1
Introduction

> . . . ex arte calcinati, et illuminato aeri seu solis radiis, seu flammae fulgoribus expositi, lucem inde sine calore concipiunt in sese; . . .
>
> *Licetus, 1640 (about the Bologna stone)*

> *[. . . properly calcinated, and illuminated either by sunlight or flames, they conceive light from themselves without heat; . . .]*

1.1
What Is Luminescence?

The word luminescence, which comes from the Latin (lumen = light) was first introduced as luminescenz by the physicist and science historian Eilhardt Wiedemann in 1888, to describe "all those phenomena of light which are not solely conditioned by the rise in temperature," as opposed to incandescence. Luminescence is often considered as cold light whereas incandescence is hot light.

Luminescence is more precisely defined as follows: spontaneous emission of radiation from an electronically excited species or from a vibrationally excited species not in thermal equilibrium with its environment.[1)] The various types of luminescence are classified according to the mode of excitation (see Table 1.1).

Luminescent compounds can be of very different kinds:

- *Organic compounds*: aromatic hydrocarbons (naphthalene, anthracene, phenanthrene, pyrene, perylene, porphyrins, phtalocyanins, etc.) and derivatives, dyes (fluorescein, rhodamines, coumarins, oxazines), polyenes, diphenylpolyenes, some amino acids (tryptophan, tyrosine, phenylalanine), etc.
- *Inorganic compounds*: uranyl ion (UO_2^+), lanthanide ions (e.g., Eu^{3+}, Tb^{3+}), doped glasses (e.g., with Nd, Mn, Ce, Sn, Cu, Ag), crystals (ZnS, CdS, ZnSe, CdSe, GaS, GaP, Al_2O_3/Cr^{3+} (ruby)), semiconductor nanocrystals (e.g., CdSe), metal clusters, carbon nanotubes and some fullerenes, etc.

1) Braslavsky, S. *et al.* (2007) Glossary of terms used in photochemistry, *Pure Appl. Chem.*, **79**, 293–465.

Molecular Fluorescence: Principles and Applications, Second Edition. Bernard Valeur, Mário Nuno Berberan-Santos.

Table 1.1 The various types of luminescence.

Phenomenon	Mode of excitation
Photoluminescence (fluorescence, phosphorescence, delayed fluorescence)	Absorption of light (photons)
Radioluminescence	Ionizing radiation (X-rays, α, β, γ)
Cathodoluminescence	Cathode rays (electron beams)
Electroluminescence	Electric field
Thermoluminescence	Heating after prior storage of energy (e.g., radioactive irradiation)
Chemiluminescence	Chemical reaction (e.g., oxidation)
Bioluminescence	*In vivo* biochemical reaction
Triboluminescence	Frictional and electrostatic forces
Sonoluminescence	Ultrasound

- *Organometallic compounds*: porphyrin metal complexes, ruthenium complexes (e.g., $Ru(bpy)_3^{2+}$), copper complexes, complexes with lanthanide ions, complexes with fluorogenic chelating agents (e.g., 8-hydroxy-quinoline, also called oxine), etc.

Fluorescence and phosphorescence are particular cases of luminescence (Table 1.1). The mode of excitation is absorption of one or more photons, which brings the absorbing species into an electronic excited state. The spontaneous emission of photons accompanying de-excitation is then called photoluminescence which is one of the possible physical effects resulting from interaction of light with matter, as shown in Figure 1.1. Stimulated emission of photons can also occur under certain conditions (see Chapter 3, Box 3.2). Additional processes, not shown, can take place for extremely high intensities of radiation, but are not relevant for luminescence studies.

1.2 A Brief History of Fluorescence and Phosphorescence

It is worth giving a brief account of the history of fluorescence and phosphorescence. The major events from the early stages to the middle of the twentieth century are reported in Table 1.2 together with the names of the associated scientists. The story of fluorescence started with a report by N. Monardes in 1565, but scientists focused their attention on light emission phenomena other than incandescence only in the nineteenth century. However, the major experimental and theoretical aspects of fluorescence and phosphorescence were really understood

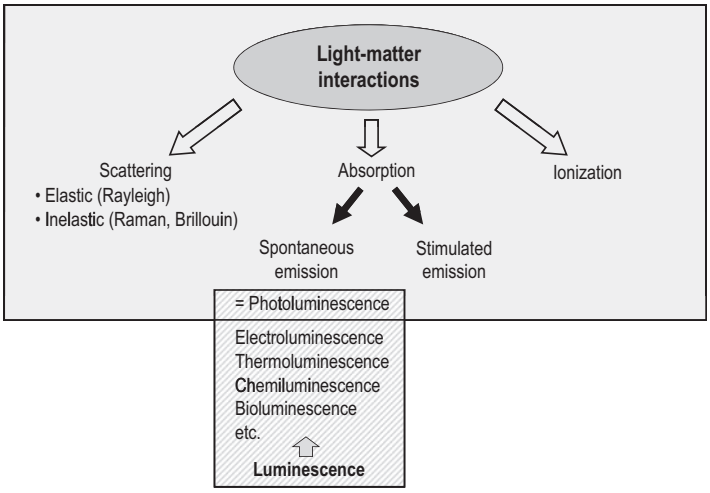
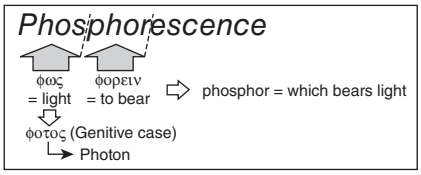


Figure 1.1 Position of photoluminescence in the frame of light–matter interactions.



Scheme 1.1

only after the emergence of quantum theory, already in the twentieth century (1918–1935, i.e., less than 20 years). As in many other areas of theoretical physics and chemistry, this was an exceptionally fecund period.

1.2.1 Early Observations

Let us examine first the origins of the terms fluorescence and phosphorescence. The term phosphorescence comes from the Greek: $\varphi\omega\varsigma$ = light (genitive case: φοτος → photon) and φορειν = to bear (Scheme 1.1). Therefore, phosphor means "which bears light." The term phosphor has indeed been assigned since the Middle Ages to materials that glow in the dark after exposure to light. There are many examples of minerals reported a long time ago that exhibit this property, and the most famous of them (but not the first one) was the Bolognian phosphor discovered by a cobbler from Bologna in 1602, Vincenzo Cascariolo, whose hobby was alchemy. One day he went for a walk in the Monte Paterno area and he picked up some strange heavy stones. After calcination with coal, he observed that these stones glowed in the dark after exposure to light. It was recognized later that the

Table 1.2 Milestones in the history of fluorescence and phosphorescence[a)].

Year	Scientist	Observation or achievement
1565	N. Monardes	Emission of light by an infusion of the wood later called *Lignum nephriticum* (first report on the observation of fluorescence)
1602	V. Cascariolo	Emission of light by Bolognese stone (first detailed observation of phosphorescence)
1640	Licetus	Study of Bolognian stone. First definition as a nonthermal light emission
1833	D. Brewster	Emission of light by chlorophyll solutions and fluorspar crystals
1842	J. Herschel	Emission of light by quinine sulfate solutions (epipolic dispersion)
1845	E. Becquerel	Emission of light by calcium sulfide upon excitation in the UV
		First statement that the emitted light is of longer wavelength than the incident light.
1852	G. G. Stokes	Emission of light by quinine sulfate solutions upon excitation in the UV (refrangibility of light)
1853	G. G. Stokes	Introduction of the term fluorescence
1858	E. Becquerel	First phosphoroscope. First lifetime measurements.
1867	F. Goppelsröder	First fluorometric analysis (determination of Al(III) by the fluorescence of its morin chelate)
1871	A. Von Baeyer	Synthesis of fluorescein
1888	E. Wiedemann	Introduction of the term luminescence
1905, 1910	E. L. Nichols and E. Merrit	First fluorescence excitation spectrum of a dye
1907	E.L. Nichols and E. Merrit	Mirror symmetry between absorption and fluorescence spectra
1919	O. Stern and M. Volmer	Relation for fluorescence quenching
1920	F. Weigert	Discovery of the polarization of the fluorescence emitted by dye solutions
1922	S. I. Vavilov	Excitation-wavelength independence of the fluorescence quantum yield
1923	S. I. Vavilov and W. L. Levshin	First study of the fluorescence polarization of dye solutions
1924	S. I. Vavilov	First determination of fluorescence yield of dye solutions
1924	F. Perrin	Quantitative description of static quenching (active sphere model
1924	F. Perrin	First observation of alpha phosphorescence (E-type delayed fluorescence)
1925	F. Perrin	Theory of fluorescence polarization (influence of viscosity)

Table 1.2 (*Continued*)

Year	Scientist	Observation or achievement
1925	W. L. Levshin	Theory of polarized fluorescence and phosphorescence
1925	J. Perrin	Introduction of the term delayed fluorescence
		Prediction of long-range energy transfer
1926	E. Gaviola	First direct measurement of nanosecond lifetimes by phase fluorometry (instrument built in Pringsheim's laboratory)
1926	F. Perrin	Theory of fluorescence polarization (sphere)
		Perrin's equation
		Indirect determination of lifetimes in solution.
		Comparison with radiative lifetimes
1927	E. Gaviola and P. Pringsheim	Demonstration of resonance energy transfer in solutions
1928	E. Jette and W. West	First photoelectric fluorometer
1929	F. Perrin	Discussion on Jean Perrin's diagram for the explanation of the delayed fluorescence by the intermediate passage through a metastable state
		First qualitative theory of fluorescence depolarization by resonance energy transfer
1929	J. Perrin and N. Choucroun	Sensitized dye fluorescence due to energy transfer
1932	F. Perrin	Quantum mechanical theory of long-range energy transfer between atoms
1934	F. Perrin	Theory of fluorescence polarization (ellipsoid)
1935	A. Jablonski	Jablonski's diagram
1943	A. Terenin	Triplet state
1944	G. Lewis and M. Kasha	Triplet state
1946–1948	Th. Förster	Theory of resonance energy transfer via dipole–dipole interaction

a) More details can be found in the following:
Harvey, E.N. (1957) *History of Luminescence*, The American Philosophical Society, Philadelphia.
O'Haver, T.C. (1978) The development of luminescence spectrometry as an analytical tool, *J. Chem. Educ.*, **55**, 423–8.
Nickel, B. (1996) From the Perrin diagram to the Jablonski diagram. *EPA Newslett.*, **58** (Part 1), 9–38.
Nickel, B. (1997) From the Perrin diagram to the Jablonski diagram. *EPA Newslett.*, **61** (Part 2), 27–60.
Nickel, B. (1998) From Wiedemann's discovery to the Jablonski diagram. *EPA Newslett.*, **64**, 19–72.
Berberan-Santos, M.N. (2001) Pioneering contributions of Jean and Francis Perrin to molecular fluorescence, in *New Trends in Fluorescence Spectroscopy. Applications to Chemical and Life Sciences* (eds B. Valeur and J.C. Brochon), Springer-Verlag, Berlin, pp. 7–33.
Valeur, B. and Berberan-Santos, M.N. (2011), A brief history of fluorescence and phosphorescence before the emergence of quantum theory, *J. Chem. Educ.*, **88**, 731–738.

stones contained barium sulfate, which, upon reduction by coal, led to barium sulfide, a phosphorescent compound. Later, the same name phosphor was assigned to the element isolated by Brandt in 1677 (despite the fact that it is chemically very different) because, when exposed to air, it burns and emits vapors that glow in the dark.

In contrast to phosphorescence, the etymology of the term fluorescence is not at all obvious. It is indeed strange, at first sight, that this term contains fluor which is not remarked by its fluorescence! The term fluorescence was introduced by Sir George Gabriel Stokes, a physicist and professor of mathematics at Cambridge in the middle of the nineteenth century. Before explaining why Stokes coined this term, it should be recalled that the first printed observation of fluorescence was made by a Spanish physician, Nicolas Monardes, in 1565. He reported the wonderful peculiar blue color (under certain conditions of observation, Figure 1.2) of an infusion of a wood brought from Mexico used to treat kidney and urinary diseases: *palo para los males de los riñones, y de urina* (later called *Lignum nephriticum*).

This wood, whose peculiar color effect and diuretic properties were already known to the Aztecs, was a scarce and expensive medicine. Therefore, it was of

Figure 1.2 Absorption and fluorescence colors of an infusion of *Lignum nephriticum* under day light. (a) taken from Safford, W.E. (1915) *Ann. Rep. Smithsonian Inst.*, 1915, 271–298. (b) mildly alkaline aqueous solution to which chips of *Eysenhardtia polystachya*—kindly provided by Dr. A. U. Acuña—were added.

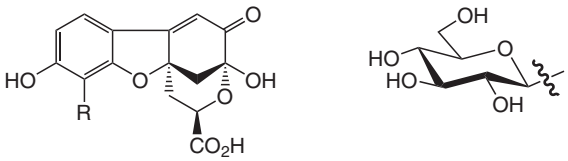


Figure 1.3 Formula of matlanine which is responsible for the fluorescence of *Lignum nephriticum*[2]. Matlali is the Aztec word for blue.

interest to detect counterfeited wood. Monardes writes on this respect: "Make sure that the wood renders water bluish, otherwise it is a falsification. Indeed, they now bring another kind of wood that renders the water yellow, but it is not good, only the kind that renders the water bluish is genuine" (in Spanish in the original). This method for the detection of a counterfeited object can be considered as the very first application of the phenomenon that would be later called fluorescence. Extracts of the wood were further investigated by Boyle, Newton, and others, but the phenomenon was not understood.

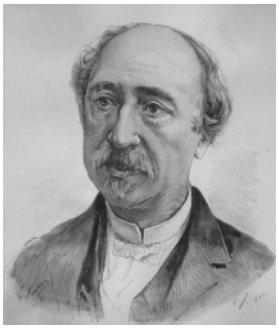
The chemical species responsible for the intense blue fluorescence was recently identified in an infusion of *Lignum nephriticum* (*Eysenhardtia*): it is a four-ring tetrahydromethanobenzofuro[2,3-d]oxacine (matlaline) (Figure 1.3).[2] This compound is not present in the plant but is the end product of an unusual, very efficient iterative spontaneous oxidation of at least one of the tree's flavonoids.

In 1833, David Brewster, a Scottish preacher, reported[3] that a beam of white light passing through an alcoholic extract of leaves (chlorophyll) appears to be red when observed from the side, and he pointed out the similarity with the dichroism of some fluorite crystals, previously reported by the French mineralogist René-Just Haüy. Both authors incorrectly viewed the phenomenon as a manifestation of opalescence (light scattering by small particles).

In 1845, John Herschel, son of the famous astronomer, considered that the blue color at the surface of solutions of quinine sulfate and *Lignum nephriticum* was "a case of superficial color presented by a homogeneous liquid, internally colorless." He called this phenomenon epipolic dispersion, from the Greek επιπολη = surface.[4] The solutions observed by Herschel were very concentrated so that the majority of the incident light was absorbed and all the blue color appeared to be only at the surface. Herschel used a prism to show that the epipolic dispersion could be observed only upon illumination by the blue end of the spectrum, and not the red end. The crude spectral analysis with the prism revealed blue, green, and a small amount of yellow light, but Herschel did not realize that the superficial light was of longer wavelength than the incident light.

2) Acuña, U., Amat-Guerri. F., Morcillo, P., Liras, M., and Rodriguez, B. (2009) *Org. Lett.*, **11**, 3020–3023.
3) Brewster, D. (1833) *Trans. Roy. Soc.*, Edinburgh **12**, 538–45.
4) Herschel, J.F.W. (1945) *Phil. Trans.*, 143–145, 147–153.

G. G. Stokes (1819–1903)



E. Becquerel (1820–1891)

The phenomena were reinvestigated by Stokes, who published a famous paper entitled "On the refrangibility of light" in 1852.[5] He demonstrated that the common phenomenon observed with several samples, both organic (including quinine) and inorganic (including fluorite crystals), was an emission of light following absorption of light. It is worth describing one of Stokes' experiments, which is spectacular and remarkable for its simplicity. Stokes formed the solar spectrum by means of a prism. When he moved a tube filled with a solution of quinine sulfate through the visible part of the spectrum, nothing happened: the solution simply remained transparent. But beyond the violet portion of the spectrum, that is, in the nonvisible zone corresponding to ultraviolet radiations, the solution glowed with a blue light. Stokes wrote: "It was certainly a curious sight to see the tube instantaneously light up when plunged into the invisible rays; it was literally darkness visible." This experiment provided compelling evidence that there was absorption of light followed by emission of light. Stokes stated that the emitted light is always of longer wavelength than the exciting light. This statement became later Stokes' law.

Stokes' paper led the French physicist Edmond Becquerel (the discoverer of the photovoltaic effect, and father of Henri Becquerel, the discoverer of radioactivity), to "réclamation de priorité" (priority claim) for this kind of experiment.[6] In fact, Becquerel published an outstanding paper[7] in 1842 in which he described the light emitted by calcium sulfide deposited on paper when exposed to solar light beyond the violet part of the spectrum. He was the first to state that the emitted light is of longer wavelength than the incident light.

In his first paper,[5] Stokes called the observed phenomenon dispersive reflexion, but in a footnote, he wrote "I confess I do not like this term. I am almost inclined to coin a word, and call the appearance fluorescence, from fluorspar, as the analo-

5) Stokes, G.G. (1852) *Phil. Trans.*, **142**, 463–562.
6) In Cosmos (1854) **3**, 509–510.
7) Becquerel, E. (1842) *Ann. Chim. Phys.*, **9** (3), 257–322.

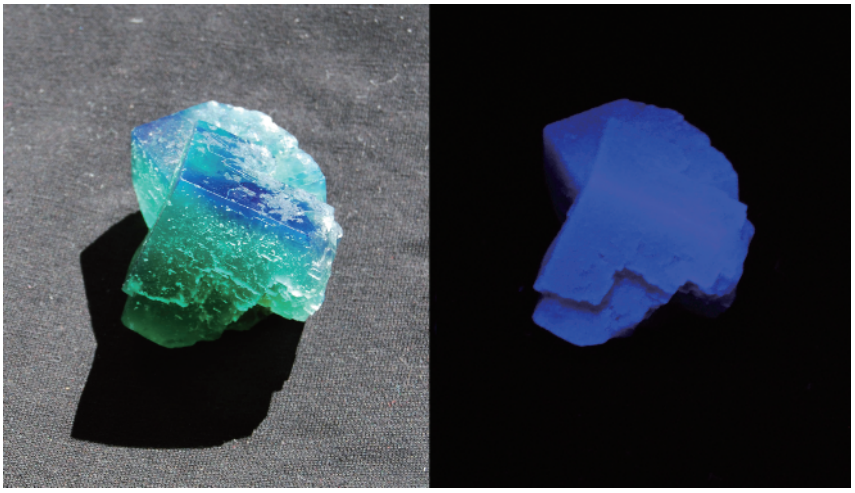
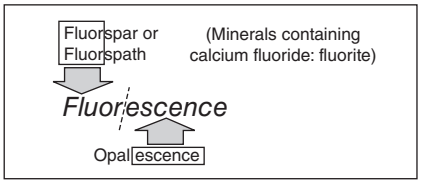


Figure 1.4 Twinned crystals of green fluorite (from Rogerley, Weardale, Durham County, England) illuminated with sunlight. A double color is apparent, as noted in 1819 by Edward D. Clarke, Professor of Mineralogy at the University of Cambridge. He reported that the finer crystals, perfectly transparent, had a dichroic ("double color") nature: the color by reflected light was a "deep sapphire blue," whereas the color by transmitted light was an "intense emerald green."



Scheme 1.2

gous term opalescence is derived from the name of a mineral." In his second paper,[8] Stokes definitely resolved to use the word fluorescence (Scheme 1.2).

In fact, not all varieties of fluorspar or fluorspath (minerals containing calcium fluoride [fluorite]) exhibit the property described above. Many are colored owing to the presence of small amounts of impurities typically from the rare-earth family, whereas pure fluorite, that is, calcium fluoride, is in fact colorless and nonfluorescent. The natural fluorite crystals from Weardale, Durham (England), the variety investigated by Stokes, offer a beautiful example of colors (Figure 1.4). The green color is due to divalent samarium absorption (in the blue and in the red),[9] whereas the deep blue color is due to divalent europium fluorescence (the states involved in the emission have seven unpaired electrons, and hence their spin multiplicity

8) Stokes, G.G. (1853) *Phil. Trans.*, **143**, 385–396.
9) Bill, H., Sierro, J., and Lacroix, R. (1967) *Am. Mineral.*, **52**, 1003–1008.

is 8).[10),11)] Both elements are present as substitutional impurities in the range 10–100 ppm.

Whatever the nature of the sample under observation, it was soon recognized that, in contrast to incandescence which is light emitted by bodies heated at high temperatures, luminescence like fluorescence and phosphorescence does not require high temperatures and does not usually produce noticeable heat. This type of emission was named "cold light" for this reason. Such a cold light was the object of an interesting controversy in the nineteenth century: does it fit into thermodynamics? This point is discussed in Box 1.1.

1.2.2
On the Distinction between Fluorescence and Phosphorescence: Decay Time Measurements

It is important to mention that Stokes viewed fluorescence as an instantaneous scattering process that ceases immediately after the exciting light is cut off. Thus, the phenomenon that he called internal dispersion would correspond in this respect to what is now known as inelastic scattering, for example, vibrational Raman scattering, and not to the post-quantum description of fluorescence as a two-step process with a finite waiting time between absorption and emission. Interestingly, such a connection can be found in the well-known terminology of Raman lines as either Stokes or anti-Stokes. Nevertheless, in vibrational Raman scattering, a characteristic and fixed emission spectrum does not exist, and it is only the shift in energy that is constant and specific of the molecular vibrations.

Becquerel, on the other hand, considered that phosphorescence and Stokes' fluorescence were one and the same emission phenomenon, always with a finite duration that was simply shorter in the case of fluorescence and longer in the case of phosphorescence. He even advocated the term fluorescence to be abandoned, considering that fluorescence was but a short-lived phosphorescence.

However, such a distinction only based on the duration of emission is not sound. In fact, we now know that there are long-lived fluorescences whose decay times are comparable to those of short-lived phosphorescences (ca. 0.1–1 μs). The first theoretical distinction between fluorescence and phosphorescence was provided by Francis Perrin (Jean Perrin's son) in his doctoral thesis[12)]: "if the molecules pass, between absorption and emission, through a stable or unstable intermediate state . . . , there is phosphorescence." This fact is a major importance in the conception of an energy diagram describing the phenomena (see the next section).

In any case, it is of great interest to measure the decay time of luminescence. In that matter, Edmond Becquerel's pioneering work deserves attention. In 1858, he started measuring the decay times of the phosphorescence of various compounds by means of a remarkable instrument called phosphoroscope[13)]: this was

10) Przibram, K. (1956) *Irradiation Colours and Luminescence*, Pergamon: London.

11) Calderon, T., Khanlary, M.-R., Rendell, H.M., and Townsend, P.D. (1992) *Nucl. Tracks Radiat. Meas.*, **20**, 475–485.

12) Perrin, F. (1929) *Ann. Phys. (Paris)*, **12**, 169–275.

13) Becquerel, E. (1867) *La Lumière. Ses Causes et ses Effets*, vol. 1, Firmin Didot: Paris.

Box 1.1 Does luminescence fit into thermodynamics? [1,2]

In the late nineteenth century, the question arose whether luminescence (cold light) violates the second law of thermodynamics according to which heat cannot flow from a colder body to a warmer body. In 1889, Wiedemann envisioned a case where the second law seems to be violated: a luminescent material could transfer radiant energy to an object having a higher temperature if this object absorbed the luminescence. To rescue the second law, Wiedemann introduced the concept of luminescence temperature that is the temperature required for the incandescent emission from a body to match the intensity of the body's luminescence. But this concept was found to be unnecessary because a fundamental distinction should be made between energy transferred from a body with a well-defined temperature (i.e., in internal thermal equilibrium) and energy transferred from a body not in internal thermal equilibrium.

What about Stokes' law in the framework of thermodynamics? At the end of the nineteenth century, the Berlin physicist Wilhelm Wien considered that this law was simply a special case of the second law. But several cases of violation of Stokes' law were reported. The first of them is due to Eugen Lommel in 1871: upon excitation of a solution of a dye (naphthalene red) with the yellow lines from a sodium flame, he was able to detect a weak green fluorescence, that is, of shorter wavelength [3]. The contamination of the light source was suspected by other researchers. In 1886, after checking carefully that no extraneous light contaminated his experiments, Franz Stenger studied not only naphthalene red, but also fluorescein and eosin: he found that all samples showed fluorescence at shorter wavelengths than excitation [4]. Wien and also Karl von Wesendonck [5] considered that in the cases where Stokes' law fails, there must be an increased absorption of energy by the fluorescent species.

Additional evidence for Stokes' law violation was provided in 1904 by Edward Nichols and Ernest Merritt, physicists at Cornell University, who were able to record the fluorescence spectra of naphthalene red, fluorescein and eosin [6]. In fact, the spectra extended beyond the short-wave limits of the exciting light. Stokes' law violation happens only in the region where the absorption and fluorescence curves overlap.

A major event in the turn of the nineteenth century was Planck's theory of quanta that Albert Einstein applied to the photoelectric effect, and also to luminescence. Considering that the energy of the absorbed and emitted light quanta (later on called photons) should be proportional to their respective frequencies, Stokes' law simply obeys the first law of thermodynamics (conservation of energy). But how can the exceptions to Stokes' law be explained? The bell-shaped intensity curves for emission suggest a statistical process. Einstein proposed that molecular motion provides the additional energy required for the violation of Stokes' law. If this assumption is correct, then the departure from Stokes' law should be larger at higher temperatures. A discussion between Einstein and Joseph von Kowalski on this topic led the latter to study the effect of temperature on the emission of rhodamine. The results showed agreement (within an order

of magnitude) with calculations based on Einstein's assumption [7]. As vibrational energy is converted into radiation, cooling of the medium can occur upon anti-Stokes emission. An interesting consequence is laser cooling of solids, a subject where significant developments occurred over the last decade [8].

1 Malley, M. (1991) *Arch. Hist. Exact Sci.*, **42**, 173–186.
2 Malley, M. (1994) *Ann. Sci.*, **51**, 203–224.
3 Lommel, E. (1871) *Ann. Phys. Chem.*, **143**, 26–51.
4 Stenger, F. (1886) *Ann. Phys. Chem.*, **28**, 201–230.
5 von Wesendonck, K. (1897) *Ann. Phys. Chem.*, **62**, 706–708.
6 Nichols, E.L. and Merritt E. (1904) *Phys. Rev.*, **18**, 403–418.
7 Kowalski, J. (1910) *Le Radium*, **7**, 56–58.
8 Ruan, X.L. and Kaviany, M. (2007) *J. Heat Transfer*, **129**, 3–10.

the very first time-resolved photoluminescence experiment. The instrument consists of two disks rotating together at variable speeds up to 3000 revolutions per second. The sample is placed between the two disks. Each disk possesses four windows in such a way that the incident light cannot go through the second disk (Figure 1.5), and therefore, there is a time lag between excitation and observation of emission that depends on the speed of rotation. By changing the latter, the intensity of emission can be measured as a function of time. Phosphorescence lifetimes shorter than 0.1 ms could be determined in this way.

Such a time resolution was however insufficient for the measurement of fluorescence lifetimes that are in the nanosecond range. Much progress in instrumentation was to be made for achieving this goal. In the 1920s, Enrique Gaviola, born in Argentina, went to P. Pringsheim's laboratory in Berlin where he built the first phase fluorometer allowing measurement of nanosecond lifetimes. He measured the lifetimes of fluorescein and rhodamine B, among other compounds.

Independently, an indirect method of determination based on steady-state fluorescence polarization was proposed by Francis Perrin in 1926 (see Section 1.2.4) and successfully applied in particular to fluorescein and erythrosin, the last one with a short lifetime (ca. 90 ps in water) below Gaviola's time resolution.

The present state of the art for measuring lifetimes is described in Chapter 10.

1.2.3
The Perrin–Jablonski Diagram

For describing the processes subsequent to light absorption by a molecule, it was found convenient to use an energy diagram in which the electronic states of the molecule are represented together with arrows indicating the possible transitions between them. Figure 1.6 displays a simplified diagram, while a modern and more detailed diagram is shown in Figure 3.1 of Chapter 3. Since the 1970s, this diagram is most often called the Jablonski diagram (from the name of the Polish physicist Aleksander Jablonski). However, it should be called the Perrin–Jablonski diagram

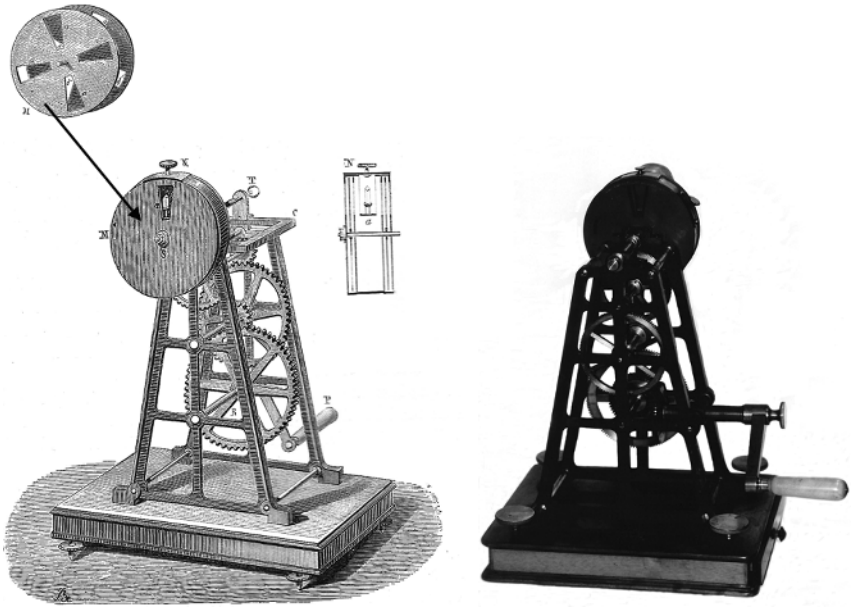


Figure 1.5 Edmond Becquerel's phosphoroscope.[13] The speed of rotation of both disks bearing four windows can reach 3000 revolutions/s, which allowed analyzing phosphorescence decays whose time constant is shorter than 0.1 ms. The phosphoroscope on the right belongs to the Musée des Arts et Métiers in Paris.

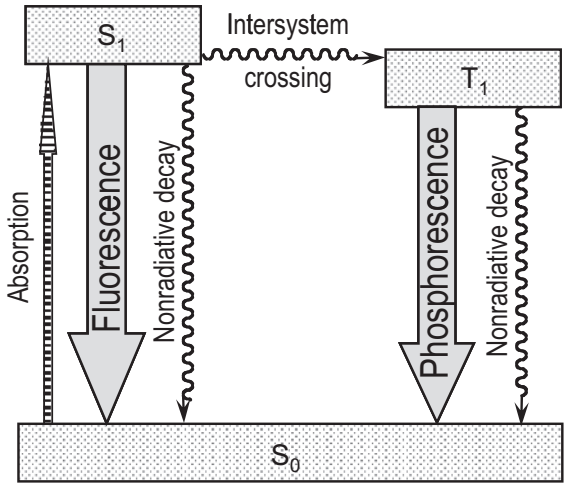


Figure 1.6 Simplified Perrin–Jablonski diagram. Fluorescence is an emission from the first excited singlet state S_1 that is reached upon light absorption. Phosphorescence is an emission from the triplet state T_1 after intersystem crossing from S_1.

in order to give appropriate credit to the contributions of the French physicists Jean and Francis Perrin. Some comments on this point are to be made.[14),15)]

It should be first noted that the diagram described for molecules is an extension of the Bohr–Grotrian diagram for atoms that was proposed in the 1920s. Regarding molecules, the first use of an energy level diagram showing the absorption and emission of light is probably due to Jean Perrin who correctly explained the phenomenon of thermally activated delayed fluorescence by including a metastable state in his diagram.[16)] In his doctoral thesis, Francis Perrin discusses in detail this model.[12)]

Surprisingly, G. N. Lewis attributed the thermally activated delayed fluorescence (called by him the alpha process) to Jablonski and not to Perrin. Moreover, he created a misnomer when he decided to refer to his own diagram as the "Jablonski diagram."[14)] In fact, most of the characteristics of the diagram, as presently perceived, are not due to Jablonski.

Regarding the diagram of Jean and Francis Perrin, it is incomplete because the metastable intermediate state cannot revert radiatively or otherwise to the ground state. That is the merit of Jablonski's work[17)] to allow such a transition, rendering possible a second emission at longer wavelengths (true phosphorescence). This is the only (and crucial) point where a difference exists between the Perrin and Jablonski schemes.[15)]

Later on, the nature of the intermediate state was established by A. Terenin (1943) and by G. Lewis and M. Kasha (1944): it is a triplet state (see the definition in Chapter 2) in contrast to the singlet excited state reached upon light absorption from the ground state. Thus, fluorescence appears to be an emission process without change in state multiplicity, in contrast to phosphorescence.

1.2.4
Fluorescence Polarization

The polarization state of fluorescence (discussed in Chapter 7) is an important aspect that was investigated almost from the beginning of fluorescence studies. In 1833, Sir David Brewster described for the first time the beautiful red fluorescence of chlorophyll, observed by passing a beam of sunlight through a green alcoholic extract of leaves. He explained fluorescence in general as light scattering by minute particles in suspension, as Haüy did before him. Herschel studied concentrated solutions of quinine sulfate in 1845. The observed fluorescence was considered by him a superficial phenomenon and named epipolic dispersion. He also found the fluorescence to be unpolarized. In 1848, Brewster rejected Herschel's interpretation but confirmed that the fluorescence was not polarized. This property contradicted Brewster's initial explanation, since light scattered by small,

14) Nickel, B., *EPA Newslett.*, (1996) **58**, 9–38; (1997) **61**, 21–60; (1998) **64**, 19–72.
15) Berberan-Santos, M.N. (2001) In: *New Trends in Fluorescence Spectroscopy. Applications to Chemical and Life Sciences* (eds B. Valeur and J.C. Brochon), Springer-Verlag, Berlin, pp. 7–33.
16) Perrin, J. (1922) *Trans. Faraday Soc.*, **17**, 546–572.
17) Jablonski, A. (1935) *Z. Phys.*, **94**, 38–46.

structureless particles is always strongly polarized. He then concluded that ". . . unless this [. . .] is a new property of light, produced by a peculiar action of certain solid and fluid bodies . . ." the scattering particles must be minute double-refracting crystals randomly oriented, with the consequence that unpolarized light is sent in all directions.

Stokes, who first recognized the true origin of fluorescence, also noticed the unpolarized nature of the fluorescence of fluid solutions, and this aspect was even used to separate fluorescence from scattered light. Nevertheless, Stokes ended his second paper on fluorescence[8)] with the observation that the green fluorescence of several solid platinum cyanides (first observed by Brewster) is polarized. He also mentioned that the respective solutions are nonfluorescent.

Gregorio Weber (1916–1997)

It was only in 1920 that Weigert found the fluorescence of dyes dissolved in viscous solvents like glycerol to be partially polarized: "The degree of polarization of the fluorescent light increases with the increase in the molecular weight, with increase in viscosity of the medium and with decrease in temperature, also with reduction of mobility of the single particle."[18)] Vavilov and Levschin[19)] proposed in 1923 that the origin of depolarization was molecular rotation. In 1926, Francis Perrin derived the equation that bears his name relating polarization with molecular size, fluorescence lifetime, temperature, and solvent viscosity[15),20)] (given in Section 7.7.1.2).

From the 1950s, Gregorio Weber made several important contributions in the area of polarized fluorescence, both theoretical and experimental, opening the way to other developments and to many applications, namely in the life sciences.[21)]

18) Weigert, F. (1920) *Verh. d. D. Phys. Ges.*, **1**, 100–102.
19) Vavilov, S.J. and Levshin, W.L. (1923) *Z. Phys.*, **16**, 135–154.
20) Perrin, F. (1926) *Compt. Rend.* **182**, 219; *J. Physique* **7**, 390–401.
21) Jameson, D.J. (2001) *New Trends in Fluorescence Spectroscopy. Applications to Chemical and Life Sciences* (eds B. Valeur and J. C. Brochon), Springer-Verlag, Berlin, pp. 35–58.

1.2.5
Resonance Energy Transfer

The first observations of the nonradiative transfer of excitation energy – also called resonance energy transfer (RET) – from an excited species to another one were reported with atoms in the gas phase: G. Cario and J. Franck showed in 1922 that upon selective excitation of mercury atoms at 254 nm in a vapor mixture with thallium atoms, sensitized emission of the latter can be detected at 535 nm. A quantum theory of resonance energy transfer via dipole–dipole interaction in the gas phase was developed by H. Kallman and F. London in 1928. The concept of critical radius (distance at which transfer and spontaneous decay of the excited donor are equally probable) was introduced for the first time.

Theodor Förster (1910–1974)

In solution, when increasing the concentration of fluorescein in a viscous solvent, E. Gaviola and P. Pringsheim observed in 1924 that the fluorescence polarization gradually decreases, but did not explain the result. It was only in 1929 that Francis Perrin correctly explained it as a consequence of homotransfer. Years before, in 1925, his father Jean Perrin proposed the mechanism of resonance energy transfer. F. Perrin developed in 1932 a quantum mechanical theory of homotransfer and qualitatively discussed the effect of the spectral overlap (between the emission spectrum of the donor and the absorption spectrum of the acceptor).

A complete theory of RET via dipole–dipole interaction was developed by Theodor Förster[22] from 1946 and based on both classical and quantum mechanical approaches (see Chapter 8). This is a very important milestone in the history of fluorescence.

Instead of RET, the term FRET first appeared in papers relevant to life sciences, as the acronym of fluorescence resonance energy transfer. But this is a misnomer because fluorescence does not intermediate resonance energy transfer, which is considered a nonradiative process (see Chapter 8, Section 8.4). However, the

22) Summaries of Förster's biography and scientific achievements can be found in: Porter, G. (1976) *Naturwiss*, **63**, 207; Kramer, H.E.A., and Fischer, P. (2011) *ChemPhysChem*, **12**, 555.

acronym FRET is so widely used that the solution to overcome this situation – and a way to acknowledge the author for an outstanding contribution to this field – is to consider that F in FRET stands for "Förster" or "Förster-type" rather than "fluorescence." However, resonance energy transfer is not limited to Förster-type transfer, that is, via dipole–dipole interaction (as shown in Section 8.4.1).

Since the end of the 1970s, (F)RET has been used as a "spectroscopic ruler": in fact, it allows one to measure the distance between a donor chromophore and an acceptor chromophore in the 1–10 nm range. It also permits monitoring of the approach or separation of two species. (F)RET has found numerous applications in photophysics, photochemistry and photobiology.

1.2.6
Early Applications of Fluorescence

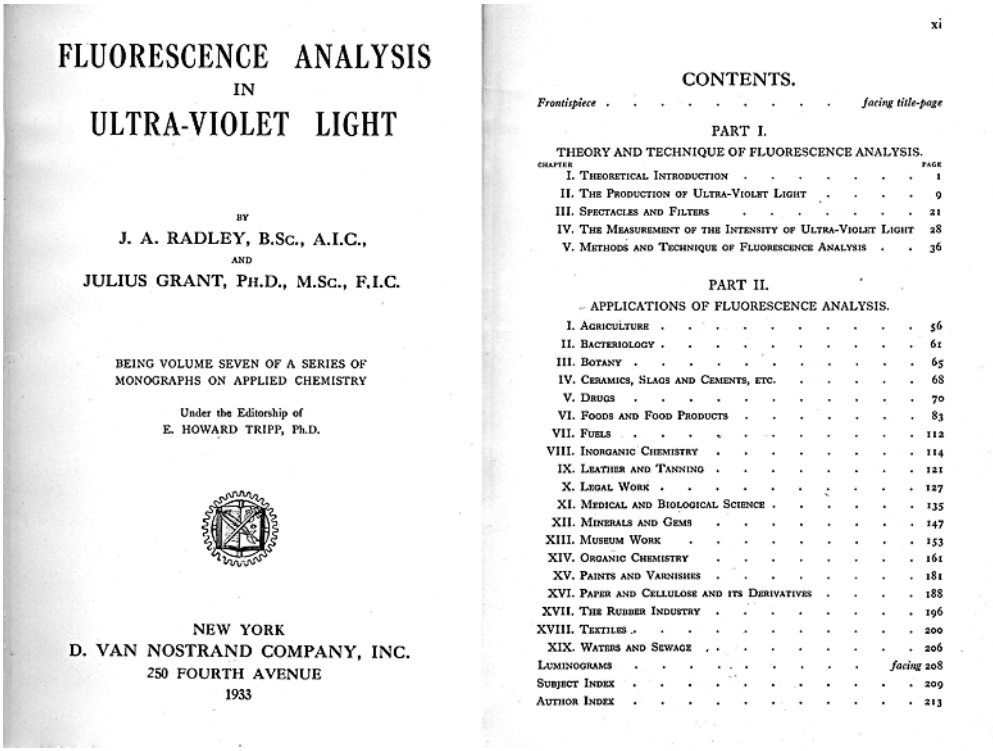
Fluorescent tubes and lamps are familiar to anybody, but who knows that the idea of coating the inner surface of an electric discharge tube with a luminescent material was conceived by Edmond Becquerel in 1857, and probably German scientists at the same time? These tubes were similar to the fluorescent tubes that are made today, but their efficiency and lifetime were insufficient for practical application to lighting. The first commercially available tubes appeared in the late 1930s and were based on the discharge in mercury vapor at low pressure that produces UV for exciting the fluorescent compounds of the inner coating. At the beginning, the latter was made of zinc orthosilicate (with varying content of beryllium) and magnesium tungstate, and was soon replaced by doped calcium halophosphates. In present tubes and compact fluorescent light bulbs, lanthanide (rare-earth) compounds such as Eu(II), Eu(III), and Tb(III) are employed: they produce blue, red, and green lights, respectively, which yields white light by additive synthesis.

Fluorescence as an analytical tool is also one of the first applications of fluorescence.[23] The first paper on this topic was published in 1862 by Victor Pierre[24] who was a professor in Prague, and later in Vienna. He studied solutions of single fluorescent compounds and mixtures: he observed that bands of fluorescent spectra were characteristic of a particular substance. He also noted the effect of solvent and acidity or alkalinity (it should be remarked that the acid/base effect on fluorescence had already been described by Boyle in the seventeenth century, although fluorescence was not understood at that time). The term *fluorescence analysis* was employed for the first time by F. Göppelsröder in 1868[25]: he described the complexation of morin (a hydroxyflavone derivative) with aluminum that is

23) O'Haver, T.C. (1978) *J. Chem. Educ.*, **55**, 423–428.

24) Pierre, V. (1862) Über die Anwendung der Fluorescenz zur Erkennung von fluorescirenden Stoffe [On the use of fluorescence for the detection of fluorescent compounds]. *Sitzber. Böhm. Ges. Wiss. Prag.*, **2**, 66–82, 82–85.

25) Göppelsröder, F. (1868) Über eine fluorescirende Substanz aus dem Kuba-holze (Fortsetzung) and über Fluorescenzanalyse [On a fluorescent substance extracted from Cuba wood and on fluorescence analysis]. *J. Prakt. Chem.*, **104**, 10–27. Note that in the first paper of the series published in 1867 (*J. Prakt. Chem.*, **101**, 408), and often erroneously cited as the first reported application of fluorescence to analysis, the application to aluminum detection was not described.



FLUORESCENCE ANALYSIS
IN
ULTRA-VIOLET LIGHT

BY
J. A. RADLEY, B.Sc., A.I.C.,
AND
JULIUS GRANT, PH.D., M.Sc., F.I.C.

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CONTENTS.

Figure 1.7 Contents of the book of Radley and Grant (1933) showing the numerous applications of fluorescence to analysis.

accompanied with a drastic enhancement of fluorescence intensity, offering thus a straightforward way to detect this metal. Then, fluorescence analysis became more and more extensively used, as demonstrated for instance by the impressive list of applications reported in the book of Radley and Grant[26] published in 1933 (Figure 1.7). Nowadays, fluorescence sensing of chemical species is still a very active field of research (see Chapter 14).

Another early application of fluorescence is the use of a fluorescent dye as a tracer in hydrogeology. In 1877, uranin (the disodium salt of fluorescein) was used for monitoring the flow of the Danube River. On all maps, it is shown that the Danube springs in the Black Forest and, after many hundreds of kilometers, flows into the Black Sea. But there are several sinks (swallow holes) in the bed of Danube. The biggest one is near Immendingen. Ten liters of a concentrated solution of uranin was poured by Knop into the bed of the upper current of the Danube, and 50 hours later, the fluorescence could be observed in the water of

26) Radley, J.A., and Grant, J. (1933) *Fluorescence Analysis in Ultraviolet Light*, Van Nostrand Co., New York.

the river Aache 12 km to the south. This river flows into the lake Constanz that feeds the Rhine. Therefore, only a small part of the water from the Danube spring arrives at the Black Sea. Most of it flows into the North Sea! Nowadays, fluorescence tracing is currently used in hydrogeology, especially to simulate and trace the discharge of pollutants.

Numerous applications of fluorescence in various fields were developed in the twentieth century and still emerge in the twenty-first century. They are presented in the third part of the present book.

1.3 Photoluminescence of Organic and Inorganic Species: Fluorescence or Phosphorescence?

The definitions of fluorescence and phosphorescence, as given in the *Glossary of Terms Used in Photochemistry* published by the International Union of Pure and Applied Chemistry,[1)] are as follows:

Fluorescence: spontaneous emission of radiation (luminescence) from an excited molecular entity with retention of spin multiplicity.

Phosphorescence: phenomenologically, term used to describe long-lived luminescence. In mechanistic photochemistry, the term designates luminescence involving change in spin multiplicity, typically from triplet to singlet or vice versa. (Note: e.g., the luminescence from a quartet state to a doublet state is also phosphorescence.)

These definitions apply to organic molecules which are the main object of the present book. However, other emitting species such as nanocrystalline semiconductors (quantum dots) and metallic nanoparticles are of great interest for applications (see Chapter 4 for their emission in relation to their structure, and other chapters for applications). The concept of spin multiplicity is not relevant to these species, but the terms "fluorescent quantum dots" and "fluorescent gold nanoparticles," for instance, are often employed in the literature. Extended definitions of fluorescence and phosphorescence are thus desirable. Returning to the early discussions on the distinction between fluorescence and phosphorescence (Section 1.2.2), it is convenient to consider that, generally speaking, fluorescence is an emission from an excited state that can be reached by direct photoexcitation, whereas phosphorescence is emitted from another excited state, with a corresponding forbidden radiative transition.

The case of semiconductors deserves special attention. Irradiation creates electrons and holes. When an electron and a hole recombine immediately, the emitted light can be called fluorescence. But they do not recombine rapidly if they are trapped in some metastable states. Then, release from the traps requires energy, and the subsequent recombination is accompanied by the emission of a photon. In that case, emission is called phosphorescence and is temperature dependent in

contrast to fluorescence. Such a temperature-based distinction between fluorescence and phosphorescence does not apply to organic species for which the fluorescence quantum yield is temperature dependent.

Regarding nanocrystalline semiconductors (quantum dots), they are often considered as fluorescent species, but the emission processes are so complex that the term "luminescent quantum dots" should be preferred to "fluorescent quantum dots."

Whenever there is a doubt on the nature of the states involved in the emission process (this is for instance the case of gold and silver nanoparticles, see Chapter 4), the term photoluminescent, or simply luminescent, should be employed.

1.4 Various De-Excitation Processes of Excited Molecules

Once a molecule is excited by absorption of a photon, it can return to the ground state with emission of fluorescence, or phosphorescence after intersystem crossing, but it can also undergo intramolecular charge transfer and conformational change. Interactions in the excited state with other molecules may also compete with de-excitation: electron transfer, proton transfer, energy transfer, excimer or exciplex formation (Figure 1.8). These de-excitation pathways may compete with fluorescence emission if they take place on a time-scale comparable with the average time (lifetime) during which the molecules stay in the excited state. This average time represents the experimental time window for observation of dynamic processes. The characteristics of fluorescence (spectrum, quantum yield, lifetime),

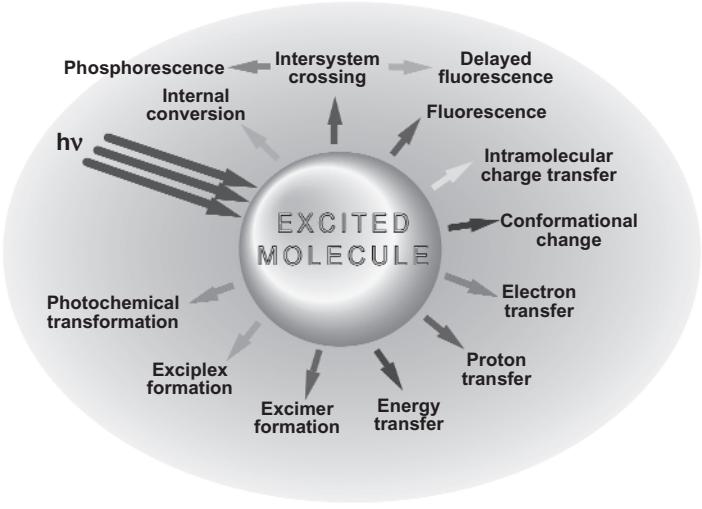

Figure 1.8 Possible de-excitation pathways of excited molecules.

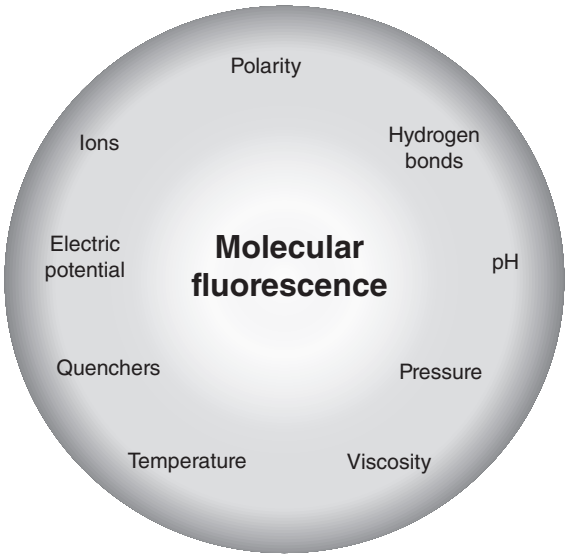

Figure 1.9 Various parameters influencing the emission of fluorescence.

which are affected by any excited-state process involving interactions of the excited molecule with its close environment, can then provide information on such a microenvironment. It should be noted that some excited-state processes (conformational change, electron transfer, proton transfer, energy transfer, excimer or exciplex formation) may lead to a fluorescent species whose emission can superimpose that of the initially excited molecule. Such an emission should be distinguished from the "primary" fluorescence arising from the excited molecule. The success of fluorescence as an investigative tool in studying the structure and dynamics of matter or living systems arises from the high sensitivity of fluorometric techniques, the specificity of fluorescence characteristics due to the microenvironment of the emitting molecule, and the ability of the latter to provide spatial and temporal information. Figure 1.9 shows the physical and chemical parameters that characterize the microenvironment and can thus affect the fluorescence characteristics of a molecule.

1.5
Fluorescent Probes, Indicators, Labels, and Tracers

As a consequence of the strong influence of the surrounding medium on fluorescence emission, a fluorescent species, usually called *fluorophore*, is currently used to get information on a local parameter that is physical, structural or chemical (Figure 1.10). The term *fluorescent probe* is commonly used, but in the particular case of a chemical parameter like pH or the concentration of a species, the term

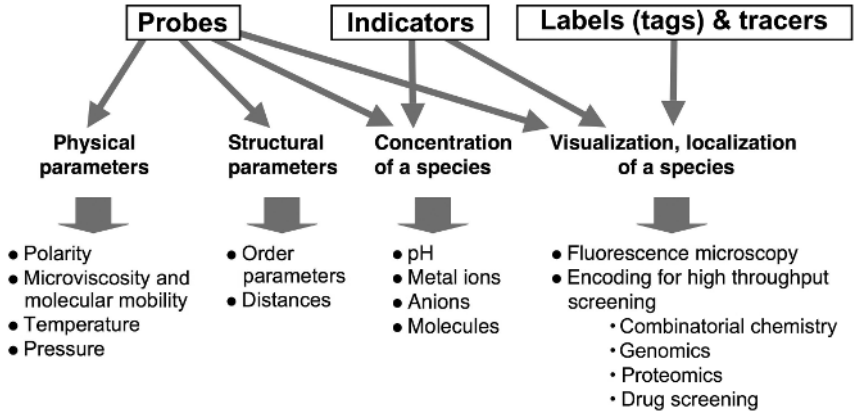

Figure 1.10 Information provided by fluorescent probes, indicators, labels (or tags), and tracers.

fluorescent indicator may be preferred (e.g., fluorescent pH indicator). On the other hand, when a fluorescent molecule is used to visualize or localize a species, for example, by using microscopy, the terms *fluorescent labels* (or *tags*) and *tracers* are often employed. This implies that a fluorescent molecule is covalently bound to the species of interest: surfactants, polymer chains, phospholipids, proteins, oligonucleotides, and so on. For instance, protein tagging can be easily achieved by means of labeling reagents having proper functional groups: for instance, covalent binding is possible on amino groups.

The hydrophilic, hydrophobic, or amphiphilic character of a fluorophore is essential. In microscopy, selective interaction of the fluorophore with specific parts of the system under study (cell, tissue, etc.), allowing their visualization, is often called staining, a term traditionally used for colored dyes.

Intrinsic fluorophores are ideal as probes and tracers but there are only a few examples found in biology (e.g., tryptophan, NADH, flavins).

Owing to the difficulty of synthesis of molecules or macromolecules with covalently bound fluorophores, many investigations are carried out with noncovalently associating fluorophores. The sites of solubilization of such extrinsic probes are governed by their chemical nature and the resulting specific interactions that can be established within the region of the system to be probed. The hydrophilic, hydrophobic, or amphiphilic character of a fluorophore is essential. A criticism often aimed at the use of extrinsic fluorescent probes is the possible local perturbation induced by the probe itself on the microenvironment to be probed. There are indeed several cases of systems perturbed by fluorescent probes. However, it should be emphasized that many examples of results consistent with those obtained by other techniques can be found in the literature (transition temperature in lipid bilayer, flexibility of polymer chains, etc.). To minimize the perturbation, attention must be paid to the size and shape of the probe with respect to the probed region. If possible, more than one probe should be used for a consistency check.

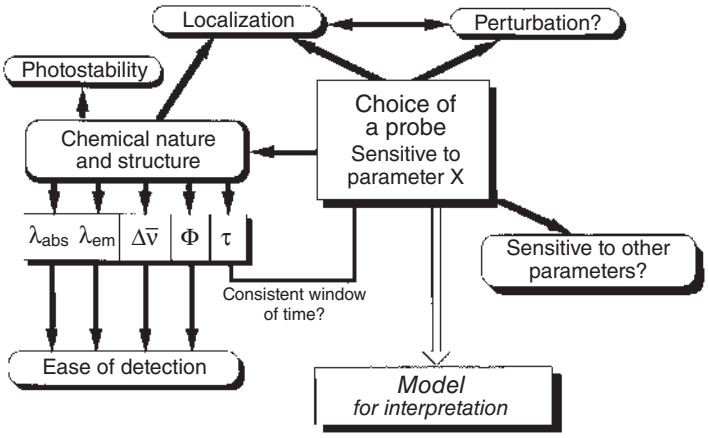

Figure 1.11 Strategy for the choice of a fluorescent probe. $\Delta\bar{\nu}$, Φ, avnd τ are the Stokes shift, quantum yield, and lifetime, respectively (see definitions in Chapter 3).

The choice of a fluorescent probe is crucial for obtaining unambiguous interpretations. The major aspects that should be taken into consideration are shown in Figure 1.11.

Fluorescent probes and tracers can offer a wealth of information in various fields, as shown in Table 1.3. The various examples described in this book will demonstrate their outstanding versatility. It should be recalled that other types of probes and tracers are used in practice: for example, radioactive tracers, with their well-known drawback of their radioactivity, and EPR (electronic paramagnetic resonance) probes that provide information mainly on molecular mobility. In contrast to fluorescent probes, they are used in rather limited fields of applications.

1.6
Ultimate Temporal and Spatial Resolution: Femtoseconds, Femtoliters, Femtomoles, and Single-Molecule Detection

The ability of fluorescence to provide temporal information is of major importance. Great progress has been made since the first determination of an excited-state lifetime by Gaviola in 1926 using a phase fluorometer. A time resolution of a few tens of picoseconds can easily be achieved in both pulse and phase fluorometries by using high repetition rate picosecond lasers and microchannel plate photomultipliers (see Chapter 10). Such a time resolution is limited by the response of the photomultiplier but not by the width of the laser pulse, which can be as short as 50–100 fs (1 femtosecond = 10^{-15} s) (e.g., with a titanium:sapphire laser). The time resolution can be reduced to a few picoseconds with a streak camera. To get an even better time resolution (100–200 fs), a more recent technique based on fluorescence upconversion has been developed (see Chapter 10).

Table 1.3 Information provided by fluorescent probes and tracers in various fields.

Field	Information
Polymers	Dynamics of polymer chains; microviscosity; free volume; oxygen permeability; orientation of chains in stretched samples; miscibility; phase separation; diffusion of species through polymer networks; end-to-end macrocyclization dynamics; monitoring of polymerization; degradation
Solid surfaces	Nature of the surface of colloidal silica, clays, zeolites, silica gels, porous Vycor glasses, alumina: rigidity, polarity and modification of surfaces
Surfactant solutions	Critical micelle concentration; distribution of reactants among particles; surfactant aggregation numbers; interface properties and polarity; dynamics of surfactant solutions; partition coefficients; phase transitions; influence of additives
Biological membranes	Fluidity; order parameters; lipid–protein interactions; translational diffusion; site accessibility; structural changes; membrane potentials; complexes and binding; energy-linked and light-induced changes; effects of additives; location of proteins; lateral organization and dynamics
Vesicles	Characterization of the bilayer: microviscosity, order parameters; phase transition; effect of additives; internal pH; permeability
Proteins	Binding sites; denaturation; site accessibility; dynamics; distances; conformational transition
Nucleic acids	Flexibility; torsion dynamics; helix structure; deformation due to intercalating agents; photocleavage; accessibility; carcinogenesis
Living cells	Visualization of membranes, lipids, proteins, DNA, RNA, surface antigens, surface glycoconjugates; membrane dynamics; membrane permeability; membrane potential; intracellular pH; cytoplasmic calcium, sodium, chloride, proton concentration; redox state; enzyme activities; cell–cell and cell–virus interactions; membrane fusion; endocytosis; viability, cell cycle; cytotoxic activity
Clinical chemistry	Fluoroimmunoassays, protease and kinase assays and other assays

Regarding spatial resolution, fluorescence microscopy in confocal configuration or with two-photon excitation (see Chapter 11) allows the diffraction limit to be approached, which is approximately half the wavelength of the excitation light (0.2–0.3 μm for visible radiation) with the advantage of three-dimensional resolution. The excitation volume can be as small as 0.1 fL (femtoliter). Compared to conventional fluorometers, this represents a reduction by a factor of 10^{10} of the excitation volume. At high dilution ($\approx 10^{-9}$ M or less), fluorophores entering and

leaving such a small volume cause changes in fluorescence intensity. Analysis of these fluctuations (which is the object of fluorescence correlation spectroscopy; see Chapter 12) in terms of autocorrelation function can provide information on translational diffusion, flow rates, and molecular aggregation. Fluctuations can also be caused by chemical reactions or rotational diffusion. The typical lower limit concentration is ~1 fM (femtomol L^{-1}). The progress of these techniques allows studying molecular interactions at the unsurpassed sensitivity of single-molecule detection.

The diffraction limit can be overcome by using a subwavelength light source and by placing the sample very close to this source (i.e., in the near field). The relevant domain is near-field optics (as opposed to far-field conventional optics), which has been applied in particular to fluorescence microscopy. This technique, called near-field scanning optical microscopy (SNOM), is an outstanding tool in physical, chemical, and life sciences for probing the structure of matter or living systems. The resolution is higher than in confocal microscopy, with the additional capability of force mapping of the surface topography, and the advantage of reduced photo-bleaching. Single molecule detection is of course possible by this technique. Recent far field techniques like stimulated emission depletion (STED) and stochastic optical reconstruction microscopy (STORM), discussed in Chapter 11, also allow breaking the diffraction limit.

The first optical detection of a single molecule was reported in 1989 by Moerner and Kador, who detected a single pentacene molecule doped into a *p*-terphenyl crystal (at liquid helium temperature) using absorption with a double modulation technique. Fluorescence excitation spectroscopy on a single molecule was demonstrated for the first time by Orrit and Bernard in 1990. The detection of a single fluorescent molecule in solution was achieved not much later. Therefore, Schrödinger's statement (in 1952) has been outspaced by reality: ". . . we never experiment with just one electron or atom or molecule. In thought experiments we sometimes assume we do, this invariably entails ridiculous consequences."

Single molecule detection offers the possibility of selecting, trapping, sorting, picking, and even manipulating molecules, especially biological macromolecules. Detection and spectroscopy of individual fluorescent molecules thus provide new tools not only in basic research but also in biotechnology and pharmaceutical industries (e.g., drug screening).

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