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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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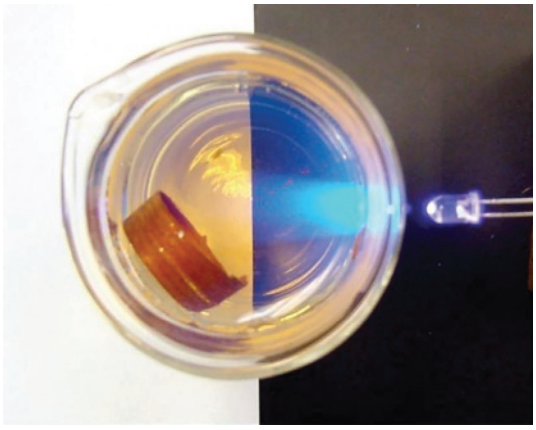

Figure 1.1 The blue fluorescence of the *Lignum nephriticum* in water. *Source*: Mark Muyskens et al. (2006)/American Chemical Society.

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].

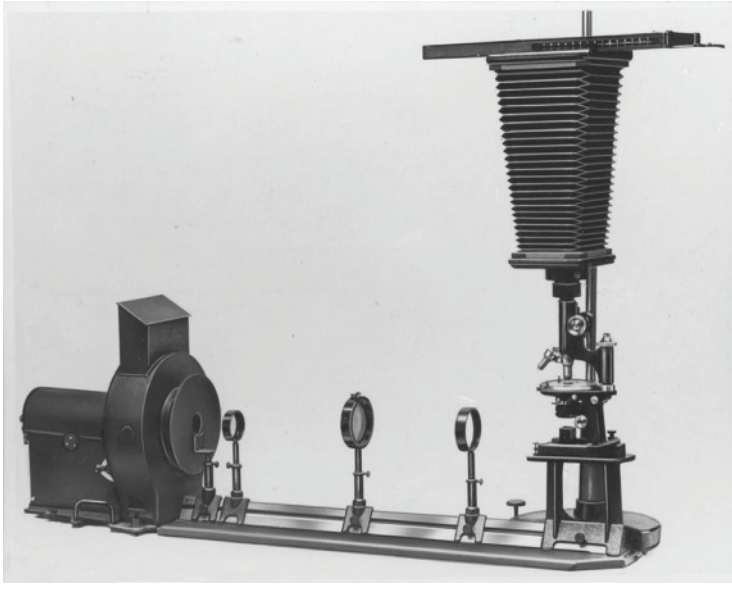

Figure 1.2 The first fluorescence microscope by Otto Heimstaedt and Heinrich Lehmann. *Source*: Carl Zeiss AG/Wikimedia Commons/Public Domain.

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

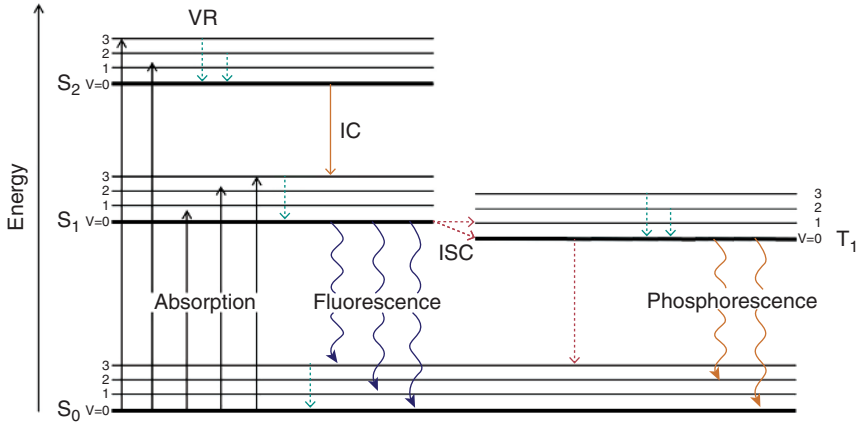

Figure 1.3 Diagram of the energy levels and different types of transitions processes.

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 φ 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 λ-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.

In addition to their usual usage in fluorescence analysis, fluorescent dyes are currently being used as functional dyes in novel applications. They are especially useful for tagging biological macromolecules, undertaking pharmacological research, drug-induced cytotoxicity tests, developing therapeutic targets, and clinical diagnostics.

Organic dyes are identified by their delocalization of π-electrons, which generates unique energy states. Light absorption and emission in these dyes occur via electronic transitions between these states, which span the whole visible spectrum and extend into the near-infrared (NIR). The large number of fluorescent synthetic organic dyes available makes it simple for scientists to select those that meet certain spectroscopic and chemical requirements. In the 1990s, Dick Haugland, along with other famous scholars such as Roger Tsien and David Waggoner, worked tirelessly to create a comprehensive library of fluorescent dyes. This fundamental endeavor accelerated the disciplines of sensing and imaging, providing the groundwork for future developments.

A wide range of models has been created within the same fluorophores' groups. Scientists have fine-tuned the spectroscopic characteristics of these dyes by making synthetic alterations, particularly through the incorporation of donor group and acceptor group (as demonstrated by Gonçalves). These developments have also resulted in the development of dyes with varying binding fidelity and reactivity index for covalent tagging, making them exceedingly adaptable. Organic dyes are ideal as molecular labels and reporters due to their tiny size and ability to integrate with minimal disturbance into biological structures such as biomembranes, proteins, and nucleic acids. Furthermore, these dyes may be engineered to have great photostability and brightness, increasing their applicability in refined imaging and sensing approaches.

Their flexibility and variety of functions have made them indispensable tools in molecular biology, diagnostics, and bioimaging.

1.2.1.1 Inorganic Pigments and Fluorescent Dyes

The animal paintings in Chauvet Cave, situated in southern France, are among the first documented applications of pigments to improve our surroundings. Carbon dating procedures confirm the hypothesis that these works of art date back to roughly 30 000 years BC. The paintings were made with charcoal, a necessary pigment used in primitive inks. Furthermore, the colorful pigment discovered at Chauvet Cave, located in the Ardeche region, was red ochre, a natural substance known as iron oxide (Fe_2O_3) [33]. Malachite ($Cu_2[CO_3][OH]_2$) and azurite ($Cu_3[CO_3]_2[OH]_2$) are popular green and blue rocks that are fairly common. However, they are difficult to utilize as pigments since they lose color when crushed into small particles. Crushed azurite was used as a blue pigment as early as 6700 BC, as evidenced by the Neolithic site of Çatalhöyük. Archil, a purplish-blue dye from lichen, was first mentioned by Greek botanists Theophrastus and Dioscorides in the 3rd century BC. It was only discovered in the 14th century. The dye requires extraction from lichen using an ammoniacal solution, has poor lightfastness, and can change color from red to blue depending on the acidic or basic solution.

Cinnabar has been discovered in Central Europe in Neolithic Vinca Culture sites dating back to the 6th millennium BCE, both on painted artifacts and in pots.

Botallackite has also been found in the Buddhist Zhongshan Grottoes in Shaanxi, China, dating back to the 11th century. Copper salts, along with comparable compounds, discovered in supergene enrichment products and copper mineralization, are bright blue and green, which makes them easily visible in sceneries and metal objects. Theophilus, a 12th-century author, invented the phrase "viride salsum," which means "salt green," to describe synthetic copper salts [34].

The emergence of Prussian Blue, one of the first synthesized pigments, was an important milestone in the development of inorganic dyes in the 18th century. This pigment was uncovered by coincidence in 1704 by German painter Johann Jacob Diesbach, who worked in Berlin [35]. In 1809, French scientist Louis Jacques Thénard produced lead chromate, now known as chrome yellow [36]. Subsequently, in the 1870s, DuPont researchers found lithopone, a white pigment [36]. Inorganic materials' shades are frequently linked to the existence of transition metal compounds, namely d-electrons and ligands, which contribute to their coloring.

Inorganic fluorescent dyes, such as quantum dots (QDs), silicon QDs, metal nanoparticles, upconversion nanoparticles, and carbon dots, have been receiving a lot of interest and are being used in a variety of fields, especially ecological and life sciences. Since 1990, studies on nanoscience and nanotechnology have demonstrated the promise of rare-earth-doped nanomaterials for a variety of uses. Initial investigations focused on luminous nanoparticles, with motivation from colloidal quantum dots and inorganic nanophosphors. The goal was to miniaturize classic luminophores for novel applications, including biolabeling and biosensing.

However, the advent of novel synthetic techniques for producing excellent crystals smaller than 100 nm has hastened advancement in this area. Recent advances in synthetic methods, such as thermal decomposition, coprecipitation, and hydro(solvo)thermal processing, have enabled the production of monodisperse rare-earth-doped nanomaterials with wonderful control over size, shape, and crystallinity. Fluorescent nanoprobes are developing as attractive biomedical tools for investigating biological systems, screening drugs, diagnosing diseases, and monitoring therapy responses.

Ekimov and Onushchenko explained the size dependency of ultrafine semiconductors' spectrum and luminous characteristics for the first time in 1981. Louis E. Brus noticed a similar dependency in colloidal CdS solutions. The word "quantum dot" was coined in 1988, and it subsequently replaced names such as ultrafine particle and nanocrystal. In 1993, Murray, Norris, and Bawendi devised a simple and successful chemical process for manufacturing quantum dots, known as high-temperature colloidal synthesis [37]. Ekimov's revolutionary discovery noticed that by varying the size of semiconductor particles, could modify the fluorescence qualities, allowing for the alteration of light coloration. This highlighted quantum dots' potential use in a variety of domains, including biological imaging and electronics. Moungi Bawendi, Louis Brus, and Aleksey Yekimov received the Nobel Prize in Chemistry in 2023 for their work on the discovery and production of quantum dots [38, 39]. "These accomplishments represent a major milestone in nanotechnology." During a news conference, Johan Åqvist, chair of the Nobel Committee for Chemistry and a biochemist at Uppsala University, claimed that quantum dots have several uses, including Quantum dot Light Emitting Diode (QLED) displays, imaging in biology and medicine, and more [40].

1.2.1.2 Fluorescent Protein Dyes

The creation of fluorescent protein dyes, often known as fluorescent proteins, represents an important accomplishment in medical imaging and molecular biology. The development of such proteins started with the finding of the first natural fluorescent protein and has resulted in the design of a vast variety of fluorescent proteins that are extensively utilized today. In 1955, Davenport and Nicol reported that the jellyfish *Aequorea victoria* had bioluminescence and emitted a green glow.

In 1962, Japanese scientist Osamu Shimomura was the first to successfully isolate and recognize a green fluorescent protein from *Aequorea victoria*. His study focused on the jellyfish's bioluminescent capabilities, which led to the discovery of GFP as the primary component accountable for the green light. Despite Shimomura's remarkable discovery, it took several decades for GFP to achieve popularity in biological studies.

In 1969, J.W. Hastings and J.G. Morin publicly designated it "green fluorescent protein." GFPs have an emission maximum of more than 500 nm. In 1994, American scientist Martin Chalfie highlighted the potential of GFP as a marker in live cells by modifying *Escherichia coli* bacteria to display GFP, proving its use in tracking the expression of genes and protein distribution in living animals.

This landmark study established GFP as an important tool in the biology of cells and molecules. In 2001, American scientist Roger Tsien achieved substantial advances in the area by creating a number of GFP mutants that produce varying shades of fluorescence. Tsien's activities included altering the GFP molecule to produce various fluorescent proteins, such as yellow fluorescent protein and cyan fluorescent protein (CFP), expanding the possibilities accessible to scientists studying complex biological procedures. His contributions were critical in improving the versatility of fluorescent proteins.

Sergey A. Lukyanov reported red fluorescent proteins (RFPs) in anthozoan corals in 1999, opening the path for further research into fluorescent proteins and chromoproteins. In 2000, Lukyanov discovered the fluorescent timer protein, an altered version of the RFP whose fluorescence shifts from greenish to red as time passes [41].

CFPs are known for their emission maxima of less than 500 nm, often between 485 and 495 nm, whereas RFPs emit exceeding 570 nm [42]. In 2008, Shimomura, Chalfie, and Tsien received the Nobel Prize in Chemistry for their collaborative efforts in the field of fluorescent protein discovery [43]. To create NIR-I FPs, bacterial phytochrome photoreceptors (BphPs), cyanobacteriochromes (CBCRs), and allophycocyanins were used as sources. In the NIR-II window, it has been shown that FPs derived from BphPs (iRFP670, iRFP682, iRFP702, iRFP713, and iRFP720) and CBCRs (monomeric miRFP670nano and miRFP718nano, for instance) have fluorescence emission endpoints.

1.2.2 The Development of Organic Fluorescent Dyes

Organic dyes comprise carbon-based chemicals that add vibrant colors to substances. They are generally aromatic structures with conjugated double bonds that absorb and reflect visible light, resulting in color. Archil, a purplish-blue dye from lichen, was first mentioned by Greek botanists Theophrastus and Dioscorides in the 3rd century BC. It was only discovered in the 14th century. The dye requires

extraction from lichen using an ammoniacal solution, has poor lightfastness, and can change color from red to blue depending on the acidic or basic solution.

Alkanet, a perennial herb with bright blue flowers, is used as a food dye and deepens port wine color. It's soluble in alcohol, turning purple in alkaline media. Kermes dye is produced from dried bodies of female Kermes family insects' family, *Coccus ilicis*, which are boiled and ground to produce a color. It takes 150 000 insects to produce 1 kg of dye. The term Kermes comes from Arabic for "little worm" and Latin for "vermiculus" [44].

The first usage of dyes stretches back to ancient civilizations, when natural colors such as indigo and madder were utilized in textile dyeing. In 1856, William Henry Perkin was working in August Wilhelm von Hofmann's lab to synthesize quinine, the only malaria treatment available from cinchona tree bark. Instead of producing colorless quinine from coal tar as he intended, his experiment resulted in a reddish powder. This unexpected outcome led to the creation of the first synthetic dye, named mauve. Before this, all pigments and fluorescent molecules came from natural sources. Recognizing its commercial potential, he established the first synthetic dye factory. Notably, Queen Victoria wore a dress dyed with mauve at her daughter's wedding. Because of these contributions, Perkin is considered the father of synthetic dyes. Runge, Fritsche, and Beissenhirtz's early research on aniline oxidation, undertaken prior to Perkin's discovery, is likely to have yielded small quantities of impure mauveine that were not identified or classified among other byproducts. The importance of the reaction was not realized until William Perkin devised an initial evaluation process to separate and purify mauveine and test its capacity for coloring silk [45]. Fuchsine, commonly known as rosaniline hydrochloride, is a magenta-colored dye developed in 1859 by French scientist François-Emmanuel Verguin [46]. In 1868, German chemists Carl Graebe and Carl Liebermann created the first natural dye, Alizarin, which considerably revolutionized organic chemistry [47]. Adolf von Baeyer, a German scientist, discovered indigo, an organic dye derived from plants, in 1882 [48]. As a result, the textile sector saw enormous growth. Heinrich Caro invented methylene blue in 1883, which is an important dye for biological staining [49]. Paul Böttiger introduced Congo red, the first azo dye, in 1884, and it has since grown to be the most versatile class of synthetic dyes [50]. Adolf von Baeyer made fluorescein in 1871 [51], and later Badische Anilin-und Sodafabrik created rhodamine dyes in 1887 (Figure 1.4), propelling the development of fluorescent dyes forward [52].

Indocyanine green (ICG) is a dye utilized in healthcare from the middle of the 1950s for a range of uses in cardiology, ophthalmology, and neurosurgery [53] produced by Kodak Research Laboratories [54]. Charles Hanson Greville Williams produced the first cyanine dye in 1856. Ethyl red and Sensitol Red (Pynacyanole) were synthesized in 1873 when H.W. Vogel used them as photosensitive substances in photography. Since then, the cyanine dye family has evolved greatly. The first dye having dual peak absorption rates and peculiar J-aggregation properties was pseudoisocyanine, which was developed in 1936 by Jelley [55]. Cyanine dyes are frequently employed in NIR imaging, notably in therapeutic settings. The FDA-approved ICG is frequently used for clinical angiography and circulatory monitoring during operations. IRDye800CW is being evaluated for protein and antibody labeling

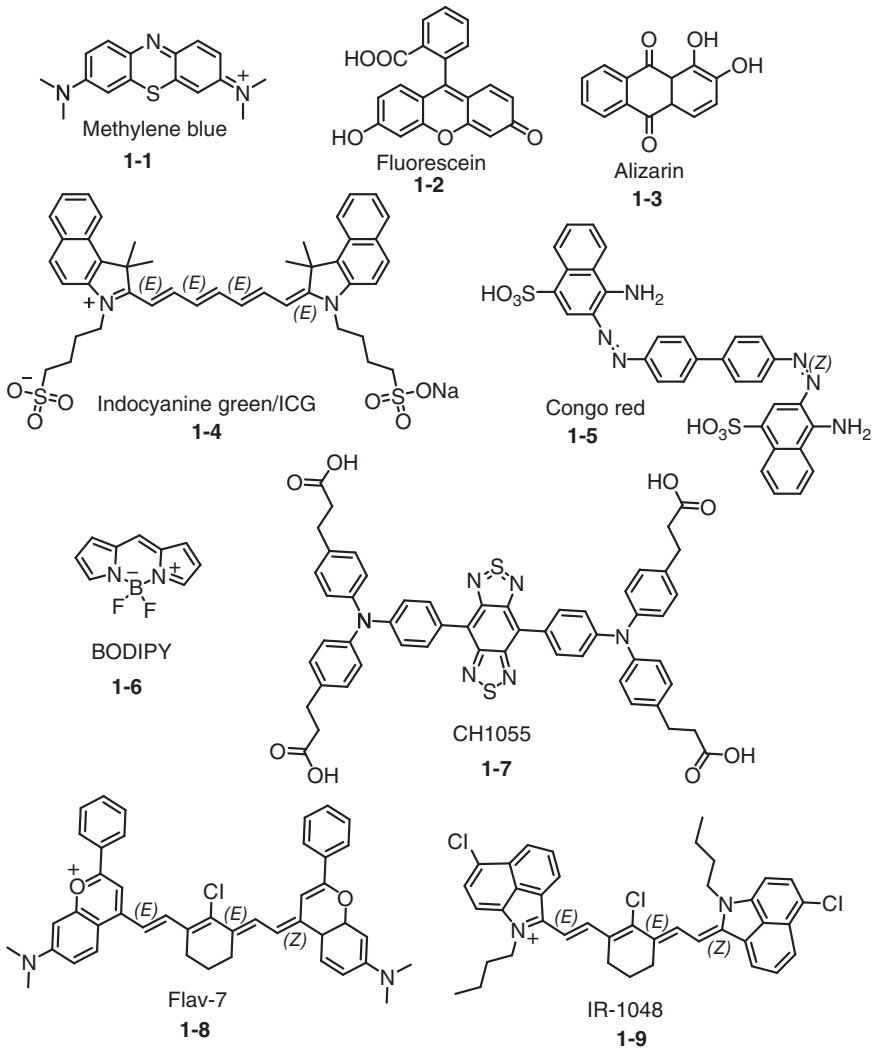

Figure 1.4 Representative fluorescent dye and corresponding chemical structures.

in molecular recognition imaging [56]. Yet, imaging depth is still limited in the NIR-I range (700–900 nm), necessitating continued work to enhance approaches for deeper tissue layers. In 1820, A. Vogel, an active member of the Royal Academy of Sciences in Munich, initially reported the isolation of coumarin [57, 58]. W. H. Perkin was the first to synthesize coumarin in 1868 by warming the sodium salt of salicylaldehyde along with acetic acid, which also opened the way for the Perkin reaction [59], which is currently utilized extensively for bioimaging and sensing. Treibs and Kreuzer discovered BODIPY (Boron-Dipyrromethene) dyes in 1968, which were an important turning point in the area of organic fluorescent dyes. BODIPY dyes have become known for their photostability and have subsequently been essential in bioimaging, sensors, and as tags in fluorescence investigations [60] resulting NIR-II (1000–1700 nm).

Since the discovery of CH1055 by Antaris et al. [61], NIR-II fluorophores with a donor–acceptor–donor (D–A–D) structural motif have been developed. Significant progress has been made with organic molecules exhibiting NIR-II emission, including CH1055, FD-1080, IR-FTAP, and Flav7 (Figure 1.4). A number of well-known NIR-II emitting molecules, such as IR-1040, IR-1048, and IR-1061, were then created by scientists throughout the course of the next 30 years; some of them have already been found commercially. By connecting with type II collagen-binding peptide, the cartilage-targeting probe CH1055-WL was created in 2019. Theranostics of early-stage osteoarthritis might benefit from this probe's potential to exhibit high-resolution cartilage focusing and degenerative imaging, according to *in vitro* and *in vivo* imaging investigations. By using molecular surgeries to replace sulfur with selenium in acceptor units, Lee et al. demonstrated IR-TT, IR-TS, and IR-SS with absorbance ranging from 600 to 1400 nm in 2020 [62]. These advancements have notably improved tissue penetration depth and imaging resolution. These characteristics have been improved by the bathochromic shift of the excitation wavelength from NIR-I to NIR-II, which lowers the absorption and scattering of excitation and emission light. Despite these improvements, there remains a pressing need for NIR-II fluorophores with longer wavelengths and higher brightness to achieve even greater imaging depth and resolution.

1.3 Fluorescent Probes for Molecular Recognition

1.3.1 Advances in Fluorescent Molecular Recognition

Devices called molecular biosensors are made to detect various analytes via biospecific identification. They serve two purposes: specific binding of the target and transduction of information into a measurable signal. By creating a complex between the biomolecule and its recognition element, biosensors allow for the simultaneous detection and measurement of biomolecules. A recordable analytical signal is produced by the transducer from changes in characteristics such as molecular mass, refractive index, or noncovalent bond formation. Enzyme-linked immunosorbent assay and other traditional bioassays do not detect the connection between the recognition unit and the target as quickly or directly as this method does. Fluorescence is a highly sensitive method for detecting intermolecular interactions, cost-effective, and easy to implement. By altering the fluorescence characteristics of a molecular recognition unit – which comprises both biological recognition and transmitting components of a biosensor – it is able to identify the target. The unit, typically a dye, shows binding affinity for a specific chemical sensor.

Biomolecular sensing involves incorporating one or two dyes in a molecular binder for specific targets. There are two options: using a single dye, which changes fluorescence parameters, or using two dyes or a dye and a quencher. Double labeling of a binder can be beneficial in certain detection technologies, allowing for increased signal variations on target binding. Different physical mechanisms can be involved in this response.

While the combination of two structurally similar dyes may result in excitation complex formation, which alters the emission spectrum, the interaction of a dye with a quencher can cause an on-off fluorescence switching effect. Fluorescence resonance energy transfer can function over distances of up to 10 nm, where the initially excited dye undergoes quenching, and fluorescence from the second dye emerges. One-electron oxidation-reduction in the excited state is made possible by intramolecular electron transfer (IET), which occurs when a single dye molecule exhibits two separate electronic regions. This process can be modulated by the binding of an analyte at the electron donor or acceptor site, influencing the strength of these components. The IET mechanism is particularly effective when the target is an ion coordinated by a chelating group. However, its use in biomolecular sensing necessitates significant electrostatic modulation of electronic properties and strong intermolecular interactions.

Quenching effects in dyes are useful for sensing applications due to their interactions with heavy and transition metal ions. These interactions can restrict the dye's intramolecular motion, leading to a significant enhancement in fluorescence intensity. Triarylmethane dyes, including crystal violet and malachite green, display intense fluorescence following adsorption on hard surfaces or coupling to proteins. The quenching mechanism involves solvent participation, and many dyes display inherently low fluorescence quantum yield in aqueous environments. Enhancing sensor-target interactions and shielding the dye from water can improve fluorescence quantum yield, making it advantageous for sensing applications. The establishment or disruption of hydrogen bonds with solvent molecules can also influence fluorescence intensity [63]. Biomolecular sensing can induce dehydration and interfere with hydrogen bonding to water, impacting the behavior of the fluorescence reporter [64].

1.3.2 Emerging Trends in Fluorescence and Molecular Recognition

Jean-Marie Lehn (Nobel Prize winner in 1987) and Fraser Stoddart (Nobel Prize winner in 2016) are notable scientists in the field of computational design of supramolecular assemblies, focusing on applications such as fluorescence and molecular recognition systems.

Walter Kohn and John Pople were both Nobel Laureates in 1998 for their contributions to the development of density functional theory and computational quantum chemistry.

Quantum chemistry and computational photochemistry involve designing molecules with desired fluorescent properties using quantum mechanical methods like time-dependent density functional theory and exploring excited-state dynamics of fluorophores.

Eric Betzig, Stefan W. Hell, and William E. Moerner were awarded the Nobel Prize in 2014 for their work on super-resolved fluorescence microscopy.

Computational approaches in nanotechnology are being used to optimize fluorescent nanomaterials like quantum dots and functionalize nanoparticles for molecular recognition. Notable scientists, including Moungi Bawendi and Louis Brus received the Nobel Prize in 2023 for their contributions to quantum dot research (Table 1.1).

Table 1.1 Nobel prize winner related to fluorescence and molecular recognition.

Year	Nobel prize winner	Work
1965	Robert B. Woodward	Achievements in organic synthesis
1987	Donald J. Cram, Jean-Marie Lehn, and Charles J. Pedersen	Development and usage of molecules with structure-specific interactions and selectivity
1998	Walter Kohn	Development of density functional theory
1998	John Pople	Computational methods in quantum chemistry
2008	Osamu Shimomura, Martin Chalfie, Roger Y. Tsien	Discovery and development of green fluorescent protein
2014	Eric Betzig, Stefan W. Hell, William E. Moerner	Development of super-resolved fluorescence microscopy
2016	Jean Pierre Sauvage, Sir J. Fraser Stoddart, Bernard L. Feringa	Design and synthesis of molecular machines
2022	Carolyn Bertozzi, Morten Meldal, K. Barry Sharpless	Development of click chemistry
2023	Moungi Bawendi, Louis Brus, Aleksey Yekimov	Discovery and synthesis of quantum dots

References

1 Lagorio, M. G., Cordon, G. B., and Iriel, A. (2015). *Photochem. Photobiol. Sci*. 14: 1538–1559.
2 Kalaji, H. M., Goltsev, V., Bosa, K. *et al.* (2012). *Photosynth. Res*. 114: 69–96.
3 Yadav, S. K. (2021). Fluorescence microscopy of organic dye, nanoparticles, quantum dots and spectroscopy. In: *Nanotechnology in Cancer Management*, 73–106. Elsevier.
4 Sharma, S. K. (2018). Persistent luminescence: Cerium-doped phosphors. In: *Phosphors*, 49–82. Jenny Stanford Publishing.
5 Roda, A. (ed.) (2011). *Chemiluminescence and Bioluminescence*, 1–50. RSC Publishing.
6 Baker, A. A. (1964). *Chymia* 9: 147–167.
7 Valeur, B. and Berberan-Santos, M. N. (2013). *Molecular Fluorescence: Principles and Applications*. John Wiley & Sons.
8 Valeur, B. and Brochon, J. C. (eds.) (2001). Introduction. In: *New Trends in Fluorescence Spectroscopy: Applications to Chemical and Life Sciences*, 3–6. Springer.
9 Garcia-Campana, A. M., Román-Ceba, M., and Baeyens, W. R. (2001). Historical evolution of chemiluminescence. In: *Chemiluminescence in Analytical Chemistry*, 1–39. CRC Press.
10 Valeur, B. and Berberan-Santos, M. N. (2011). *J. Chem. Educ*. 88: 731–738.
11 Jameson, D. M. and Ross, J. A. (2010). *Chem. Rev*. 110: 2685–2708.
12 Ceredig, R. (2020). *Philos. Trans. R. Soc. A* 378: 20200105.

13 Kasten, F. H. (1989). 1 - The origins of modern fluorescence microscopy and fluorescent probes. In: *Cell Structure and Function by Microspectrofluorometry* (ed. E. Kohen), 3–50. Academic Press.
14 Meyer, R. (1987). *Zeit. Phys. Chem*. 24: 468–508.
15 Witt, O. N. (1876). *Ber. Dtsch. Chem. Ges*. 9: 522–527.
16 Vavilov, S. (1922). *Lond. Edinb. Dubl. Phil. Mag*. 43: 307–320.
17 Tang, Y., Dai, L., Zhang, X. *et al.* (2015). *Sci. Rep*. 5: 11073.
18 Hassdenteufel, S. and Schuldiner, M. (2022). *Curr. Opin. Chem. Biol*. 66: 102102.
19 Stern, O. and Volmer, M. (1919). *Phys. Z*. 20: 183–188.
20 Demasa, J. and Crosby, G. (1968). *J. Chem. Phys*. 48: 4726.
21 Jameson, D. M. and Croney, J. C. (2003). *Comb. Chem. High Throughput Screen*. 6: 167–176.
22 Jameson, D. M., Croney, J. C., and Moens, P. D. (2003). *Methods Enzymol*. 360: 1–43.
23 Masters, B. R. (2010). The development of fluorescence microscopy. In: *Encyclopedia of Life Sciences*, 1–9. John Wiley & Sons.
24 Emerson, R. and Rabinowitch, E. (1960). *Plant Physiol*. 35: 477–485.
25 Arnold, W. A. (1991). *Photosynth. Res*. 27: 73–82.
26 Itoh, T. (2012). *Chem. Rev*. 112: 4541–4568.
27 Demchenko, A. P., Tomin, V. I., and Chou, P.-T. (2017). *Chem. Rev*. 117: 13353–13381.
28 Braun, G., Borges, Jr. I., Aquino, A. J. A. *et al.* (2022). *J. Chem. Phys*. 157: 154305.
29 del Valle, J. C. and Catalán, J. (2019). *Phys. Chem. Chem. Phys*. 21: 10061–10069. https://doi.org/10.1039/C9CP00739C.
30 Jang, S. J. (2021). *J. Chem. Phys*. 155: 164106.
31 Rubin, M. B. and Braslavsky, S. E. (2010). *Photochem. Photobiol. Sci*. 9: 670–674. https://doi.org/10.1039/B9PP00182D.
32 Wong, K.-L., Bünzli, J.-C. G., and Tanner, P. A. (2020). *J. Lumin*. 224: 117256.
33 Abel, A. (2020). *Colour Des*, 557–587. *Woodhead Publishing*.
34 Siddall, R. (2018). *Minerals* 8: 201.
35 Guichard, C., Le Hô, A.-S., and Williams, H. (2023). *Art Hist*. 46: 154–186.
36 Habashi, F. (2016). *InterCeram: Int. Ceram. Rev*. 65: 156–165.
37 Brichkin, S., Spirin, M., and Razumov, V. (2024). *High Energy Chem*. 58: S10–S23.
38 Razumov, V. (2024). *High Energy Chem*. 58: S1–S3.
39 Viswanatha, R. (2024). *Resonance* 29: 69–81.
40 Wegner, K. D. and Resch-Genger, U. (2024). *Anal. Bioanal. Chem*. 416: 3283–3293.
41 Sivakumar, K. (2009). *Adv. Biosyst*. 24–29.
42 Ferreira, G., Bollati, E., and Kühl, M. (2023). *Front. Mar. Sci*. 10: 1204843.
43 Zimmer, M. (2015). Oxford University Press.
44 Abel, A. (2012). 24 – The history of dyes and pigments: From natural dyes to high performance pigments. In: *Colour Design*, 557–587. Woodhead Publishing.
45 Plater, M. J. and Raab, A. (2016). *J. Chem. Res*. 40: 758–762.
46 Cooksey, C. and Dronsfield, A. (2009). *Biotech Histochem*. 84: 179–183.
47 Fieser, L. F. (1930). *J. Chem. Educ*. 7: 2609.
48 Prasad, R. (2018). *Indian J. Hist. Sci*. 53: 296–301.
49 Krafts, K., Hempelmann, E., and Oleksyn, B. (2011). *Biotech Histochem*. 86: 7–35.

50 Franck, H. G. and Stadelhofer, J. W. (2012). *Springer Science & Business Media*.
51 Anwar, M., Nadeem, F., Faiz, S., and Alam, M. (2018). *Int. J. Chem. Biochem. Sci*. 14: 120–141.
52 Schnermann, M. J. and Lavis, L. D. (2023). *Curr. Opin. Chem. Biol*. 75: 102335.
53 Reinhart, M. B., Huntington, C. R., Blair, L. J. *et al.* (2016). *Surg. Innov*. 23: 166–175.
54 Staurenghi, G., Bottoni, F., and Giani, A. (2013). Clinical applications of diagnostic indocyanine green angiography. In: *Retina*, 51–81. London: Elsevier Saunders.
55 Jelley, E. E. (1936). *Nature*. 138: 1009–1010.
56 Zhu, S., Hu, Z., Tian, R. *et al.* (2018). *J. Adv. Mater*. 30: 1802546.
57 Pereira, T. M., Franco, D. P., Vitorio, F. *et al.* (2018). *Curr. Top. Med. Chem*. 18: 124–148.
58 Abernethy, J. L. (1969). *J. Chem. Educ*. 46: 561.
59 Cao, D., Liu, Z., Verwilst, P. *et al.* (2019). *Chem. Rev*. 119: 10403–10519.
60 Bodio, E. and Goze, C. (2019). *Dyes Pigm*. 160: 700–710.
61 Antaris, A. L., Chen, H., and Cheng, K. (2016) *Nat. Mater*. 15: 235–242.
62 Li, L., Dong, X., Li, J. *et al.* (2020). *Dyes Pigm*. 183: 108756.
63 Pistolis, G., Paleos, C., and Malliaris, A. (1995). *J. Phys. Chem*. 99: 8896–8902.
64 Altschuh, D., Oncul, S., and Demchenko, A. P. (2006). *J. Mol. Recognit*. 19: 459–477.