	0.456	0.198
Δk_{0-1} (cm^{-1})	3758	3678	1597
Average Δk (cm^{-1})		3300	

peaks with three components, one for each of the vibrational modes of water. In most experiments, only the ground to a first excited state transition is observed. These peaks have their width form rotational states and since the relative intensities of these peaks change the center of the visible spectral shape appears to change. However, $\Delta\mathcal{E}_{\text{vibration}}$ is always constant and is not wavelength dependent per each vibrational mode. This also explains the change in the spectral shapes as we change the excitation wavelength. The 3300 cm^{-1} wavenumber is commonly used to describe this shift, it is a linear combination of the three peaks. The observed spectral shape is always shifted by the same amount of energy thus the change in wavelength increases as the excitation wavelength increases.

Raman scattering is a useful tool for studying molecular and protein systems. Molecules which do not have fluorescence may have Raman, as is the case for water. This allows for their detections spectroscopically. A Raman signal requires many more Raman active molecules than an emission measurement requires fluorophores.

Vibrations which occur in a solid lattice such as semiconductors also have a Raman signal.

Another situation when virtual states are used to explain a phenomenon is two-photon excitation. In this process, two photons of the same energy, $\mathcal{E}_\gamma$, excite a chromophore with $\Delta\mathcal{E} = 2\mathcal{E}_\gamma$. The first photon absorbed excites the chromophore to an excited virtual state, and if the chromophore absorbs another photon of the same energy while in this virtual state, it will excite to a real excited state. This is the reason why high intensity is needed for two-photon excitation because the virtual state is very short-lived and the second photon needs to be absorbed very quickly after the first. This idea also works for three-photon excitation where $\Delta\mathcal{E} = 3\mathcal{E}_\gamma$ and so on. When a chromophore is excited by absorbing multiple photons (two or more) the process is called multi-photon excitation.

SUMMARY

This section is meant to remind the reader of which quantities are important to consider while conducting experiments. Each of the quantities is measured as a function of wavelength.

- $I(\lambda)$ – The intensity is measured as a function of the instrument and setup parameters. It measures how many photons at a certain wavelength reach the detector, useful to determine spectral shapes. This observation alone is not always enough to make a convincing case in support of a hypothesis since it strongly depends on the equipment used. For these reasons, most emission spectra use arbitrary units (a.u.) or are normalized. Relative intensities in the same spectrum (one intensity measurement/spectrum) can make a compelling case, however.
- $I(\lambda)/I_0(\lambda)$ – The ratio of intensities is a common way to communicate data because it is reproducible among different instruments. This ratio (normalization) is also often calculated by instruments.
- $P(\lambda)$ – The power of emitted light measured in Watts (W, mW). Usually relates to a light source. The power of a light wave relates to the number of photons per second as $\frac{N_\gamma}{s} = P\frac{\lambda}{hc}$.

- $N(t,\lambda)$ – The quantity measured is the number of photons emitted per time interval and directly corresponds to the number of chromophores in the excited state. For steady-state experiments, $N(t,\lambda) = N(\lambda)$ and is a constant in time. Lifetimes and relaxations rates are calculated from this quantity thus a lifetime measurement will result in $N(t,\lambda)$ data which is then fitted to find the lifetime, τ.
- $Ab(\lambda)$ – The absorbance is a quantity often measured to characterize chromophores and provides information on many types of other samples. Although this quantity is a difference and a ratio of intensities it is important enough to consider separately.

CHAPTER 2

Experimental Basics

Most of the data in this book will be acquired as a function of wavelength, meaning there will be multiple data points, one for each wavelength. This wavelength scale may be converted to other scales such as wavenumber or frequency (see Table 1-1.1). Plotted optical data such as emission intensities or absorption versus wavelengths as the independent axis is called a spectrum. Sometimes, instead, the wavelengths wavenumbers are used. Both presentations are equivalent and often both scales are used simultaneously.

Real instruments have random errors resulting from a dark current of the photodetector and other limitations such as threshold current, which affects the reading. Perhaps one of the most dissatisfying limitations is the difficulty of measuring light intensity on a meaningful unit scale. This is the reason why many equations in Chapter 1 use ratios of intensities. Fluorescence intensities are usually presented in arbitrary units (a.u.).

The term *fluorescence* was originally introduced by Stokes and referred to Sir John Herschel's experiment reporting the celestial blue color glowing from the solution of quinine sulfate. Now, the term fluorescence describes the process of light emitted when a molecule, called a fluorophore, transitions from its excited singlet electronic state to its electronic ground state. Usually, fluorophores achieve the excited state via stimulation by light (absorption). Therefore, fluorescence belongs to a family of photoluminescence, together with phosphorescence which is an effect of the transition from an excited triplet state to the ground state. However, experimentalists from various fields seldom limit their tools by strict definitions because instruments will report any light they see no matter of its origin.

In this book, we will use term "fluorescence" strictly referring to light emitted from a molecule (chromophore) when it transitions from the singlet excited state to a singlet ground state after a finite duration of time subsequent to light absorption. The light emitted when transiting from a triplet state to a singlet ground state will be called phosphorescence.

In this chapter, we will introduce the most fundamental concepts that are frequently needed when running and describing specific experiments. The term "Experiment" will refer to physical measurements of light absorbed or emitted by the sample, basic data analysis, and results in interpretation. To familiarize a new researcher, not yet familiar with

fluorescence technology, how absorption and fluorescence experiments should be done correctly we will introduce basic concepts and terms ("fluorescence slang"). Starting from the description of optical cuvettes, optical filters, principles of absorption/transmission and emission measurements, most crucial corrections, signal-to-noise ratio, and statistical data analysis.

2-1 TYPES OF CUVETTES

A cuvette is a vessel used for spectroscopic measurements. Cuvettes come in different shapes, volumes, and are made of different materials, and should be transparent for the wavelengths of interest. An ideal cuvette would only hold samples (most commonly liquid) and does not interact with the light used in the experiment. This is often an acceptable approximation and it is often worthwhile to obtain a cuvette that satisfies this approximation. Real cuvettes transmit light of a limited spectral range, present dielectric mismatch of refractive index (different refractive index than air and solvent), and can have wear and tear damage such as scratches (some may be small and go unnoticed). All of these factors can adversely affect the outcome of an experiment.

The material a cuvette is made of determines which wavelengths of light it is suitable for. The cuvette should have a sufficient transmission such that light attenuation on the cuvette walls will not affect the outcome of an experiment. Typical optical cuvettes do not have a uniform transmission for all wavelengths, and most often the transmission in the UV or IR is the limiting factor. There is no universal agreement upon minimum transmission to determine the suitability of a cuvette for use at a specified wavelength, and manufacturers tend to use different standards (between 10% and 90%). Table 2-1.1 Transmission Ranges of Different lists some materials cuvettes are made from and their theoretical (usable) transmission range.

Table 2-1.1 should not be taken literally nor be used as a guide. It is presented only to make the reader aware that the transmission ranges of cuvettes may vary greatly. It is important to be sure that the cuvette is adequate for the wavelengths of interest in your experiment. There are plenty of plastic cuvettes that work with wavelengths lower than 380 nm and there are hundreds of different transparent plastics not suitable for fluorescence or absorption measurements. In reality, the fabrication process (quality of surface and the purity of the cuvette walls) contribute to light attenuation (its absorption). Thus, cuvette performance may vary across different brands. Furthermore, many manufacturers

TABLE 2-1.1 Usable Transmission Ranges of Different Cuvettes

Material	Theoretical Transmission (nm)
Far UV quartz	170–2700
Near IR quartz	220–3800
UV silica	220–2500
Optical glass	320–2500
Plastic	380–850
UV plastic	290–900

use their own proprietary materials and surface coating to increase the transmission range and/or decrease the price of their cuvettes. A high transmission may be required to yield meaningful results, thus working at the boundaries of cuvette transmission can cause problems. For a protocol for finding the transmissions of cuvettes, see Experiment 3-3. The only way to truly know the quality of your cuvette is to measure it in your laboratory as described in Experiments 3-3 and 3-4.

The most common absorption and fluorescence cuvettes have an external base of 12.5 mm × 12.5 mm and a height of 45 mm and internal dimensions 10 mm × 10 mm. This is a standard size for which most spectrophotometers and spectrofluorometers holders are designed. There are also many types of larger and smaller cuvettes. Some vendors sell adaptors which allow placing smaller cuvettes into the standard holder that accept 12.5 mm × 12.5 mm square cuvettes. Unless otherwise stated, it should be assumed that a cuvette used in the experiment has external dimensions 12.5 mm × 12.5 mm and internal dimensions 10 mm × 10 mm. These are the so-called standard 1 cm × 1 cm cuvettes (Figure 2.1-1a). Cuvette sizes often refer to the volume of liquid they are designed to hold. A 1 cm cuvette holds 1 mL of solution per 1 cm of height. So, a 45 mm tall cuvette may hold up of 4.5 mL of solution. For many biological experiments, a few milliliters of the volume is difficult to achieve. The volume needed to reach a certain height of solutions can be changed by making two or four of the cuvette walls thicker inward. For example, a "0.4 cm cuvette" ("4 mm cuvette") is a cuvette with external dimensions of 12.5 mm × 12.5 mm and internal dimensions of 4 mm × 10 mm. Such a 4 mm cuvette will require a solution volume of 0.4 mL per ~10 mm of height; similarly, a 1 mm cuvette corresponds to a solution volume of 0.1 mL per ~10 mm of solution height. It is important to remember that due to the surface tension and the formation of a meniscus, the height

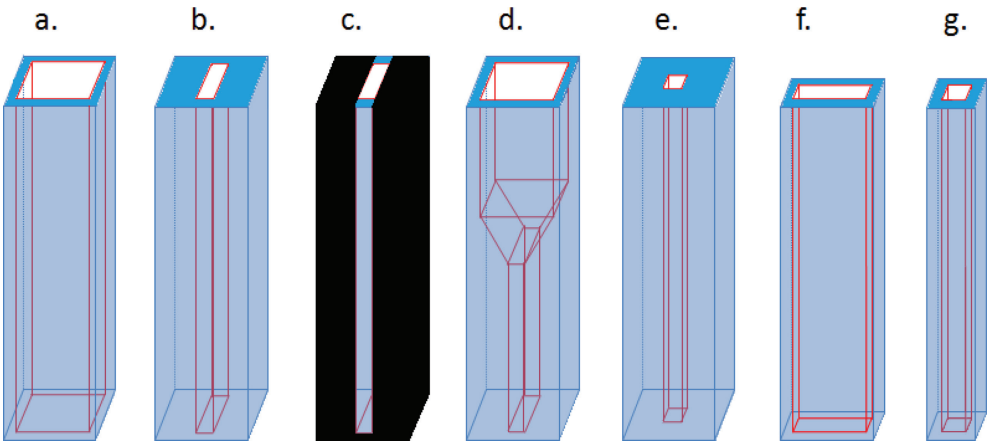


FIGURE 2-1.1 Schematics of cuvettes, a–e are standard size. Red lines inside represent internal volume holding the liquid sample. All cuvettes are drawn to be 12.5 mm × 12.5 mm × 400 mm external dimensions. a: 10 mm × 10 mm fluorescence cuvette; b: 2 mm × 10 mm fluorescence cuvette; c: 10 mm × 2 mm absorption cuvette; d: 2 mm × 10 mm cuvette; e: 2 mm × 2 mm fluorescence cuvette. Cuvettes f and g are examples of not standard dimension cuvettes.

is only approximate. Also, cuvettes with different exterior base dimensions are used, for example, 4 mm × 12.5 mm. Such cuvettes are more difficult to mount in standard spectrophotometers without a proper adapter. Figure 2-1.1 shows the schematics of several different cuvettes.

The path length of the cuvette is the length light travels through the sample inside the cuvette. Cuvettes a and c in Figure 2-1.1 have 1 cm path lengths. Cuvettes b, c, and f have the same dimensions (2 mm × 10 mm) and cuvettes e and g have the same internal dimensions (2 mm × 2 mm) but different external dimensions. Cuvettes f and g have non-standard exterior dimensions and are not referred to in this text unless specifically described. However, those who are comfortable with non-standard sizes cuvettes should certainly use them, though with the appropriate adaptors or specially designed sample holders.

Cuvette c is a small volume absorbance cuvette. It has two dark (black) sides that do not transmit any light. This is useful because a 1 cm path length may be used with a smaller volume and any light not traveling through the sample will be blocked from reaching the detector. Importantly, using cuvette b instead of c in an absorbance measurement will result in getting an incorrect result even when the background has been measured with this cuvette (b). For example, see Experiments 3-11 and 3-12. Cuvette c, however, cannot be used for an emission measurement, which is typically done in a different geometry. Fluorescence cuvettes are cuvettes that are transparent on all four sides (some specialized cuvettes may have three transparent sides or only two orthogonal sides).

2-2 TYPES OF FILTERS

A filter is an optical element that selectively allows a specific range of wavelengths of light to pass through while blocking or attenuating others. Filters are typically labeled for the wavelengths they work for. Filters can be categorized in the following ways:

- Short (low) pass filters: Allow shorter wavelengths to pass while blocking longer wavelengths.
- Long (high) pass filters: Allow longer wavelengths to pass and block shorter wavelengths (lower energy).
- Bandpass filters: Allows a range of wavelengths (band) to pass and block shorter and longer wavelengths.
- Neutral density filters: Allow all wavelengths to pass but attenuates all of them. Figure 2-2.1 shows the schematics of each type of filter mentioned above.

A λ long pass filter allows wavelengths above λ to pass and blocks shorter wavelengths than λ. For example, a 540 long-wave pass filter means that wavelengths above 540 nm are transmitted and below 540 nm are blocked. Similar, a λ short pass filter allows wavelengths below λ to pass and blocks wavelengths longer than λ. A bandpass filter will typically have defined central wavelength for the light transmittance and a bandwidth of the transmitted band. So, a λ bandpass filter allows wavelengths centered at around λ to pass, blocking

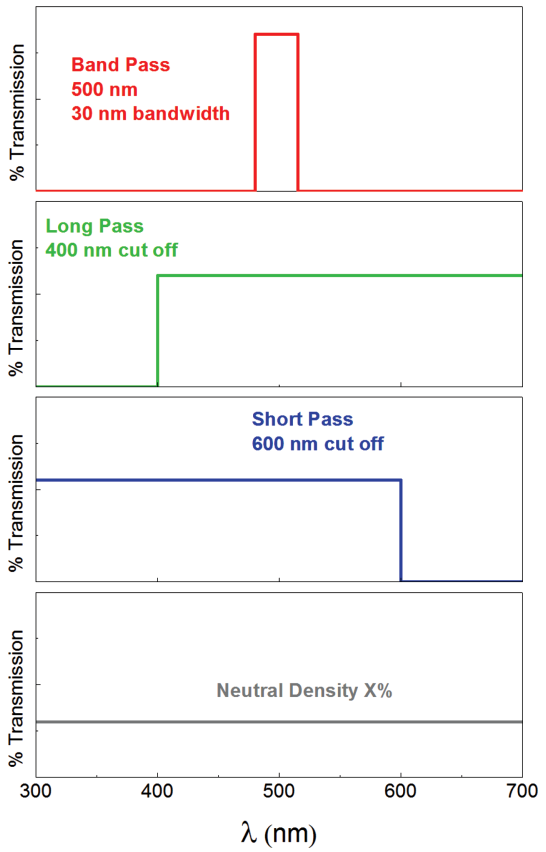


FIGURE 2-2.1 Schematics of idealized filters.

wavelengths longer than $\lambda + \Delta\lambda/2$ and shorter than $\lambda - \Delta\lambda/2$. The filter passes a band of width $\Delta\lambda$ thus wavelengths passed are in the range $\lambda - \Delta\lambda/2$ to $\lambda + \Delta\lambda/2$. A neutral density filter is a filter that in the broad range of wavelengths attenuates the transmitted light by a specified amount. For example, a neutral density filter 1 will have absorption 1 and will attenuate transmitted beam about 10-fold (will transmit 10% of light).

Various filters can be made from different types of materials and work differently. Most general types are dichroic filters and absorptive filters. A dichroic filter is typically an interference thin film deposited on glass/quartz which reflects certain wavelengths and allows other to pass, and an absorptive filter is typically dyed glass or plastic which absorbs certain wavelengths and allows others (outside dye's absorption) to pass. Some absorptive filters can also be based on liquid solutions of chromophores.

Either type of filter is effective for most fluorescence experiments; however, there are some differences that may prove relevant for certain experiments. Since dichroic filters reflect light instead of absorbing it they are less prone to be damaged via heating. This is only a concern when using high-intensity laser light or high power xenon lamps. Absorptive filters frequently have a residual fluorescence that can perturb measurements. When using such filters (called sometimes colored glass), we need to be alert that filter intrinsic

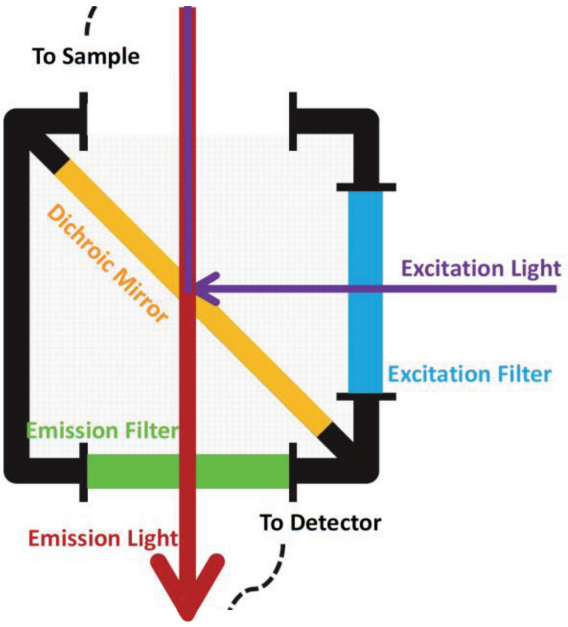


FIGURE 2-2.2 Microscope cube with two filters and a reflective dichroic filter (mirror).

fluorescence may corrupt our measurement. So, for some experiments, it can be important to what filters we will use, and sometimes also the filters sequence can be important. For example, to eliminate short-wavelength scattering (scattered excitation light) when measuring a weak fluorescence, we prefer to put first an interference filter and the absorptive filter next. The interference filter that strongly reflects unwanted light will attenuate short-wavelength scattering limiting intrinsic emission of the absorptive filter. The opposite sequence will first expose to the scattering absorptive filter that would emit a longer wavelength that is not attenuated by interference filter (it overlaps with emission we want to detect).

One common microscopy component that uses filters is called a cube. A schematic of a cube is presented in Figure 2-2.2. There are excitation and emission filters (typically dichroic) at right angles to each other and a third dichroic filter (mirror) at a 45° angle to both of them. The dichroic filter at 45° acts as a mirror that reflects a range of wavelengths and very efficiently transmits the other wavelength. In a standard microscope, the emission light from the sample travels the same path as the excitation light between the sample and the dichromic mirror only in the opposite direction. The longer wavelength emission light that is transmitted by the dichroic mirror and leaves the cube at the bottom.

2-3 MEASURING TRANSMITTANCE/ABSORBANCE

Before doing a fluorescence experiment, the first step should typically be a measurement of sample absorption/transmittance. This will let us know the spectral range where the sample absorbs and what is the optical density of the sample. It is important to realize that

the absorbance and transmittance are related and it is easy to convert from the sample absorbance to sample transmittance.

An instrument for measuring sample transmittance or absorbance (sometimes called also transmission or absorption) spectra in the UV/VIS range is called a *spectrophotometer*. A spectrophotometer typically consists of a light source, an optical system including a spectral apparatus (monochromator), a sample compartment/holder, a radiation (light) detector, and a system for data acquisition and data processing (connection to a computer). Figure 2-3.1 shows schematics of two concepts for spectrophotometers, a dual-beam spectrophotometer, and a single beam spectrophotometer. The dual-beam spectrophotometer is shown in Figure 2-3.1a. In the dual-beam spectrophotometer, a special pair of cuvettes with identical (or very similar) optical properties should be used. The dual-beam spectrophotometer divides the light beam into two parts that travel through two parallel trajectories into both cuvettes. One cuvette holds the sample while the other is the reference (or blank) cuvette containing the solvent or a reference solution. The light passes simultaneously through both cuvettes, and the detection system compares the intensities of the two beams, converting them into transmission or absorption readings. The second type of spectrophotometer, more commonly used today, is a single beam spectrophotometer (Figure 2-3.1b). In the single-beam instruments, the cuvettes are moved sequentially into a single beam of light. First, the cuvette containing a solvent (reference) is used for collecting the baseline, which is stored and remembered by the

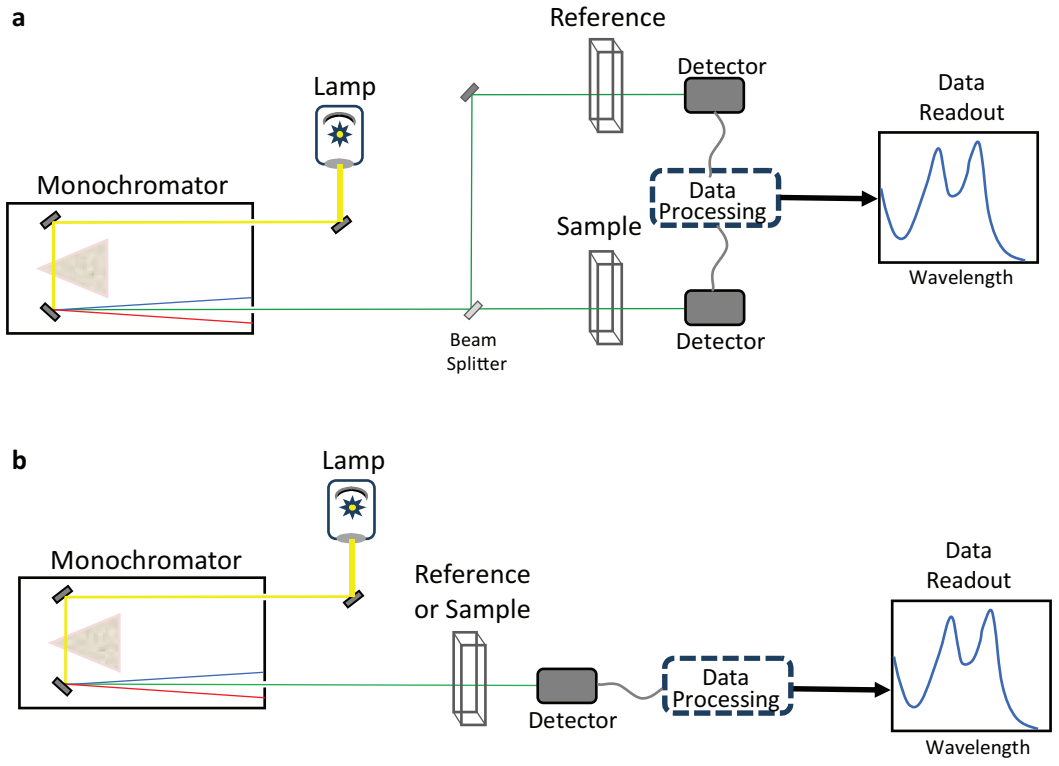


FIGURE 2-3.1 Schematics of dual-beam (a) and single beam (b) spectrophotometers.

operating computer. Next, the baseline cuvette is replaced with an identical cuvette filled with the sample of interest. Then, after setting the measurement software from baseline to sample, readings for each consecutive sample can be taken and will be corrected with the previously measured baseline.

As the light travels through the sample (in cuvette), photons are absorbed, scattered, and reflected, decreasing the intensity of the transmitted light. If the intensity of the initial beam is I_0, its intensity when it reaches the detector will be I, which will, of course, be lower than I_0. The fraction of incident light that passes through the sample is called transmitted light—*light transmittance*. The light transmittance, T is defined as a percentage of the light that has passed through the sample and is:

$$T = \frac{I}{I_0} \times 100\,\% \tag{2-3.1}$$

Or, as a wavelength-dependent transmittance in terms of absorbance:

$$T(\lambda) = \frac{I(\lambda)}{I_0(\lambda)} = \frac{1}{10^{Ab(\lambda)}} \times 100\% \tag{2-3.2}$$

For the definition of absorbance, please revisit Section 1-3 "Interaction of Light and Chromophores" (p. 9).

Light transmittance is measured as the percentage of light from the light source that was ultimately received by the detector. Typical samples used in fluorescence spectroscopy are liquid solutions, but occasionally solid samples (polymer films or glasses) are used. It is important to realize that there are other ways in which light intensity may be lost apart from being absorbed by the sample. For example, light passing through any interface of two media (such as air and glass wall) will be partially reflected. In practice, even the cleanest, highest-quality optical glass will still reflect a few percents (typically about 1–3%) on each interface (total on two cuvette walls 5–10%). A typical cuvette has two glass walls and the traveling light beam crosses four interfaces (air/glass, glass/solvent, solvent/glass, and glass/air), significantly attenuating the beam intensity due to the multiple reflections. These reflections are due to a dielectric mismatch (refractive index mismatch) and are the reason why an empty cuvette attenuates light more than a cuvette filled with water (water has a higher refraction index than air). The refractive index of air is practically 1 and the refractive index of normal glass is about 1.5. Since the refractive index of water is 1.33, the reflection on glass/water and water/glass interfaces are significantly smaller than on-air/glass interfaces (for more details on this, see Experiments 3-1 and 3-2). When measuring the baseline, one must remember that the solvent itself may absorb light independently of the chromophore dissolved in it (this is why a baseline measurement is necessary). Also, some samples containing larger particles like proteins may scatter light via Rayleigh scattering. In order to evaluate the true absorption by the chromophore, the measurement has to be corrected for light reflections, light scattering, and solvent absorption. This is typically done using matched (or the same) cuvettes filled with solvent only.

Special Cases of Measuring Absorption

On many occasions, we must deal with samples that would not be considered typical. A common case when working with modified and recombinant proteins is that the sample volume is limited. Frequently, we also may have to measure solid samples that have a limited size or atypical shape. A good example would be filters that are used for laser beam "cleaning" which can be as small as 4 mm in diameter. In this case, where the sample is small, we have to remember a few important factors such as beam diameter and beam position (how high, in relation to the cuvette bottom, the beam passes through the sample) to properly position the sample.

Measurements of Absorption of Low Volume Samples

When preparing samples of concentrated proteins, DNA, or other biomolecules we will be forced to measure absorptions using a very low volume of samples. In these cases, we will typically have less than 1 mL of total solution volume. Putting half a milliliter of the sample into a standard (1 cm × 1 cm) cuvette will give us a sample layer only 0.5 cm high, and due to the meniscus, the layer thickness in the center will be even less than 0.5 cm. For a typical spectrophotometer, direct measurements of the absorption of such a sample will be very difficult or impossible for the following reason. The light beam size (diameter) could be larger than 5 mm and part of the beam will miss the sample. A practical solution is using a smaller cuvette, for example, a cuvette that is 0.2 cm × 1 cm (as shown in Figure 2-1.1). A variety of such cuvettes are available today with different thicknesses ranging from 0.5 to 5 mm. Half of a milliliter of the sample in the cuvette that has a thickness of 2 mm will have a height of 2.5 cm, which would be very easy to measure in any spectrophotometer.

Another problem is when the small volume sample has low absorbance. To increase the measured absorption, we can use the 1 cm path of a 0.2 cm × 1 cm cuvette, but to do this, we have to consider the beam shape/diameter. In most spectrophotometers, the beam diameter (beam cross-section) is much larger than 2 mm, and using the 1 cm path will create significant problems. Typically, these problems can be solved by using a cuvette made for absorption measurements that has two black walls (not transparent to light) as shown in Figure 2-1.1c. One must measure the baseline with such a cuvette first, and it is always necessary to use identical (matched) cuvettes (or the same one, cleaned of course). Also, when using such absorption cuvettes, it is important to always place the cuvette in the holder in exactly the same way—a small displacement on one of the sides may strongly change the reading. This error is manifested by an artificially high or low (negative) absorption reading outside the absorption spectrum (wavelength where there is no absorption), as will be shown in Experiments 3-5 and 3-11. Of course, such black-walled cuvettes cannot be used for fluorescence measurements. The sample will need to be transferred to a different type of cuvette (like in Figure 2-1.1b) that does not sacrifice transparency on any of its walls. What happens if someone, trying to speed-up the experiment, will use this cuvette (2-1.1b) for the absorption? This will create a significant error in the measured absorption. Making a blank out of such a cuvette filled with a buffer will **not** protect against error and the absorption reading will be distorted (see Experiment 3-11). To understand this type of situation, one could consider a sample that has very high absorption. In this case, practically no light

should reach the detector, with absorption readings higher than 3 (limits of most typical spectrophotometers) corresponding to light intensity attenuation 10^3-fold or the transmission of $T = 0.1\%$. However, a significant part of the light traveling through the glass side of the cuvette will always reach the detector. If 50% of the beam is traveling through the glass and is not absorbed the apparent total beam attenuation detected by the spectrophotometer will be about 50% of what corresponds to absorption only 0.3, which is obviously incorrect. We can solve this problem by limiting the beam size below 2 mm or adopting a fluorescence cuvette for absorption measurements as shown in Experiment 3-12. For a quick fix, one may use a pinhole or vertical slit to limit the beam cross-section. In this case, a significant part of the light will be cut off, thus sacrificing the overall spectrophotometer sensitivity.

Measurement of Absorption of Small (Solid) Samples

There are a few examples where we will deal with small samples. One is a sample in the form of a small piece of glass or plastic. This for example could be an optical filter used to "clean" laser beams. Other small sample types are microstructures deposited on large glass supports. Some other common examples of small samples are cells deposited/grown on a microscopic slide, surface deposited proteins (as seen in an immunoassay), or dyes deposited on a small surface.

Typically, such surface depositions are not uniform and only a small fragment of the area is sufficiently uniform, usually less than 1–2 square mm. In such cases, limiting the beam size with a pinhole can be problematic since it may dramatically reduce the beam intensity; especially for array type spectrophotometers where the beam size could be 4–5 mm diameter. For such small samples, the spectrophotometer beam can be collimated/focused to a small spot. In Figure 2-3.2, we present the concept for focusing the beam to a small spot using two lenses and a pinhole. We and others used such a set-up for measuring absorption from plasmonics nanostructures (500 µm × 500 µm) deposited on glass surfaces. The first lens focuses the beam and the second re-focuses the beam to fit it into the spectrophotometer detection system. In this case, the beam intensity is not significantly attenuated and spectrophotometer sensitivity is not compromised, but the correct baseline and appropriate reference must be made with the lenses and the pinhole/slit in place.

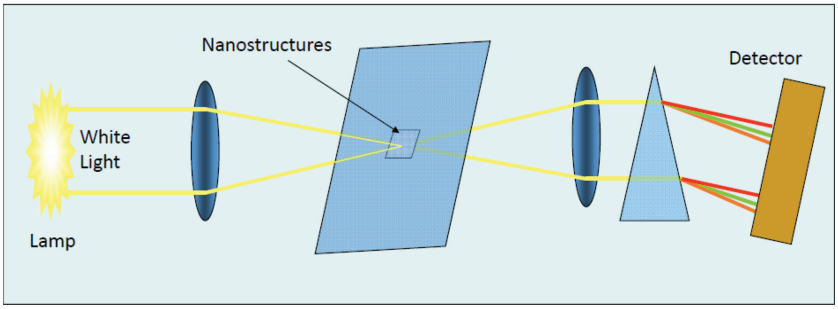


FIGURE 2-3.2 Concept for collimating/focusing the light beam to measure absorption from a small sample deposited on transparent background (glass).

2-4 FACTORS AFFECTING THE PRECISION OF ABSORPTION MEASUREMENT

There are many vendors of spectrophotometers and each vendor will typically offer a few different models. However, there are a few rules of thumb in place to help maximize precision. Typically, the highest precision is obtained for absorbance measurement, *Ab*, when in the range of 0.1–1.0. The theoretical minimum error of an absorbance measurement depends on the type of detector used. Detectors that are thermal noise-limited (i.e., phototube/photodiode) are the most precise for $Ab = 0.43$, while for detectors that are shot noise-limited (i.e., photomultiplier), maximum precision is achieved at $Ab = 0.86$. This corresponds to 2.7- and 7.2-fold light attenuation, respectively, for thermal noise and shot noise-limited detectors.

In typical experiments, the absorption may vary from 0.02 to 2 and can be measured with good precision. For small absorptions, where light attenuation is minimal, we are trying to extract a small difference from a high light intensity. For high absorptions, the attenuation is very strong and the intensity of light reaching the detector is very low, which will disturb the precision. In this case, the actual precision is limited by the random fluctuations of the detection system. Stray light, or light not from the spectrophotometer's light source, can also be a limiting factor in absorption measurements. Typical detectors are broadband detectors (detect light in a broad range like 200–900 nm) and respond to all light reaching them. For high concentrations, when the absorption is very high, the contribution of stray light can be significant, leading to the instrument reporting an incorrectly lower absorbance. It is a good practice to test the empirical detection limit of the spectrophotometer. This can be done by a calibrated increase/decrease of the sample concentration. When absorption is so high that it reaches a point where an increase in sample concentration will not result in linear (proportional) increase in the reported absorbance, the detector is not detecting enough light or/and is simply responding to stray light. In practice, the concentration of the sample or the optical path length must be adjusted to place the unknown absorbance within a range that is valid for the instrument. Most recent spectrophotometers use modulated/flashlight sources with a "lock-in" type detection mode and are not significantly affected by the stray light, but keeping a spectrophotometer in a dark place is a good practice that helps to limit stray light. Additionally, one must be aware that a computer monitor may be an unintended source of stray light perturbing absorbance measurements. This is a much more significant problem for fluorescence measurements where very weak light signals are detected.

Besides the above limitations, there are a few other factors that can decrease the accuracy and precision of absorption measurements. The most common are: (1) sample scattering, (2) optical beam distortion, (3) sample photostability, (4) defected or inadequate cuvette, and (5) sample fluorescence.

1. *Sample scattering.* Light scattering by the sample is due to the presence of particulate matter, emulsions, air bubbles, micelles, or large proteins that may cause scattering. Extensive sample scattering will result in the attenuation of the transmitted beam and the measured absorbance will be higher than expected. Typical scattering by small particles (Rayleigh scattering) strongly depends on the wavelength (λ^{-4}) and

quickly increases at shorter wavelengths. Knowledge of how much light is scattered will help determine a more accurate absorbance reading. This is a typical problem for measuring proteins solutions, cells, or even quantum dots. When the size of scattering particles is much smaller than the wavelength of light, the correction for Rayleigh scattering can be determined (calculated) directly from the measurement. In cases of larger objects like micelles or cells, the correction can only be made by measuring the proper baseline, which is in most cases very difficult or just impossible (Experiments 3-17 through 3-19).

2. *Optical beam distortion.* There are many factors that may contribute to beam distortion. A simple reflection from the cuvette walls depends on the angle of incidence. For an incident beam orthogonal to the cuvette walls, the reflection is minimal. As the angle of incidence increases (angle measured from the normal to the cuvette wall surface), the reflection increases. The reflection strongly depends on light polarization. Incidentally, a cuvette held not orthogonal to the beam during measurement will have different reflections than a properly positioned cuvette. In addition, a twisted cuvette will displace the beam in a way that may significantly distort intensity readings by the detector. See Experiments 3-1 and 3-2.

3. *Sample photostability.* To measure absorption it is necessary to expose the sample to the light of different wavelengths. This may lead to chromophore photodegradation. Byproducts of degradation may have different absorptions. This is a minor problem for absorption measurements, but it will be much more serious for fluorescence experiments. In general, the absorption/fluorescence measurements should be done with the smallest possible illumination.

4. *Defected or inadequate cuvette.* Using a cuvette that has two parallel flat walls is very important. Not parallel walls and/or the cuvette position not orthogonal to the light direction will result in light banding and redirecting the beam from its original trajectory. The other obvious problems are cracked or dirty cuvettes that are easy to see and should be avoided.

5. *Sample fluorescence.* Chromophores with high fluorescence quantum yields will emit strong fluorescence light. A small part of this fluorescence emission will reach the spectrophotometer detector, which may distort the absorption reading at the longer wavelengths. This is a minor problem, but sometimes may lead to surprising results, manifesting as negative or decreased absorption. This effect is detectable for spectrophotometers that illuminate the sample with white light (all wavelength) and the monochromator selects the wavelength later just before the detector. Typically, diode-array spectrophotometers separate the wavelength before the array detector.

2-5 EFFECT OF SAMPLE SCATTERING ON MEASURED TRANSMISSION AND ABSORBANCE

As we mentioned before, typical samples will have some residual scattering that in many cases can be neglected. However, working with biological samples (proteins, DNA, cells), we frequently will see significant scattering that may considerably alter results. A typical

suspension of cells will have high scattering that is manifested by whitish/yellowish apparent color of the suspension. Even working with protein solutions that visually appear perfectly clear in reality are not free of scattering. We need to remember that proteins are in fact particles, and may have (especially in the UV spectral region) significant scattering contributions to transmitted light attenuation. Such scattering is often difficult to measure or even to estimate.

Precise scattering measurements require specialized systems. In Figure 2-5.1, we present systems we have been using to monitor sample scattering. In this case, a solution is placed in the center of the sample holder in-between two hemi cylinders and the intensity of scattered light is collected to the fiber optics mounted on a movable arm. In many instances, scattering will be a directional phenomenon and it is necessary to measure the relative intensity of scattered light as a function of the direction of observation (in reference into the direction of incoming light) and polarization of incoming light.

For the purpose of our discussion of absorption and emission, we do not need to study the details of the special distribution of scattered light. In the case of absorption, it is important to realize what contributes to observed light attenuation—increase of apparent absorption. In this case, we need to realize that light that is scattered out will not reach the spectrophotometer detector, and will be counted as beam attenuation. For large objects (comparable to or larger than the wavelength of light) like very large protein aggregates, cells, etc., the effect is significant but has limited wavelength dependence in the optical range and will result in overall baseline increase. For objects much smaller than the wavelength of light, the effect is rather small but quickly increases toward shorter wavelengths. For particles smaller than 20–30 nm (practically all proteins), scattering should behave as Rayleigh scattering with the overall dependence as λ^{-4}. So, for a dye-labeled protein that

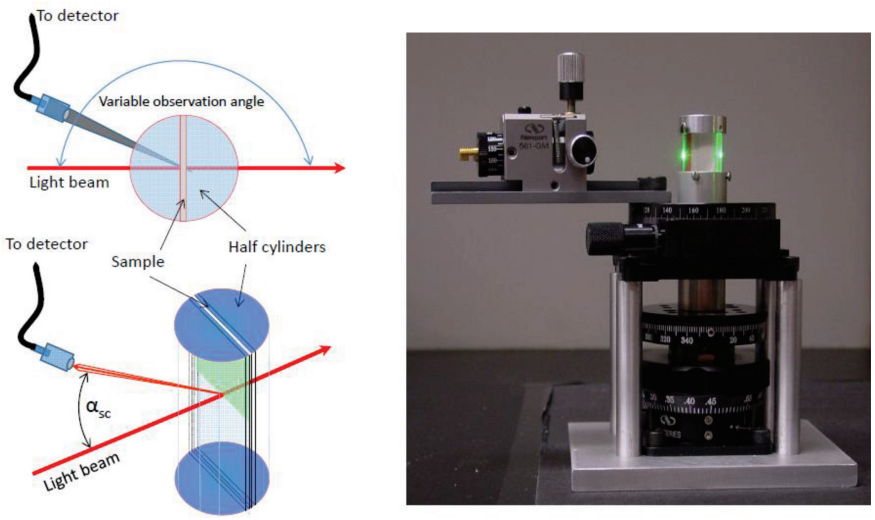


FIGURE 2-5.1 Design for the variable angle scattering detection. Left: concept for the design; Right: real photograph of the device.

absorbs above 400 nm, the effect can be negligible, but moving to a range near 300 nm, the scattering contribution becomes significant. See Experiments 3-17 through 3-19.

It is important to realize how scattering can affect absorption measurement. As an example, we will consider silica particles (called Ludox) that are 20–30 nm in size. Such particles are frequently used as scatterers (references) for fluorescence lifetime measurements. Silica has no significant absorption above 250 nm and when suspended in water it is visually clear. For high concentrations, a bluish opalescence can be seen. The Rayleigh scattering cross-section is given by Equation 1-5.1. We can approximate that the intensity of out-scattered light will depend on the dielectric constants of solvent and particles, particle size, and most importantly, wavelength. Simplifying the Rayleigh Equation 1-5.1, we can approximate scattered light intensity:

$$I_s(\lambda) \cong \frac{B}{\lambda^4} \tag{2-5.1}$$

where B substitutes for all constant parameters in Equation 1-5.1. The observed attenuated intensity due to sample scattering will be $I(\lambda) = I_0(\lambda) - I_s(\lambda)$. We can recalculate this to measured transmittance T and absorbance for thin samples as:

$$T(\lambda) = \frac{I_0(\lambda) - I_s(\lambda)}{I_0(\lambda)} 100\% \quad \text{or} \quad Ab(\lambda) = -\log \frac{I_0(\lambda) - I_s(\lambda)}{I_0(\lambda)} \tag{2-5.2}$$

Figure 2-5.2 shows the expected wavelength-dependent transmittances (solid lines) and absorbance (dashed lines) as a function of wavelength for two different values of B corresponding to 20 nm (thick lines) and 30 nm (thin lines) particle sizes. In Figure 2-5.2, we can see the characteristic behavior of measured transmittance or absorbance due to

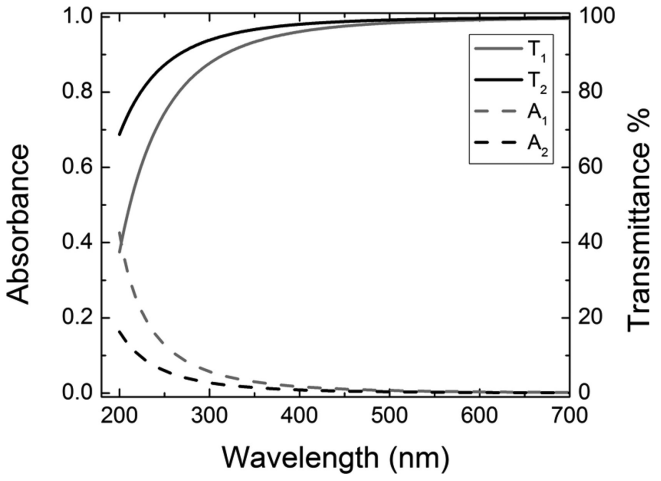


FIGURE 2-5.2 Calculated absorbance and transmittance of silica particles (Ludox) suspended in water. The thicker dark lines are calculated for 20 nm particles; the thinner light lines represent larger, 30 nm particles.

light scattering by small particles. It is important to stress that such sample behavior is only expected if the scattering particles are much smaller than the wavelength of light. In the case of particles absorbing in the UV spectral range, we can estimate scattering contribution by measuring absorbance/transmittance at longer wavelengths. A few examples are presented in Experiments 3-17 through 3-19, where we measure absorption spectra in the presence of small silica particles or absorbance of large proteins.

2-6 MEASURING EMISSION

In contrast to absorbance/transmittance measurements, measurements of emission are more complicated and frequently more prone to errors due to improper configurations and sample preparation. Figure 2-6.1 presents a schematic configuration of a typical instrument for fluorescence measurements—*spectrofluorometer.* This is a T-format instrument configuration where the emission excitation line and emission detection line form a 90° angle (so-called *square geometry*). There are two emission detection channels (so-called left and right detection lines), but in most common instruments only one detection line (left or right) is present. Single line detection configurations are called L-format configurations.

A full-spectrum light from the excitation lamp goes to the excitation monochromator (equipped with a diffraction grating) where an appropriate wavelength can be selected.

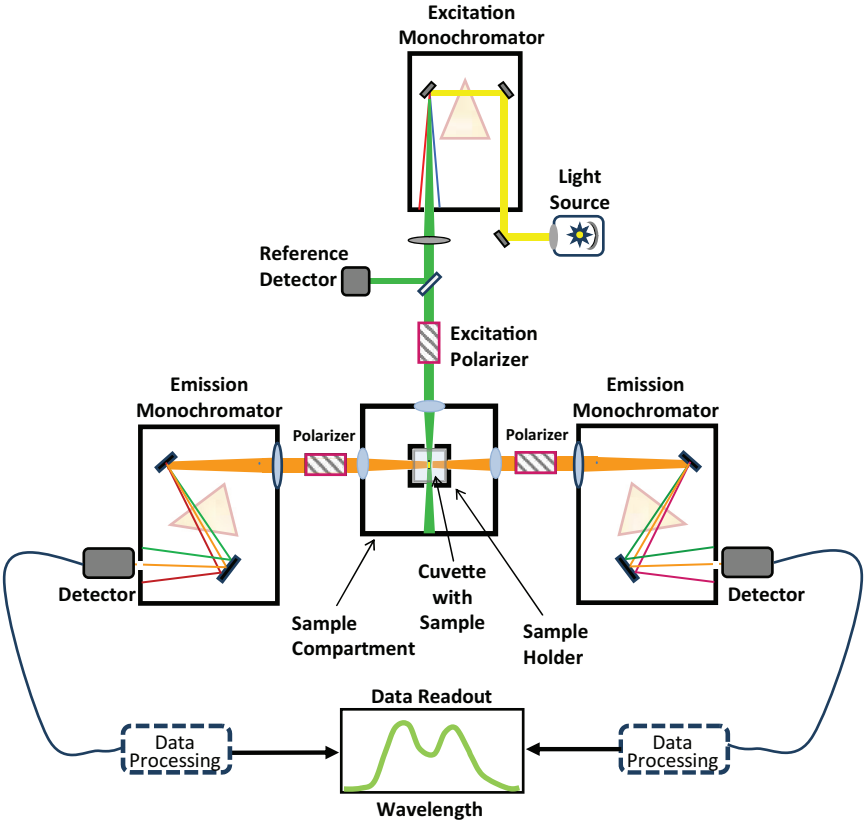


FIGURE 2-6.1 Schematic of a T-format spectrofluorometer (square geometry).

One needs to remember that selecting a given wavelength from a monochromator really means selecting a bandwidth of wavelengths at each step. Depending on the selected slits (or slit width settings), the bandwidth can typically vary from 1 nm to 20 nm. Next, the excitation beam passes through the polarizer and is focused on a sample. The fluorescence is collected through the lens, passes the emission polarizer, and is focused on a diffraction grating in the emission monochromator. The monochromator scans the selected wavelength range through the emission spectrum. Again, depending on the slit openings, a bandwidth of wavelengths is selected at each step. Most diffraction grating-based monochromators select the bandwidth in nanometers, which is *kept constant* through the entire scan. A prism-based monochromator may select the bandwidth in an energy scale (e.g. 500 kK) that is kept constant through the scan. The light from the monochromator is focused on the detector, typically a photomultiplier (PMT). The signal from the PMT is measured at each wavelength point and graphed as an emission spectrum by the data collection electronic system. The measurements are done by scanning the emission monochromator with a fixed excitation wavelength within the absorption of the sample (emission spectra collection). Another possibility is to scan wavelengths on an excitation monochromator with a fixed observation wavelength (within the fluorescence of the sample) on the emission monochromator (excitation spectra collection). Sometimes, we can also use a synchronous scan where both monochromators are scanned with a fixed wavelength separation (excitation at λ and emission at $\lambda + \Delta\lambda$). In Figure 2-6.2, we present

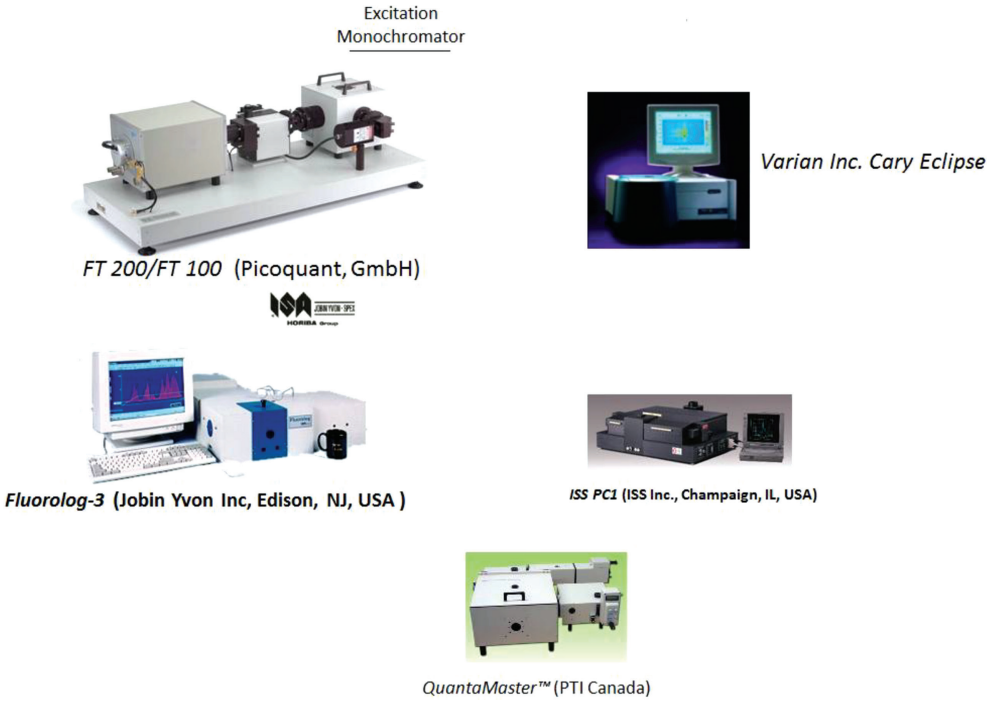


FIGURE 2-6.2 Photographs of spectrofluorometers that have been used by us. FT200/FT100 instruments use laser/LED excitation sources.

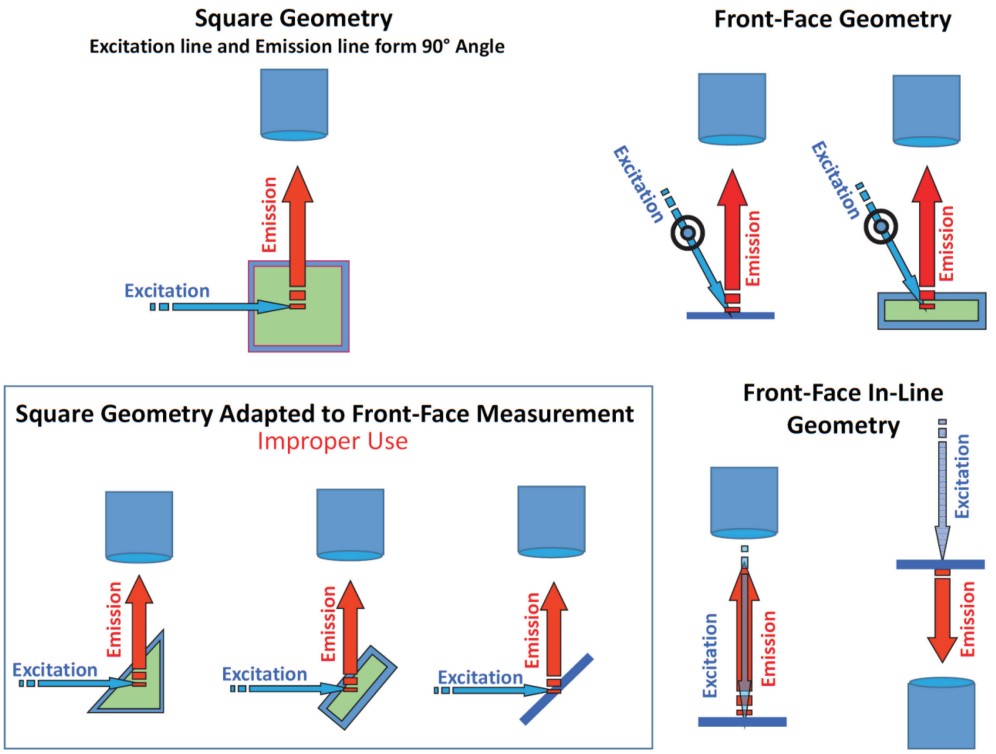


FIGURE 2-6.3 Most common emission geometries used for fluorescence measurements (in the box are improper geometries).

photographs of a few fluorescence instruments we have been using. These are only a few examples and many other instruments are on the market, but all of them fall to these few basic categories.

Fluorescence measurements can be done in many different configurations that are defined by relative orientations of excitation and emission lines as well as sample orientation. Figure 2-6.3 presents three commonly used configurations for fluorescence measurements. Most common is the *square geometry* configuration, where the excitation line and emission line form a 90° angle (Figure 2-6.3 (top)). In this case, the excitation beam passes through the sample while the emission is observed in the orthogonal direction. Since a free-space emission is equally distributed in all directions it does not matter which way, left or right, we measure the 90° angle. The main advantages of the 90° angle are: (1) an easy way to obtain the G-factor when measuring polarization; (2) contribution from the scattered excitation light is minimal. In addition, square cuvettes are typically used, and in the right-angle configuration, we do not have uneven perturbations due to reflections from the cuvette walls. If the walls of the cuvette form an angle other than 90°, reflections on the two interfaces (solvent/glass and glass/air) will strongly depend on light polarization and transmitted light will have not only lower-intensity but more importantly will have a distorted polarization.

It is important to keep a proper orientation of excitation polarization and observation polarization, to fulfill the so-called magic angle (MA) condition. A fluorescence signal

will depend on the dynamics of chromophores and the polarizer orientation can play an important role. The MA conditions will be discussed in detail in the "Introduction" of Chapter 4.

In some experiments, the researcher may need to work with highly concentrated solutions (in other words, solutions that have very high optical density—high absorption at the excitation wavelength) and using square geometry will not permit such measurements. The excitation beam will simply not penetrate the solution and the emission can only be observed from the surface. Also, sometimes the sample can be deposited on a surface and square geometry cannot be used even if absorption is very low. In some cases, the manufacturer will suggest the configuration as marked in Figure 2-6.3 (bottom-left) as a potential solution. This configuration uses a triangular cuvette or twisted thin 2 mm cuvette. The excitation happens only on the surface (excitation light does not penetrate the solution) and emission emerges only from the surface. This approach may give an approximate value for overall fluorescence response, but it is completely wrong for polarization measurements.

To understand why this is a problem, let us consider a schematic of a triangular cuvette in Figure 2-6.4. The excitation comes from the left and is vertically polarized (a light electric vector is perpendicular to the page surface). Light approaches the glass wall of the cuvette where it is partially reflected and the beam is refracted according to Snell's Law. Then, it enters the sample where it is again partially reflected and refracted. For marked light polarization (orthogonal to the plane), penetrating light changes the direction of propagation but does not change the light polarization. However, for light polarized in the figure plane, both direction of propagation and the direction of polarization will change. Excited sample emits fluorescence and emission needs to exit the cuvette. During this process, the light again encounters two interfaces (liquid-glass and glass-air) where it is

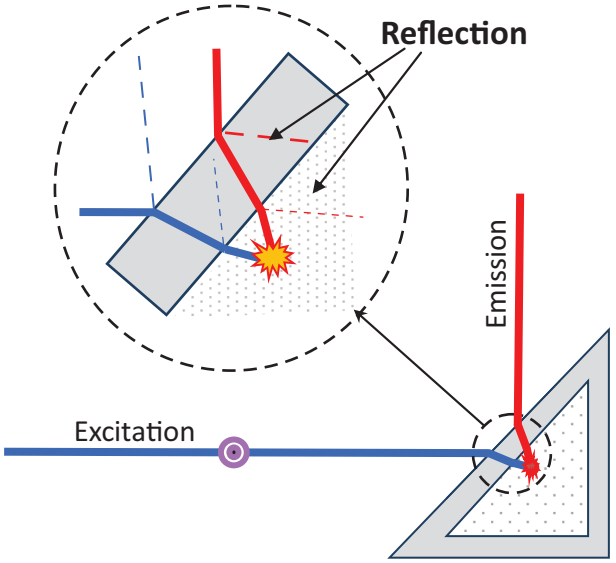


FIGURE 2-6.4 Reflections and refractions of the light on a tilted cuvette wall.

partially reflected and refracted (as marked in the inset of Figure 2-6.4). We will lose some intensity of the transmitted light, but more importantly, these two reflections strongly depend on the light polarization. Since the wall of the cuvette forms an angle of about 45° that is close to the Brewster angle for a glass–air interface, the polarization distortion of transmitted light can be very significant. Certainly, such a configuration should not be used for any polarization measurements and may also generate problems for lifetime measurements.

The proper front-face configuration is shown in Figure 2-6.3 (top-right) where the excitation line and emission line are in the plane orthogonal to the light polarization and form an angle smaller than 90°. *The important requirement is that the direction for emission observation is orthogonal to the front surface of the sample.* Just twisting the cuvette in the square geometry or using the triangle cuvette as shown at the top in Figure 2-6.3 for reasons mentioned above, may lead to significant errors. In a proper front-face configuration, the magic angle conditions are as in the square geometry (vertically polarized excitation and observation polarizer oriented at 54.7°). A significant limitation for a front-face configuration is the fact that we cannot evaluate the G-factor by just rotating the excitation polarization to horizontal.

The front-face configuration will typically not be supported by the instrument manufacturer, but sample compartments can be easily adopted for front-face measurements. In Figure 2-6.5, we present schematics of such an adaptor made for SLM and ISS sample compartments with the picture of the sample compartment. It is important to remember that in the front-face configuration we use only vertical excitation, with the sample surface orthogonal to the emission line. Only in such conditions, the polarization can be properly measured.

The third geometry that we present in Figure 2.6-3 (bottom) is the in-line geometry as used in microscopy and high-throughput screening (HTS). In-line is the front-face

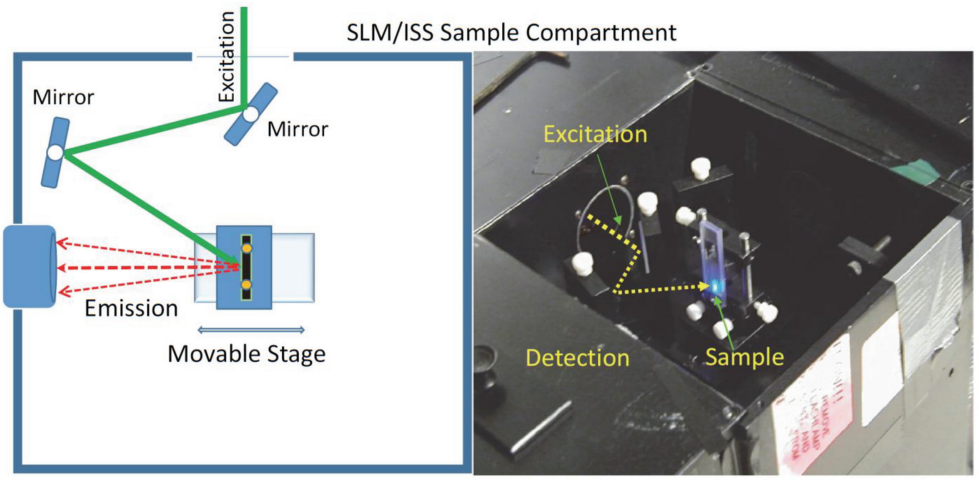


FIGURE 2-6.5 Front-face configuration: left: schematic representation; right: photograph of an open sample compartment with the front-face adapter.

geometry where the angle between excitation and emission is 0°. This geometry is proper for intensity and lifetime measurements (it is possible to achieve magic angle condition), however, this configuration requires special precautions in order to avoid a straight excitation light leak to the detector.

2-7 EFFICIENCY OF COLLECTING EMISSION: MEASURING QUANTUM YIELD

When comparing the emission of fluorophores in different solvents, we need to consider solvents refractive indexes. A typical observation system detects fluorescence intensity from one well-defined direction. The only light coming through a collecting lens will reach the detector. The amount of collected light depends on the solvent refraction index. As schematically presented in Figure 2-7.1, light exiting the cuvette undergoes refraction that affects the collection angle that the lens can see. This directly affects the detected number of photons and may distort measurements of quantum yield that rely on a direct comparison of two different samples that may have a different index of refraction. Below, we discuss how this influences quantum yield calculations.

Quantum Yield Calculation

A common example where we should be careful how to analyze the results can be calculating the quantum yield (QY) of unknown fluorophores using a known standard (reference). This is the most common way of evaluating the quantum yield for unknown dyes, and we will discuss how it should be done properly. Equation 1-3.16 gives a general definition for quantum yield as a ratio of emitted photons to absorbed photons by the sample or equivalently defines QY through the radiative and non-radiative rates. Using these definitions, there is no simple way to evaluate/calculate the quantum yield of an unknown fluorophore. The reason for that is that we will need to have a perfectly calibrated excitation lamp to know exactly the number of photons emitted by the lamp per second and a perfectly calibrated detection system that will be able to exactly evaluate the total number of emitted photons (photons emitted in all directions). For evaluating the total number of emitting photons, sometimes integrated spheres are used that does not always give satisfactory

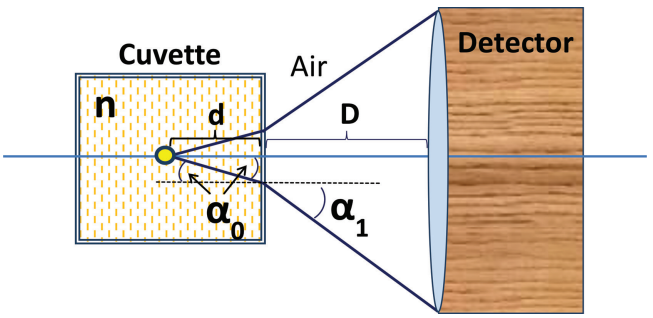


FIGURE 2-7.1 Refraction of light exiting a cuvette. At a higher refractive index, the light exits the cuvette at a bigger angle and less light is collected by the lens.

results. But the most convenient and efficient approach for quantum yield evaluations is to use a reference standard of known quantum yield. In general, emission of the fluorophore (number of emitted photons) is proportional to the number of absorbed photons and the fluorophore quantum yield. Photons are emitted in all directions (and only a small part reaches the detector), and additionally, photons are spread through the emission spectrum. Using a known standard/reference in exactly the same configuration as our unknown sample, we can safely assume that the portion of photons emitted toward the detector from our sample is the same. *Caution! Remember to use MA detection to avoid distortion due to sample polarization.* So, in principle, it is sufficient to measure absorptions for the reference and sample and then emission spectra in identical conditions (if the spectral range is different we need to properly recalculate the number of detected photons). The number of absorbed photons is equal to the number of excited molecules per unit of time and is proportional to the change in the intensity of light going through the sample. According to the Beer–Lambert law (1-3.7), the number of absorbed photons (excited molecules) of the standard and sample will be:

$$N_R \sim \Delta I_R = I_0\left(1-10^{-OD_R}\right) \text{ and for sample } N_S \sim \Delta I_S = I_0\left(1-10^{-OD_S}\right) \tag{2-7.1}$$

where OD_R and OD_S are optical densities (absorbances) for the reference and sample, respectively. The number of emitted photons for the reference and sample will be:

$$N_R^{Ph} = N_R\, QY_R \quad \text{and} \quad N_S^{Ph} = N_S\, QY_S \tag{2-7.2}$$

A typical system does not collect all the photons, but for the same detection configuration (same system and the same/identical cuvette), the fractions of collected photons for the reference and sample should be identical if the samples have the same refractive indexes. As it is relatively easy to keep the detection geometry constant, quite frequently our sample and reference can be in different solvents that may have different refractive indices. To discuss how this may affect our signal and how to correct for this effect, let us consider the configuration presented in Figure 2-7.1. The reference is in the solution that has a refractive index, n_R, and the sample refractive index n_S. The emitting point in the center of the cuvette is located at a distance d from the cuvette wall and emits uniformly in all directions. The detector is at a distance D ($D>>d$) from the cuvette. For a fixed geometry, the system will always see the light entering into the detector direction (collecting lens) under the angle α_1. Due to refraction, this will correspond to the angle α_0 that will depend on the refractive index, n, of the solvent in the cuvette. Only the light emitted from a point source into the cubical angle α_0 will reach the detector. At any given distance, the intensity I is distributed on a surface $4\pi d^2$ and the portion of energy flowing to the detector is proportional to the surface area covered by the angle α_0, $s = \pi r^2 = \pi(d^*\sin\alpha_0)^2$, and using the notation as in Figure 2-7.1 for sample and reference, we will get:

$$S_0^R = \pi D^2 \mathrm{tg}^2 \alpha_0^R \quad \text{and} \quad S_0^S = \pi D^2 \mathrm{tg}^2 \alpha_0^S \tag{2-7.3}$$

We assumed $D \approx D + d$. Snell's law lets us calculate angles for reference $\sin\alpha_0^{\mathrm{R}} = n_{\mathrm{R}} \sin\alpha_1$ and for a sample $\sin\alpha_0^{\mathrm{S}} = n_{\mathrm{S}} \sin\alpha_1$. We can now write a number of photons emitted toward the detector at a given wavenumber (wavelength) by reference and sample respectively:

$$N_{\mathrm{R}}^{\mathrm{Ph}}(\nu) = \frac{N_{\mathrm{R}}\, QY_{\mathrm{R}}}{4\pi R^2} \pi D^2 n_{\mathrm{R}}^2 \mathrm{tg}^2\alpha_1 \quad \text{and} \quad N_{\mathrm{S}}^{\mathrm{Ph}}(\nu) = N_{\mathrm{S}}\, QY_{\mathrm{S}} \pi D^2 n_{\mathrm{S}}^2 \mathrm{tg}^2\alpha_1 \tag{2-7.4}$$

We can use the wavelength or wavenumber scale to calculate the total number of photons (energy) emitted by the sample. The total number of emitted photons is proportional to the surface area under the emission spectrum. We can enter it to Equation 2-7.4 and by comparing a signal measured for reference and sample we can calculate the quantum yield of the sample:

$$QY_{\mathrm{S}} = QY_{\mathrm{R}} \frac{\left(1 - 10^{-\mathrm{OD_R}}\right) n_{\mathrm{S}}^2 \int F_{\mathrm{S}}(\nu) d\nu}{\left(1 - 10^{-\mathrm{OD_S}}\right) n_{\mathrm{R}}^2 \int F_{\mathrm{R}}(\nu) d\nu} \tag{2-7.5}$$

where $F_{\mathrm{S}}(\nu)$ and $F_{\mathrm{R}}(\nu)$ are integrated intensities (areas under the emission spectra profile) for sample and reference respectively. The spectrum profile will generally include a detector sensitivity factor (correction for different sensitivity to a different wavelength—later discussed in "Measuring Emission Using Different Detectors—Detector Sensitivity"). Equation 2-7.5 lets us calculate the quantum yield of a sample if we know the quantum yield of reference. In this general representation, we do not have to worry about differences in sample and reference absorptions.

For most recent monochromators (with gratings as dispersing elements), the linear step will be in wavelength and we will use the wavelength version of Equation 2-7.5:

$$QY_{\mathrm{S}} = QY_{\mathrm{R}} \frac{\left(1 - 10^{-\mathrm{OD_R}}\right) n_{\mathrm{S}}^2 \int F_{\mathrm{S}}(\lambda) d\lambda}{\left(1 - 10^{-\mathrm{OD_S}}\right) n_{\mathrm{R}}^2 \int F_{\mathrm{R}}(\lambda) d\lambda} \tag{2-7.6}$$

We need to remember that when converting spectra between wavelength and wavenumber we need to use the proper conversion.

How to Convert from Wavenumbers to Wavelengths?

Let assume that we measure the intensity of $F(\lambda)$ with the bandpass of $\Delta\lambda$. The signal will be proportional to the number of photons in the $\lambda - \Delta\lambda$ wavelength range, $F(\lambda)\Delta\lambda$. In the wavenumber representation, this will be $F(\nu)\Delta\nu = F(\nu)\left(1/\lambda - 1/\lambda + \Delta\lambda\right)$. Of course, both representations must provide the same number of photons, therefore $F(\lambda) = F(\nu)/\lambda(\lambda + \Delta\lambda)$. For $\Delta\lambda$ much smaller than λ, this dependence can be written as $F(\lambda)\lambda^2 = F(\nu)$.

Sometimes to evaluate QY, a simplified version of the Equation 2-7.6 is used:

$$QY_S = QY_R \frac{OD_R n_S^2 \int F_S(\lambda) d\lambda}{OD_S n_R^2 \int F_R(\lambda) d\lambda} \tag{2-7.7}$$

A significant error can be made when the absorption of the sample and reference are a different and even larger error when the emission spectra of reference and sample are in different spectral ranges and not using the proper conversion. We will briefly analyze how big of an error it can be made when the simplified equation is used.

Absorption

Generally, we would recommend the absorption for a sample and reference to be low (below 0.1) and they should be adjusted (if possible) to be as close as possible. This is not always possible, so we would like to know how much error we can make by using the simplified Equation 2-7.7. The variation will be in the difference of an exact ratio $R_e = (1-10^{-OD_R})/(1-10^{-OD_S})$ versus approximate ratio $R_a = OD_R/OD_S$. For discussion, we will define variance in both values as:

$$V = \frac{R_e - R_a}{R_e} \tag{2-7.8}$$

In Figure 2-7.2, we are plotting the dependence for variance V as a function of OD_R for a few values of OD_S. For graphing purposes, we consider the absorption range from 0 to 0.5. We do not recommend measurements when absorptions are greater than 0.1 due to significant excitation beam attenuation and limited sample penetration that may alter the geometry. The deviation (difference between exact and approximate value Equation 2-7.6)

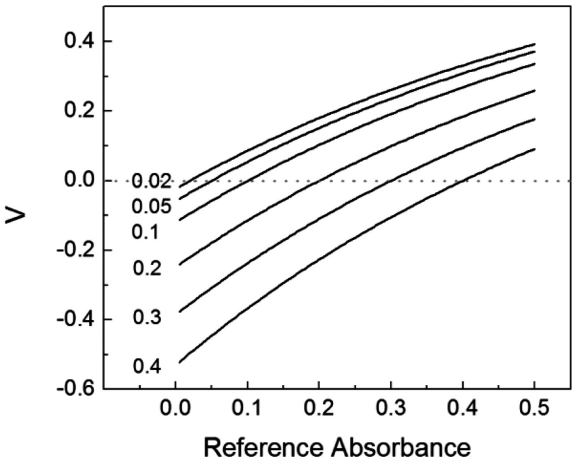


FIGURE 2-7.2 Dependence for variance in experimental error as a function of reference absorption.

depends on the absorption difference between the sample and reference in an almost linear fashion (curves in Figure 2-7.2 are almost parallel). So, if the differences in absorption between the sample and reference are less than 0.1 the expected error is below 0.1 (10%). For example, when sample absorption is 0.2 and reference absorption is 0.3 the error will be within 0.1 (10%). Obviously, for absorptions below 0.1, the difference between the sample and reference is much smaller than 0.1 and the error will be well below 10%. But still, for the absorption of reference 0.1 and sample 0.05, we will make 5% error.

Area under the Emission Spectra

As we discussed earlier, to calculate quantum yield we need to evaluate the area under the emission curves (total number of photons) for reference and the sample, and the integration can be done for the wavelength or wavenumber scale $\left(\int F(\nu)d\nu \text{ or} \int F(\lambda)d\lambda\right)$. Depending on the type of the used monochromator, the proper integration will be with wavelength or wavenumber. Since most, if not all, modern spectrofluorometers use gratings, we will use wavelength representation. In principle, if a proper conversion between wavelength and wavenumber has been used ($1/\lambda^2$), the result will be correct. In many cases, the emission spectrum of the reference and sample are shifted, sometimes significantly and we should remember about the proper conversion. Let us consider a simple example of two emission bands as shown in Figure 2.7-3. One centered at 25 kK (400 nm) and the other at 20 kK (500 nm) with a width of 3 kK (note: 1 kK = 1000 cm^{-1}, K stands for Kajzer). As a 5 kK (100 nm) shift may sound large, just looking on spectra in the wavelength scale

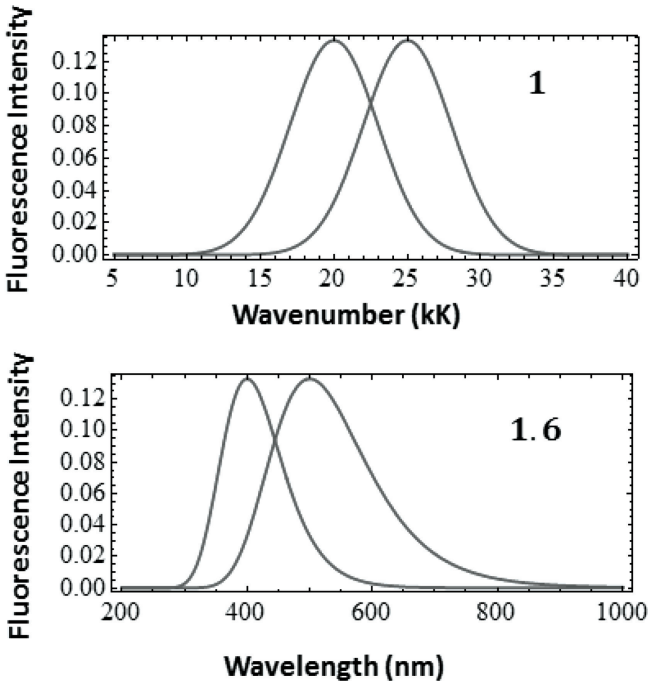


FIGURE 2-7.3 Two emission spectra presented in a wavenumber scale (top) and wavelength scale (bottom).

in Figure 2-7.3, one would realize it is not that uncommon. An evaluation of the area under the emission curve using the wavenumber scale gives the same values for the reference and sample, and the ratio between areas of the sample and reference is 1. However, measuring the same spectra in the wavelength scale and evaluating areas in the wavelength scale (**no** $1/\lambda^2$ correction factor) the areas are very different, giving the ratio between the area of sample and reference of 1.6. This means a colossal error in evaluating the quantum yield of 60%. It is clear that even a small spectral shift between the reference and sample may lead to a significant mistake in quantum yield evaluation. Obviously, when integrating the area under the curves when using $1/\lambda^2$ correction factor the ratio is 1, as it should be.

There are more examples where the surface area under the spectrum is used and improper integration may result in significant mistakes. To get a perspective of how large the effect is, let us consider a symmetrical bell-shaped spectra of fixed width in the energy scale (wavenumber) with precisely defined peaks as presented in Table 2-7.1. All bell-shaped spectra have identical shapes (width and peak intensity) and they are shifted by multiples of 5 kK (kilo Kaisers). These spectra are illustrated in Figure 2-7.4 in wavenumber (top) and wavelength (bottom). In the wavenumber, they are all equally spaced and the area under the peaks are equal. In the wavelength representation, the peaks are not evenly spaced and the area under each curve grows as it moves to longer wavelengths. Furthermore, the order of peaks is reversed when plotted in wavenumber/wavelength; the peak with the lowest wavenumber has the longest wavelength.

Thus, in the wavenumber representation, it may be concluded that all peaks represent emissions of the same intensity (area under the curve), that they are symmetric, and that they are equally spaced. The above conclusions would not be reached using the wavelength representation.

The top and bottom spectra of Figure 2-7.4 present exactly the same data, computer-generated normal distributions as shown in Table 2-7.1. They look quite different because the independent axis of each plot is an inverse transformation of the other as shown in Table 2-7.1. In reality, when generating the wavelength data, each point (x, y) was

TABLE 2-7.1 Sample Peaks and Some Properties Relative to the First Peak Computed in Wavenumber and Wavelength

Peak (kK)	Ratio of Area under Peak to Area under First Peak in Wavenumber	Ratio of Area under Peak to Area under First Peak in Wavelength	Shift from First Peak (kK)	Shift from First Peak (nm)
$\frac{1}{2\sqrt{2\pi}} e^{-\frac{(\lambda-18)^2}{2*2^2}}$	1	1	0	0
$\frac{1}{2\sqrt{2\pi}} e^{-\frac{(\lambda-22)^2}{2*2^2}}$	1	1.5	4	−101
$\frac{1}{2\sqrt{2\pi}} e^{-\frac{(\lambda-26)^2}{2*2^2}}$	1	2.1	8	−170.9
$\frac{1}{2\sqrt{2\pi}} e^{-\frac{(\lambda-30)^2}{2*2^2}}$	1	2.8	12	−222.2
$\frac{1}{2\sqrt{2\pi}} e^{-\frac{(\lambda-34)^2}{2*2^2}}$	1	3.7	16	−261.4

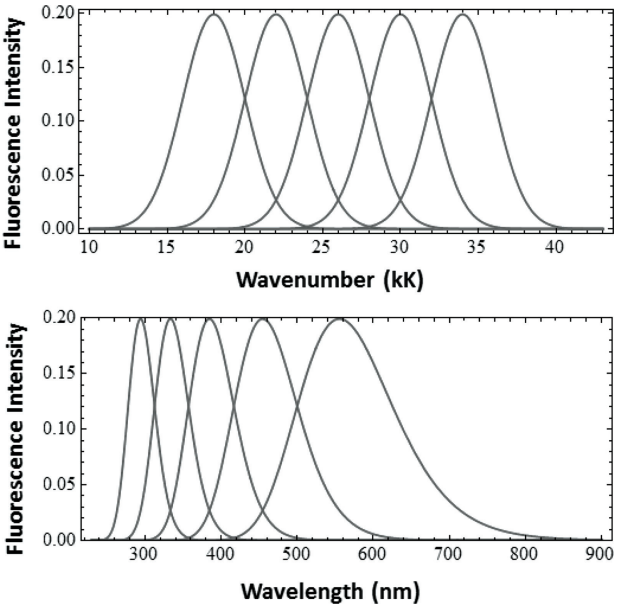


FIGURE 2-7.4 Sample peaks presented in wavenumber (top) and wavelength (bottom).

transformed to (10,000/x, y). Notice that the dependent axis value (intensity) is not transformed at all. Surface areas under each curve in Figure 2-7.4 (top) are identical, but comparing the surface area under the first curve (short wavelength) in Figure 2-7.4 (bottom) to the last one (long wavelength), we can see that the change is over 3-fold (over 300%).

We presented this example as a warning on how big errors can be made when improperly converting from wavenumber to wavelength representation.

2-8 WAVELENGTH CALIBRATION

A spectrum (transmission or emission) is an intensity reading as a function of wavelength. We typically consider error associated with the measured intensity, but we should be aware of possible errors in the wavelength reading. Most error analysis will not consider error for an independent variable and it is assumed that this error is statistically negligible. However, even if statistical error for reading each particular wavelength is very small it is possible (quite frequent in fact) that systematic error could be a big problem. Typically mechanical use of the monochromator or the entire system may lead to small mirror or diffraction grading displacement that shifts all wavelength reading toward smaller or higher values. In the case of wavelength, this is a problem of monochromator calibration. Monochromators are used to separate a beam of light into its wavelength components using refraction (prism) or diffraction (grating). The detector then performs a reading on each separate wavelength. Most monochromators use movable diffraction gratings to separate different wavelengths of light. We often rely on the manufacturer for service to calibrate the instrument and almost always such calibrations are reliable. However, an instrument may lose calibration over time or due to inadvertent misuse (e.g., sending the monochromator to an out of range wavelength). In some gratings, this may happen when the stepping motor slips or somebody

selects an impossible to reach wavelength (i.e., −1 nm or 100,000 nm). A slight change in entering beam alignment may cause a prism-based monochromator to lose calibration.

There are a few methods that can be used to check the calibration. All of these methods rely on spectral features that are known to the experimentalist. Most frequently today, we depend on widely available lasers or other discreet line sources such as mercury, sodium, or xenon lamps. Laser light is typically monochromatic and has a well-defined wavelength. Widely available laser pointers are good sources of monochromatic light at 405 nm (violet laser diode), 532 nm (green laser diode), and 635 nm (red laser diode). Using these laser diodes, we can easily calibrate the monochromator in the visible spectral range. But we encourage the experimentalist to verify independently that the nominal wavelength specified for the laser is correct. Some more sophisticated spectroscopy labs will also have lasers with many other wavelengths, such as 224 nm (HeAg), and 325 nm (HeCd).

However, there are two problems with this. As it is easy to calibrate a monochromator externally, typically it is not easy to incorporate a laser beam into a spectrophotometer. The best way of doing this is to use scattering in the place of a sample. Second, UV lasers are not always easily accessible. In this case, we recommend using known spectral features of some fluorescent compounds. For example, benzene is a common solvent available in most chemistry labs. A drop of benzene closed in a quartz cuvette evaporates quickly, and gaseous benzene has very well defined and structured absorption/emission spectra that can be very easily used for calibration. It may be a surprise to some less-experienced researchers, but the absorption and emission of such vapors are quite significant. In Experiment 3-14, we present a simple routine to perform calibrations of the spectrophotometer and spectrofluorometer using this approach.

A small calibration error may be readily fixed with a single wavelength. However, when the calibration is off by a large amount in an unknown direction, a single wavelength is not enough. Oftentimes line sources such as lamps, which have multiple distinct emission lines, are used. Identifying these lines is most often done by finding the distance between them or their relative heights. Figure 2-8.1 shows the spectra of two commonly used lamps with mercury and xenon sources.

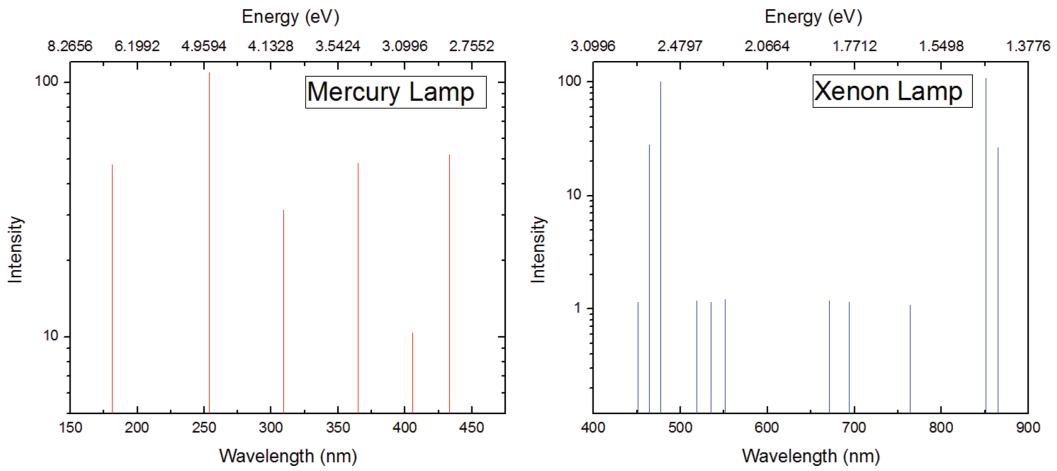


FIGURE 2-8.1 Spectra of lamps used for alignment.

Comparing the wavelength scales (bottom) and energy scales (top), it can be noticed that if the separation of two spectral lines in wavelength is 10 nm, and their separation in energy will differ based on the location of the two spectral lines. Thus, if the wavelength scale is shifted by 20 nm the distances between these spectral lines will be the same in wavelength but not in energy. The energy-to-wavelength conversion can also be calculated as $\mathcal{E} = 1239.8/\lambda$. Often while calibrating an instrument, the operational software asks for three-wavelength lines. These lamps provide more than enough peaks of known location. If the calibration is correct, the spectral lines plotted in an evenly spaced wavelength and evenly spaced energy will be mirror images of one other.

2-9 INSTRUMENT RESPONSE

Real instruments have photodetectors that have different sensitivities to different wavelengths; they have light sources that emit different intensities at different wavelengths, and they have mirrors that have different reflectivity at different wavelengths. A spectrophotometer or spectrofluorometer corrects for these differences in its software when they are set to do so (usually by default). However not all data acquisition modes do these corrections automatically, and the internal calibration has to be redone. As an example, Figure 2-9.1 presents two typical relative response functions of two different photodiodes. The dependent axis has been normalized and shows how much less sensitive the detector is at different wavelengths. The 1 on the scale corresponds to the maximum efficiency of the detector which is usually around or less than 80%, or it will detect four (or less) out of five photons. It is common for a certain detector to be five times as sensitive to blue light than to red light. Thus, the measured intensity of 500 a.u. in blue wavelengths and 100 a.u. in red wavelength would mean that there were the same amount blue and red wavelength photons emitted/detected. This conversion is typically done in the detector unit itself and data corrected for the relative response is a readout. Alternatively, software from the detector manufacturer may correct the data in the time of measurement.

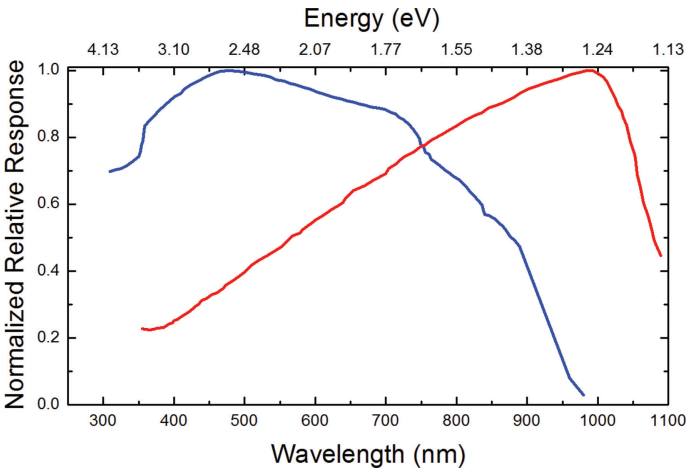


FIGURE 2-9.1 Relative responses of two photodiodes.

The intensities of excitation lamps per wavelength are just as varied as the detector sensitivity/response. When measuring excitation spectra, some instruments may automatically correct for the excitation light intensity. However, we recommend regularly checking such calibrations since the lamp profile typically changes with time. Such procedures will be discussed in Experiment 5-4.

2-10 LAMP PROFILE

Most instruments use various types of lamps (light sources) to generate light that can be used for measuring absorbance/transmittance or for excitation in fluorescence experiments. In the case of absorption that is a relative measurement in light intensity change, the only limiting factor is sufficient light intensity. So, there is no need to calibrate a lamp since we are always measuring the baseline. Also, there is no universal lamp that will cover the entire spectral range with sufficiently high light intensity and will have constant or known intensity. The spectrophotometers use two lamps, one to cover UV (usually a deuterium lamp) and one to cover the visible spectral range (usually a halogen lamp). The instrument will automatically use and change appropriately the lamp for the requested range.

A bigger problem is the emission measurement because the fluorescence signal directly depends on the intensity of the excitation lamp, and in addition, all lamps will have different spectral profiles. Also, the apparent lamp profile may depend on the monochromators and optics being used. A xenon lamp has good intensity in UV but in many lamp constructions, the glass used for the lamp production limits the UV range to only ~300 nm. One additional problem is the fact that the lamp profile depends on many factors, like sufficient warming time for the lamp and age of the lamp. In this case, comparing fluorescence signals of two chromophores with different excitation wavelengths can be a real challenge, especially when measuring and calibrating the lamp profile. We will discuss some examples of determining the lamp profile in the experimental part.

2-11 PEAKS AND SPECTRAL SHAPES

Typically observed absorption and emission spectra are a composition of multiple peaks. A peak is a normal distribution or a spectral profile (envelope). Often, what is called a peak is really a sum of a few normal distributions. In this section, some basic ideas of recognizing peaks are presented. Most commonly assumed spectral profile for a single peak is a Gaussian distribution. Since absorption and fluorescence spectra represent distribution in an energy scale, to properly represent the Gaussian profile we should use an energy-equivalent scale (eV or wavenumber, ν). The peak can be represented with a function P, given by:

$$P(\nu) = \frac{A_0}{\sigma\sqrt{2\pi}} e^{-\frac{(\nu-\nu_0)^2}{2\sigma^2}} \tag{2-11.1}$$