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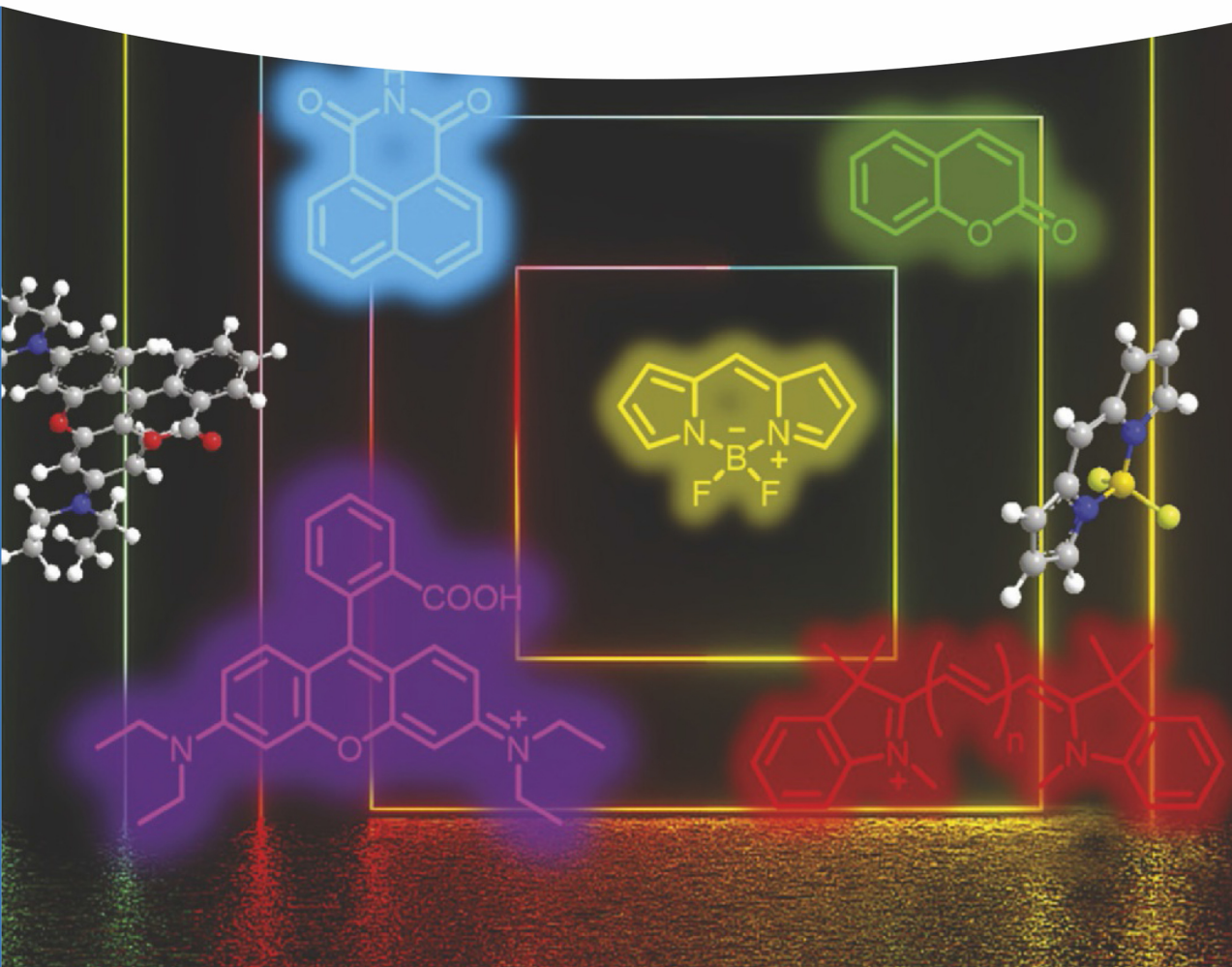
# Fluorescent Probes for Bioactive Species

## Design, Synthesis, and Biomedical Applications



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# Preface

As a bridge between chemistry and cell biology, as well as life science, fluorescent probes are driving the innovations in analytical testing techniques and biological imaging technologies at an unprecedented speed. From the discovery of fluorescence by Stokes in the 19th century to the breakthrough of super-resolution fluorescence microscopy in the 21st century, fluorescent probes have evolved from simple signal tools to "intelligent eyes" for real-time, dynamic, and in situ analysis of the behavior of biologically active species. This book *Fluorescent Probes for Bioactive Species: Design, Synthesis, and Biomedical Applications* focuses on this cutting-edge field, systematically reviewing the design principles, mechanisms of action, and imaging applications of fluorescent probes, and provides a panoramic perspective from basic theory to application practice for interdisciplinary researchers.

Bioactive species (such as reactive oxygen species, reactive sulfur species, reactive nitrogen species, etc.) play crucial roles in cell signal transduction and disease development. However, their short half-life, low concentration, high activity, low content, as well as the heterogeneity and complexity of the cellular microenvironment impose strict requirements on the detection technology. Fluorescent probes have become the core means to solve this problem due to their advantages of high spatial and temporal resolution, real-time dynamic monitoring, and in situ imaging capabilities. Based on the interdisciplinary nature of fluorescent probes in chemistry, biology, and medicine, this book not only presents the physical and chemical basis of fluorescence theory (such as Stokes shift and quantum yield, etc.), but also analyzes the recognition mechanism of probes to biological targets (such as covalent bond cleavage and electron transfer, etc.), and also summarizes the application of these probes in solving specific biological and medical problems. The book is compiled based on the "theory-design-application" model.

The basic theory (Chapters 1 and 2) deeply analyzes the chemical principles of fluorescent molecular recognition, including excitation mode (single-photon/two-photon/upconversion, etc.), fluorescence reporting mechanism (turn on type/ratiometric type/lifetime type, etc.), and optical modulation principle (PeT/ICT/FRET, etc.), which lays a physical and chemical foundation for probe design.

The section of design and application of probes (Chapters 3–10) is divided into chapters according to the category of bioactive species, from reactive oxygen species, reactive sulfur species, reactive nitrogen species, reactive carbon species, oxidoreductase enzymes, neurotransmitters, and others.

At present, the field of fluorescent probes is developing in the direction of "high selectivity, deep tissue penetration, and intelligent response." The latest research included in this book (such as near-infrared probes and organelle-targeting design) not only shows technological breakthroughs, but also provides insights into possible future development directions, such as optimizing the probes from the perspective of molecular engineering, combining computational chemistry to predict the active sites, integrating them into the practical needs of biomedical scenarios, and so on. Whether it is to analyze the signal pathway in basic research or to capture early biomarkers in clinical diagnosis, the ideas and cases provided in this book have forward-looking guiding significance.

This book can be used as a reference for teachers and students majoring in chemistry, biology, and medicine, and also a technical manual for researchers. The systematic theoretical framework and rich application examples in the book help more researchers to explore and innovate in the interdisciplinary field of fluorescent probes and bioactive species, and bring new breakthroughs to life science research and disease diagnosis, and treatment.

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China

## Acknowledgment

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# 1

# Fluorescence and Molecular Recognition

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During the past decade, there has been a remarkable growth in the use of fluorescence for molecular recognition. A general concept of this method was called "fluorescent sensor or probe," which could identify molecules with a concomitant fluorescence signal. Nowadays, such fluorescent sensors have been widely used in the fields of *in vivo* imaging, surgical navigation, immunochemistry, clinical diagnosis, environmental analysis, criminal investigation, food safety, and other fields because of their simplicity, high selectivity, and sensitivity in fluorescent assays. Recognition at the molecular level is a fundamental characteristic to glean insights into the processes of biology and chemistry. In this chapter, we present the principles of fluorescence and molecular recognition, highlight recent work that uses these methods, and discuss the applications and future directions as they apply to molecular recognition.

## 1.1 Advancing Fluorescence Theory

### 1.1.1 The Discovery of Fluorescence

For many years, fluorescence has been an intriguing scientific technique that enables researchers to examine minute aspects of live organisms by revealing hidden components through brilliant hues. Understanding life beneath the microscope has been made easier by the discovery of fluorescence. In the Florentine Codex, Franciscan missionary Bernardino de Sahagún (1499–1590) recorded the use of a wood called "coatli" that had medicinal qualities and could alter the color of water. Because the water looked bluish in the sun, they utilized this wood to make drinking cups that assisted individuals with urinary issues [1]. In 1565, Spanish scientist

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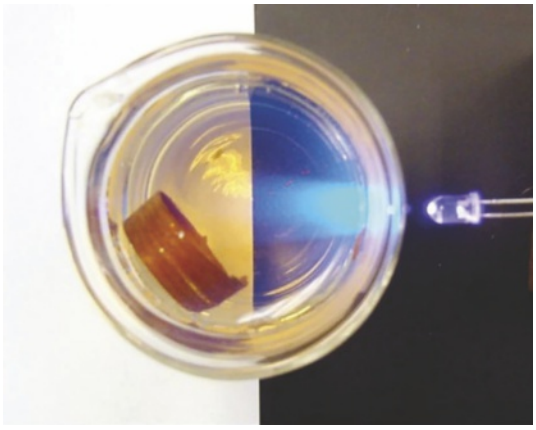

**Figure 1.1** The blue fluorescence of the *Lignum nephriticum* in water. *Source*: Mark Muyskens et al. (2006)/American Chemical Society.

Nicolás Monardes wrote in "Historia medicinal de las cosas que se traen de nuestras Indias Occidentales" of the blue-tinted, sparkling look of water when mixed with the Mexican plant *Lignum nephriticum* [2]. Monardes' paper, which highlighted the special optical qualities of kidney wood, *Lignum Nephriticum*, was translated into Latin in 1574 by the Flemish botanist Charles de L'Écluse (Figure 1.1). When Vincenzo Casciarolo from Bologna burned a stone he named "lapis solaris" in 1603, it turned out to be barium sulfate, which gave off purple-blue light [3]. The Bolognian stone's ability to emit light was identified by Galileo Galilei in 1612 and was subsequently given the name phosphorescence [4, 5].

In 1646, German Jesuit priest Athanasius Kircher published "Ars Magna Luciset Umbrae" (translation: The Grand Mastery of Brightness and Gloom), which explains that light passing through a wood infusion appears yellow, but when reflected, it appears blue. Robert Boyle extended Monarde's research in 1670 and found that some salts caused wood to lose its capacity to alter the color of water after repeated applications. Additionally, he found that adding vinegar eliminated the tint, while potassium carbonate restored it. Boyle was the first to employ fluorescence as a measure of pH [6].

Scottish scientist David Brewster made the first observation of fluorescence in concentrated solutions in 1833, when he found chlorophyll fluorescence, which happens when sunlight is absorbed by a combination of alcohol and leaves, turning the light orange, yellow, and greenish [7].

In 1842, Edmond Becquerel made the remarkable discovery that calcium sulfate emitted ultraviolet light with a wavelength greater than the light it absorbed [8]. In 1858, he developed the first phosphoroscope, which measured the duration of phosphorescence [9].

This phenomenon was understood through research on quinine. Once thought to be a powerful medication for treating infectious disorders like malaria, quinine is an alkaloid that is extracted from Cinchona plants in South America. In 1845, Sir John Frederick William Herschel discovered the luminescence in a quinine solution and coined this effect as "epipolic dispersion" [10].

He reported a distinct blue hue in two articles that were published in the *Journal of the Royal Society of London*. At that time, scientific understanding was insufficient to accurately identify this color. Though fully transparent and colorless when placed between the eye and a light source, the blue color was seen under specific lighting circumstances and angles.

In his 1852 work "On the Change of Refrangibility of Light," George Gabriel Stokes first proposed the idea of "dispersive reflection" in the sunlight spectra [11]. He proved that fluorescence only happens when UV light is reflected onto a quinine solution, causing a Stokes shift – longer wavelengths of light being emitted than absorbed.

After proposing the use of fluorescence for investigation in 1864, Gabriel Stokes was known as the "Father of Fluorescence" [12]. In 1875, Eugen Lommel proposed the notion that fluorescence can only happen when a body absorbs radiation [13]. The words "fluorophore" and "chromophore" were later coined by R. Meyer in 1897 [14] and O.N. Witt in 1876 [15], respectively. E. Merritt and E. Nichols investigated in 1905 how dyes absorb light at various wavelengths to get excited and then release fluorescent light [16].

Heinrich Lehmann and Otto Heimstaedt invented the first fluorescence microscope between 1911 and 1913 (Figure 1.2), which was used to investigate autofluorescence in a variety of biological materials [17]. Using a microscope, Stanislav von Prowazek investigated dye binding in live cells in 1914 [18]. Theoretical explanations of how certain mechanisms might reduce fluorescence brightness were provided by Stern and Volmer in 1919 [19]. This understanding is important for applications like sensing small changes in concentration. S.J. Vavilov and W.L. Levshin's 1923 study on the interaction of polarized light with fluorescent materials yielded significant details for polarization microscopy methods [11]. Energy efficiency in transforming absorbed energy into fluorescent light output was measured by S.J. Vavilov in 1924, and it is essential for photonic applications [20]. The foundation for the study of dynamic systems incorporating fluorescent materials was established by F. Perrin's in his 1925 study on polarized light emission from excited dye molecules [21]. For theoretical models and experimental designs in a variety of domains, E. Gaviola's 1926 measurement of the nanosecond range of electronic excitations' duration following energy absorption is essential [22].

Fluorescent dyes employed in biological staining to cause secondary fluorescence in tissues are referred to as fluorochromes, a name Haitinger first used in 1934 [13]. In 1935, the Jablonski diagram, which showed electronic states and their transitions, was created by Alexander Jablonski, who also established fluorescence lifetimes [23]. In order to better understand fluorescence intensity, Francis Perrin extended Jablonski's work by adding the ideas of fluorescence polarization and quantum yield [7]. The efficiency of energy transmission between donor–acceptor pairs spanning nanometers, as shown in resonance energy transfer, is the main emphasis of T. Förster's 1948 quantum mechanical concepts, which also explain dipole interaction and energy transfer in biochemistry research. Knowledge of photosynthesis was greatly improved by pioneering research on delayed fluorescence in photosynthetic plants by Robert Emerson and William Arnold [24, 25].

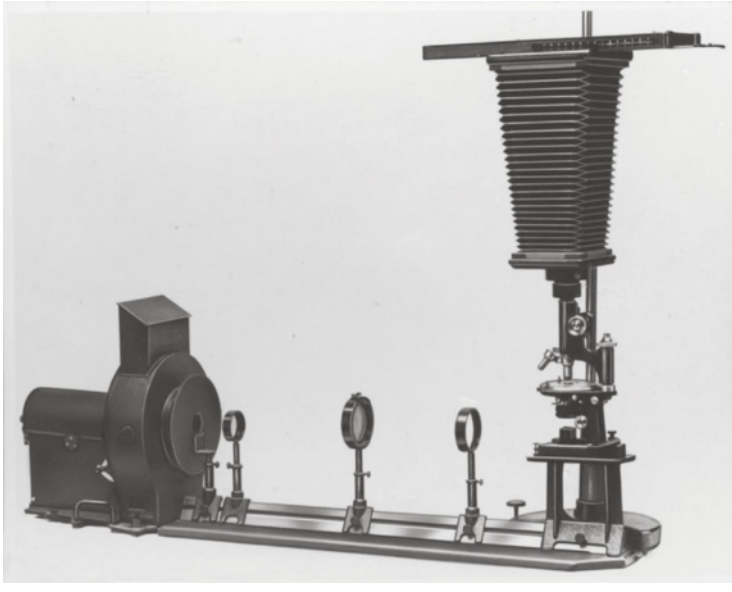

**Figure 1.2** The first fluorescence microscope by Otto Heimstaedt and Heinrich Lehmann. *Source*: Carl Zeiss AG/Wikimedia Commons/Public Domain.

Biological imaging was transformed after 1970 by the advancement of fluorescence probe technology, which included the revelation and manufacture of green fluorescent protein (GFP). Modern techniques like live-cell imaging and super-resolution microscopy were developed, and these are now essential resources for life sciences research.

### 1.1.2 The Mechanism of Fluorescence

The term "fluorescence" has a long history, going all the way back to its first discovery, which was detailed in the historical research mentioned above. Fluorescence is the result of a fluorescent molecule, known as a fluorochrome, absorbing light at one wavelength and releasing energy as radiation at another. The crucial relationship between energy and wavelength is that each fluorochrome interacts with particular wavelengths to produce fluorescence.

Photon-excited molecules undergo relaxation processes, which fall into two categories: radiative and nonradiative. Fluorescence and phosphorescence emission are examples of radiative processes, but infrared emission between vibrational levels is frequently regarded as a nonradiative activity. Whereas phosphorescence moves between states of differing multiplicity, fluorescence moves between states of the same multiplicity. Intramolecular nonradiative reactions, which include internal conversion, intersystem crossover, vibrational relaxation, and photochemical transformations, can take place without collisions. Intersystem crossover is the transition between states of different multiplicity, whereas internal conversion is the nonradiative transfer between distinct electronic states [26].

A molecule's many electronic states and the activities that take place during their transition are depicted in Professor Alexander Jablonski's illustration. $S_0$, $S_1$, and $S_2$ stand for the singlet ground state, first excited state, and second excited state, respectively. These levels, denoted by 0, 1, 2, etc., represent the different vibrational energy levels in which fluorophores can reside. When a molecule absorbs a photon of light, it matches the energy difference between its excited singlet states ($S_n$) and ground state ($S_0$), causing fluorescence. This change takes place in the stimulated state, where vibrational relaxation takes place, and it happens in femtoseconds. The molecule releases part of its excess energy to the surroundings during this stage, mostly in the form of heat and other nonradiative events. Energy is increases along the vertical axis in this mechanism.

As shown in Figure 1.3, when a molecule descends to the first excited singlet, the lowest vibrational level of the $S_1$ state, vibrational relaxation occurs, resulting in fluorescence, a photon. This procedure doesn't produce any light and takes place in picoseconds ($10^{-12}$ seconds). A Stokes shift occurs when the molecule returns to the ground state and emits a photon that is less energetic than the one that was absorbed. The variation in energy among captured and emitted photons, as well as the consequent wavelength difference, is known as the Stokes shift. Fluorescence emission, leading to visible light, happens rapidly – typically within $10^{-9}$ seconds.

Emission time after excitation was first used to distinguish between fluorescence and phosphorescence. Long-lived phosphorescence and short-lived fluorescence have comparable lifetimes; thus, this is inadequate. Before emission, the excited species goes through an intermediate stage, which is when phosphorescence is seen. Phosphorescence changes spin multiplicity, usually from triplet to singlet, but fluorescence sustains it [10].

Applications such as fluorescence microscopy benefit from fluorescence's short lifetime, direct transition from $S_1$ to $S_0$. It makes it possible to watch biological processes in real time. It follows Planck's rule: according to Planck's law, photon energy and wavelength are inversely related. The average time between excitation

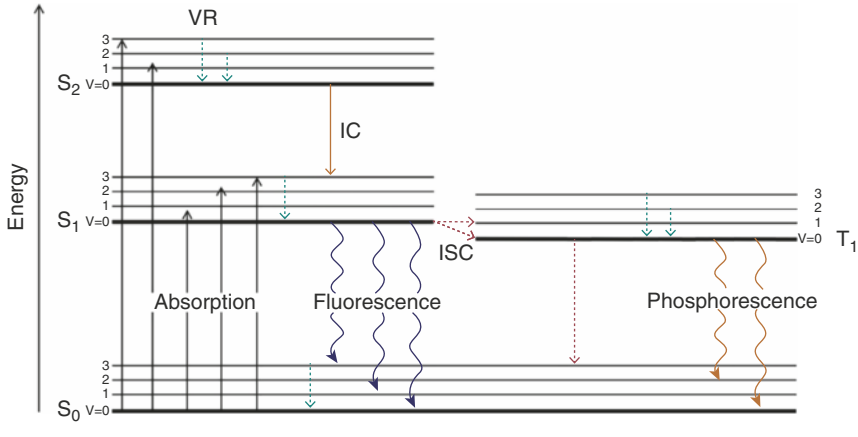


**Figure 1.3** Diagram of the energy levels and different types of transitions processes.

and ground state is known as the fluorescence lifetime. The $T_1$ triplet state is the outcome of intersystem crossover ISC to the triplet state when the thermal population is equal to vibrational levels.

### 1.1.3 Fundamental Rules of Fluorescence Mechanism

#### 1.1.3.1 Kasha's Rule

A key idea in fluorescence, Kasha's rule was initially proposed by American scientist Michael Kasha in 1950. Irrespective of the higher excited state attained during light absorption, it claims that the lowest excited singlet state ($S_1$) is the primary source of fluorescence emission. Higher-energy states lose energy through nonradiative processes, which are caused by the Stokes shift phenomenon. Phosphorescence is equally covered by Kasha's Rule, which states that luminescence always results from the singlet or triplet, the lowest excited electronic state of a given multiplicity. This rule is most noticeable in complicated compounds and compressed materials. It offers an essential foundation for planning, analyzing, and refining fluorescence investigations in a range of fields, such as visualization, spectroscopy, and molecular research [27].

#### 1.1.3.2 Exceptions to Kasha's Rule

Molecular systems whose fluorescent emission comes from higher excited states rather than the lowest excited state of a given multiplicity are exceptions to Kasha's rule. A few examples are metalloporphyrins, medium to large acenes, all-trans polyenes, and compounds such as pyrene, biphenylene, and azobenzene. These anomalies arise when there is a significant enough energy difference between the $S_2$ and $S_1$ states to lower the internal conversion rate and favor $S_2$ fluorescence. Despite highlighting complications or misclassifications rather than actual breaches, notable instances such as $C_{70}$, pyrene, and azulene serve to illustrate these variances [28].

#### 1.1.3.3 Kasha–Vavilov Rule

One of the main principles of photochemistry that provides constant light emission efficiency is the Kasha–Vavilov rule. According to this theory, the fluorescence quantum yield is independent of the wavelength of light used for excitation, which means that as long as a molecule is stimulated to an electronic state, the color of the light has nothing to do with how much light it emits. The chemical methyl salicylate serves as an example of how the Kasha and Kasha–Vavilov rules are related. The nonradiative channel of the molecule causes different fluorescence quantum yields in the gas phase, which is in opposition to the Kasha–Vavilov rule. The rule is not applicable in these circumstances because the molecule's nonradiative channel loses some energy without producing light, resulting in a fluctuation in fluorescence quantum yield depending on the excitation wavelength. Kasha's rule is still valid because the fluorescence emission always originates from the lowest excited electronic state ($S_1$), despite the excitation wavelength. This demonstrates how the two rules differ in that the Kasha–Vavilov rule deals with emission efficiency, which is impacted by molecular environments and energy dissipation routes, whereas Kasha's rule concentrates on the source of fluorescence emission [29].

#### 1.1.3.4 Energy Gap Law

The energy gap (EG) Law is a fundamental concept in fluorescence, providing a framework for understanding how molecules lose energy in excited states. Introduced by Englman and Jortner in 1970, this law describes the exponential relationship between the nonradiative decay rate of excited states and the energy gap between electronic states. The EG law basically says that nonradiative decay rates rise sharply as the energy variation between the excited state ($S_1$) and the ground state ($S_0$) falls. This means that molecules with narrower energy gaps are more likely to lose energy through nonradiative processes, such as internal conversion or intersystem crossing, rather than emit it as light (fluorescence). Conversely, molecules with larger energy gaps tend to have higher fluorescence quantum yields, as they are less prone to wasting energy nonradiatively.

The foundation of the EG rule is twofold: first, the electronic transition of interest is weakly connected to ambient degrees of freedom and molecular vibrations, guaranteeing that certain vibrational modes are the main mediators of energy dissipation. Second, the peak-frequency vibrational modes dominate the vibronic transition mechanism, efficiently channeling energy loss. These assumptions simplify the complex interactions between electronic and vibrational states, making the EG law a powerful tool for predicting fluorescence efficiency and analyzing energy dissipation pathways.

The EG law is derived using the stationary phase approximation applied to the Fermi Golden Rule (FGR) rate expression, which describes quantum transitions. This derivation highlights the exponential increase in nonradiative decay rates as the energy gap narrows, reflecting the enhanced probability of vibrational energy dissipation. The EG law has been widely applied in various fields, including estimating the energy levels of elusive "dark states," optimizing luminescent materials for light-emitting devices, and improving solar energy conversion systems.

Despite its utility, the simplicity of the EG law imposes limitations, particularly in scenarios involving strong electronic-vibrational coupling or alternative energy dissipation pathways. Recent advancements have extended the EG law to account for additional environmental effects, such as Ohmic environments, and alternative quantum transition mechanisms, enhancing its applicability to more complex molecular systems. Nonetheless, the EG law remains an essential principle in photophysics and photochemistry, offering valuable insights into the factors that govern fluorescence efficiency and energy loss in molecular systems [30].

#### 1.1.3.5 Quantum Yield

The Einstein postulate (1905, 1912) revolutionized photochemical reactions by allowing researchers to talk about the quantity of photons and molecules in an operation. Bodenstein suggested in 1929 that Avogadro's number of photons be referred to as Einstein and represented by the letter "E." This would result in an Einstein of absorbed photons that would give rise to a mole of stimulated molecules. Even though this suggestion is still valid today, photochemists would rather use Einstein without E. capital. The symbol $\varphi$ was adopted by E.E. Warburg, an early researcher in quantified photochemistry, to represent the specific photochemical effect, which is the proportion of moles reacting or produced divided by the energy absorbed.

Additionally, he utilized the symbol p to denote the number of gram-moles needed to absorb one gram calorie of $\lambda$-wavelength luminescence. After Einstein, this ratio was converted to the *Güteverhältnis*, which is now known as the quantum yield. Vavilov coined the term "fluorescence yield" in 1924 to describe the percentage of absorbed rays that changed into fluorescence rays, paralleling Warburg's "photochemical productivity." Quantum yield and efficiency were not employed by Perrin, although he did use the idea of the quantity of molecules fluorescing per quantum absorbed. Kistiakowski was the first chemist to exploit quantum efficiency. The impact behind defining and elucidating the terms and symbols used by photochemists was exerted by Warburg [31].

Quantum yield determines how well a fluorescence mechanism operates by comparing the number of photons released as fluorescence to those absorbed by the molecule. It represents the equilibrium of radiative and nonradiative decay mechanisms. A high quantum yield indicates that fluorescence is the dominant channel, implying that absorbed light is efficiently converted into emitted light. A decreased yield, on the other hand, indicates that nonradiative processes predominate, reducing fluorescence efficacy.

The (integral) quantum yield, $\Phi(\lambda)$, is defined by IUPAC for photophysical phenomena such as luminescence, electron ejection in photomultipliers, intersystem crossover, and photochemical reactions. Quantum yield gives information on the probability of fluorescence emission and the use of molecules across different fluorescent applications. Photons are produced from excitation energy by luminescent materials, although this process is not always 100% effective because of losses from alternative deactivation pathways. Two parameters – quantum yield and quantum efficiency (energy) – are established to guarantee effectiveness. A decreased quantum efficiency is the outcome of the luminous material in photoluminescence converting excitation photons into lower-energy photons. It is necessary to improve the quantum yield if the excitation is not direct, as in a structure loaded with an alloy of metals or a luminous activator. This phenomenon is called "downshifting" and is applicable to other wavelength-converting materials exhibiting downconversion or upconversion [32].

## 1.2 Advancing Fluorescent Dyes

### 1.2.1 Introduction and Classification of Fluorescent Dyes

Fluorescent dyes, a class of functional dyes with a lot of potential, have sparked the interest of researchers due to their high emission intensity, vibrant colors, and strong fluorescence. In 1871, Adolf von Baeyer created the first synthetic fluorescent dye by bringing together phthalic anhydride and resorcinol, resulting in fluorescein, which displays a bright green luminescence when subjected to ultraviolet light. Several fluorescent substances have been developed, including organic fluorescent dyes, fluorescent proteins, and inorganic fluorescent dyes. These dyes are widely utilized in several sectors, including electronics (display technology), lighting, communications, printing and dyeing, and preventing counterfeiting.