Fluorescence Sensing

SPECTRAL OBSERVABLES FOR FLUORESCENCE SENSING

The fluorescence intensity can be used to measure the concentration of the fluorescent species. The goal is to measure the concentration of some analyte, not the amount of fluorophore. In the case of blood gases these analytes are pH, pCO_2, and pO_2. Blood electrolytes include Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^-, and many additional analytes that are measured in the clinical laboratory. Fluorescence sensing requires a change in a spectral property response to the analyte. Changes can occur in the intensity, excitation spectrum, emission spectrum, anisotropy, or lifetime of the sensing probe.

The most direct sensing method is when the fluorescence intensity of the probe changes in response to the analyte . Such changes often occur for fluorophores that are subject to collisional quenching by a relevant species, such as oxygen. While conceptually simple, collisional quenching is only useful with a few clinically relevant analytes. It is often inconvenient or unreliable to use intensity changes, which can occur for a wide variety of reasons. For instance, the use of fiber optics is desirable as a means to locate the sensor at the site of interest, and to have the light source and detector remotely located. However, it is difficult to perform quantitation intensity measurements through fibers. Fluorescence microscopy is another instance where intensity measurements are difficult. It is not possible to control the fluorophore concentration at each location in the cell, and the local probe concentration changes continually due to diffusion and/or photobleaching. For such

applications it is important to use measurements which are independent of fluorophore concentration. This can be accomplished using wavelength-ratiometric probes (Figure 1), which display shifts in the absorption or emission spectra upon binding of the analyte. Wavelength- ratiometric probes are desirable because the ratios are independent of the probe concentration. The analyte concentration can then be determined from the ratio of fluorescent intensities measured at two excitation or emission wavelengths.

Another ratiometric method is fluorescence polarization or anisotropy. In this case the analyte causes a change in the anisotropy of the label. Anisotropy measurements are frequently used in competitive immunoassays (a procedure for detecting or measuring specific proteins or other substances) , in which the actual analyte displaces labeled analyte that is bound to specific antibody. This results in a decrease in the anisotropy. Anisotropy values are calculated using the ratio of polarized intensity measurements. The use of an intensity ratio makes the anisotropy measurements independent of fluorophore concentration as long as the measurements are not distorted by autofluorescence or poor signal-to-noise. Fluorescence lifetimes can also be used for sensing (Figure 1). The lifetimes can be measured using either time-domain (TD) or frequency-domain (FD) methods. A few years ago lifetime measurements were regarded as too complex for sensing applications. However, advances in electrooptics technology now make it feasible to perform nanosecond decay time measurements using small inexpensive instruments. The use of

lifetimes for sensing may be the next step in making sensors that display the long-term stability needed in real-world applications.

Optical Properties of Tissues

The design of fluorescence probes for clinical applications is determined in part by the optical properties of water and tissues. In general, the autofluorescence (is the natural emission of light by biological structures such as mitochondria and lysosomes when they have absorbed light, and is used to distinguish the light originating from artificially added fluorescent markers (fluorophores)). from tissues or any biological sample is lower for longer excitation wavelengths.

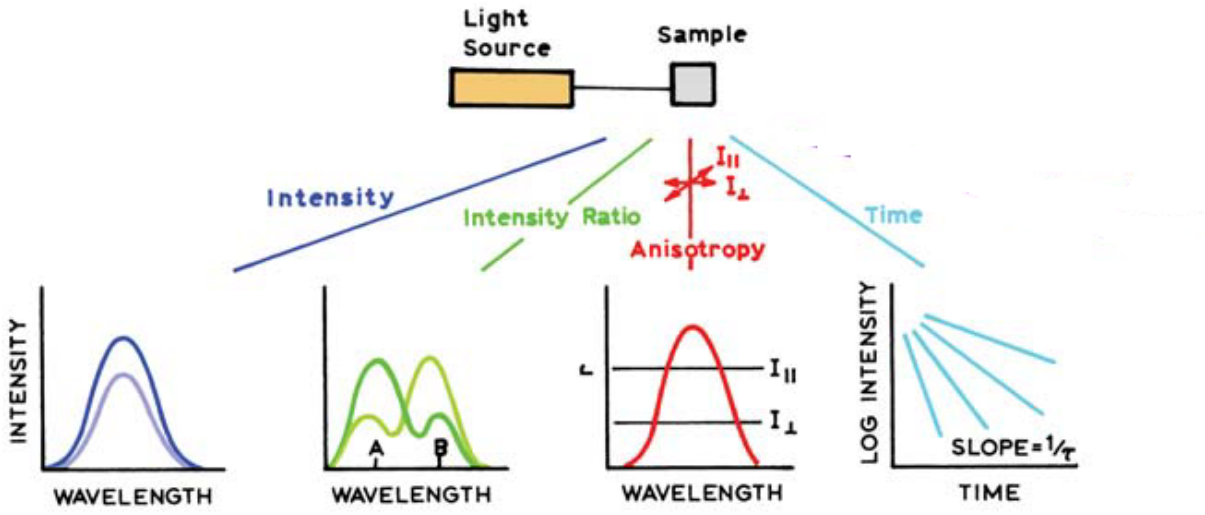


Figure 1. Spectral observables for fluorescence sensing. From left to right, sensing is performed using intensities, intensity ratios, anisotropies, time-domain lifetimes.

The use of longer wavelengths also avoids light absorption by hemoglobin and melanin (Figure 2). In the past there has been a limited number of fluorophores that emit at long wavelengths.

At present there is a growing number of fluorophores that emit between 700 and 1000 nm. This range is useful because water absorption increases above 1000 nm. The region of low absorption from 600 to 1000 nm is sometimes called the therapeutic range. Fortunately, a variety of lasers and solid-state lasers are available for excitation in this range of wavelengths.

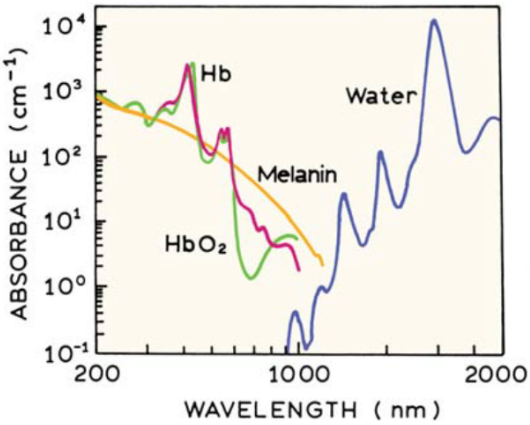


Figure 2. Optical absorbance of tissues and water. Hb, hemoglobin.

MECHANISMS OF SENSING

Any phenomenon that results in a change of fluorescence intensity, wavelength, anisotropy, or lifetime can be used for sensing. The simplest mechanism to understand is collisional quenching, where the fluorophore is quenched by the analyte . Collisional quenching results in a decrease in the intensity or lifetime of the fluorophore, either of which can be used to determine the analyte concentration. Static quenching can also be used for sensing, but the lifetime would not change. Resonance energy transfer (RET) is perhaps the most general and valuable phenomenon for fluorescence sensors. Any

process that brings the donor and acceptor into close proximity will result in a decrease in the donor intensity and/or decay time. Since energy transfer acts over macromolecular distances, it can be used to detect association of proteins as occurs in immunoassays. However, the applications of RET are not limited to detection of protein association. RET has also been used as the basis for pH and cation sensors. Sensors were developed that contain acceptors whose absorption spectra are dependent on pH. A change in pH results in a change in absorbance of the acceptor, which in turn alters the donor intensity.

Another mechanism for sensing is available when the fluorophore can exist in two states, if the fractions in each state depend on the analyte concentration (Figure 3). Typically there is equilibrium between the fluorophore free in solution and the fluorophore bound to analyte. One form can be nonfluorescent, in which case emission is only seen in the absence or presence of analyte, depending on which form is fluorescent. Probes that act in this manner are not wavelength-ratiometric or lifetime probes. Alternatively, both forms may be fluorescent but display different quantum yields or emission spectra. This type of behavior is often seen for pH probes, where ionization of the probe results in distinct absorption and/or emission spectra. Spectral shifts are also seen for probes that bind specific cations such as calcium. Such probes allow wavelength-ratiometric measurements. In this case the change in intensity or shift in the emission spectrum is used to determine the

analyte concentration. Probes that bind specific analytes are often referred to as probes of analyte recognition.

There are many mechanisms that can be used to design probes that exhibit changes in fluorescence in response to analytes. Fluorescence probes can form twisted intramolecular charge-transfer (TICT) states. Another mechanism is photoinduced electron transfer (PET), which has been used to develop sensors for metal ions. These sensors often rely on the well known quenching by amines due to PET. Figure 4 shows a PET-based zinc sensor. In the absence of zinc the anthracene is quenched by exciplex formation with the amino groups. Upon binding of zinc, the nitrogen lone pair of electrons is no longer available for PET. As a result charge transfer no longer occurs, and the anthracene becomes fluorescent. While the mechanism of this particular sensor is understood, this is not true of all sensors. In many cases spectral changes are seen but the mechanism is not certain.

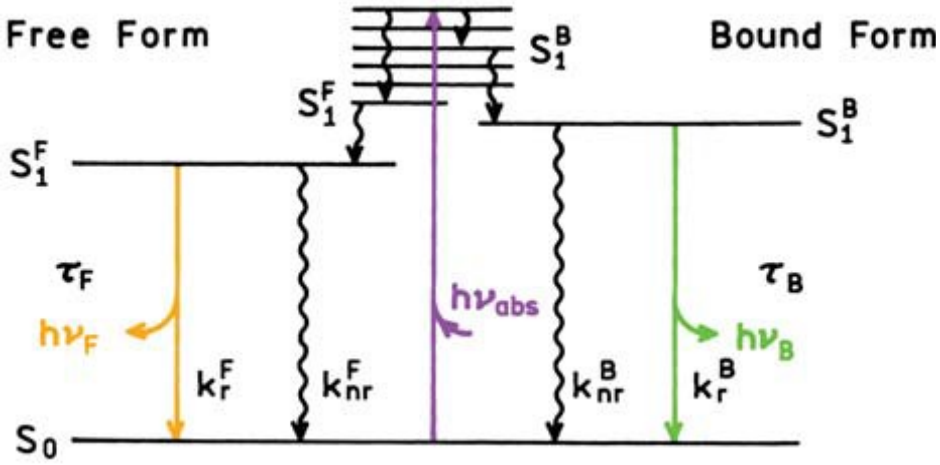


Figure 3. Jablonski diagram for the free (F) and bound (B) forms of a sensing probe.

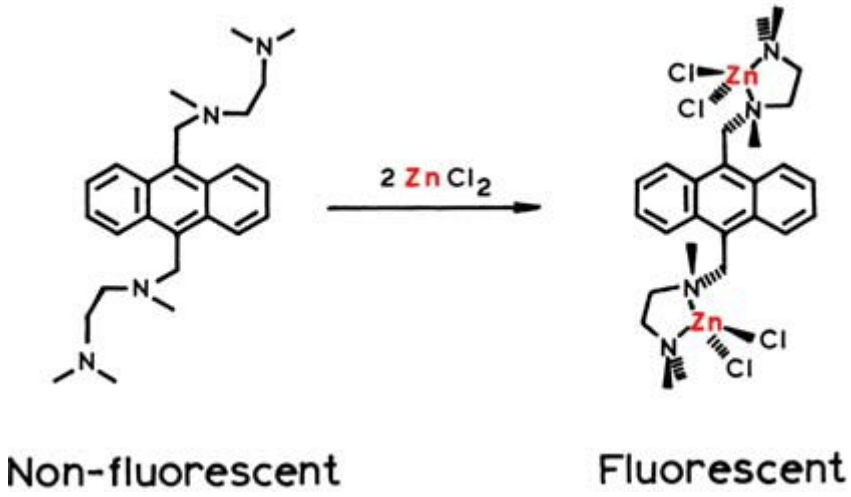


Figure 4. A zinc probe based on photoinduced electron transfer.

Chloride Sensors

It is well known that heavy atoms like bromine and iodine act as collisional quenchers. For sensing applications chloride is more important because it is prevalent in biological systems. However, chloride is a less effective quencher, and relatively few fluorophores are quenched by chloride. A hint for developing chloride-sensitive probes was available from the knowledge that quinine is quenched by chloride. Quinine contains a quinolinium ring, which can be used to make a variety of chloride-sensitive probes. These chloride-sensitive probes can be used to measure chloride transport across cell membranes. Erythrocyte (a red blood cell)ghosts are the membranes from red blood cells following removal of the intracellular contents. The ghosts were loaded with SPQ and 100 mM chloride. The ghosts were then diluted into a solution of 66 mM K_2SO_4. Sulfate does not quench SPQ. When diluted into sulfate-containing buffer the intensity of SPQ increased due to the chloride. This transport is due to an anion exchange

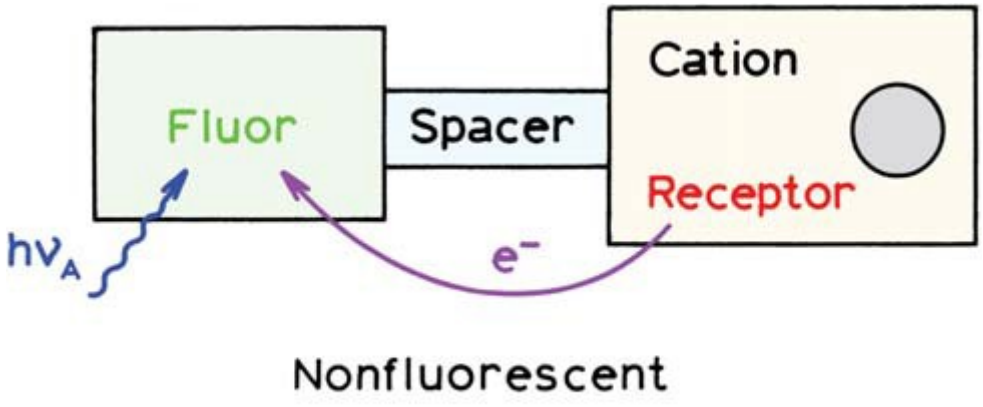
pathway. Chloride transport could be blocked by dihydro-4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (H_2DIDS), which is an inhibitor of anion transport. Hence, the chloride probes can be used for physiological studies of ion transport.

Figure 5. Representative chloride probes. These probes are collisionally quenched by chloride

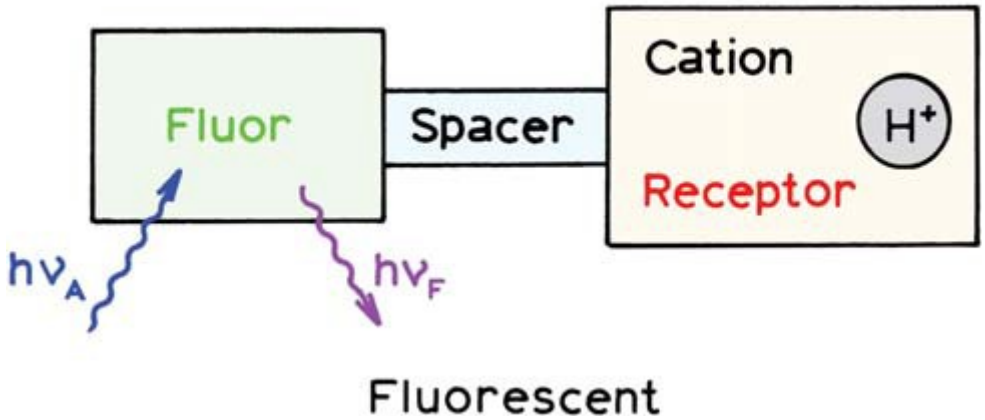
PHOTOINDUCED ELECTRON TRANSFER (PET) PROBES FOR METAL IONS AND ANION SENSORS

probes could be designed based on reversible ionization of a group in conjugation with the aromatic ring (Figure 6). Another mechanism for sensors is the quenching interaction of a linked side chain with the fluorophore. The origin of these probes can be traced to the early studies of exciplex formation of amines with aromatic hydrocarbons. This phenomenon has been exploited to develop sensors based on quenching of fluorophores by amines. The basic idea is that quenching by amines requires the lone pair of electrons localized on the nitrogen (Figure 7). When the fluorophore is in the excited state

these lone pair electrons are in a higher-energy orbital (HOMO, top) than the energy of the vacancy left by the excited electron. Hence, an electron from the nitrogen enters this lower-energy orbital, effectively quenching the fluorescence. If the lone electron pair binds a proton or a cation the energy of this pair is lowered (bottom). Electron transfer is then inhibited and the fluorophore is not quenched. Such probes are said to undergo photo induced electron transfer (PET), which is the light induced transfer of electrons from the nitrogen into the aromatic ring. A simple example of a PET sensor is the alkylamino anthracene shown in Figure 8. At low pH the amino group is protonated and does not quench the anthracene. As the pH is increased, the amino group becomes unprotonated, and the fluorescence decreases due to PET.

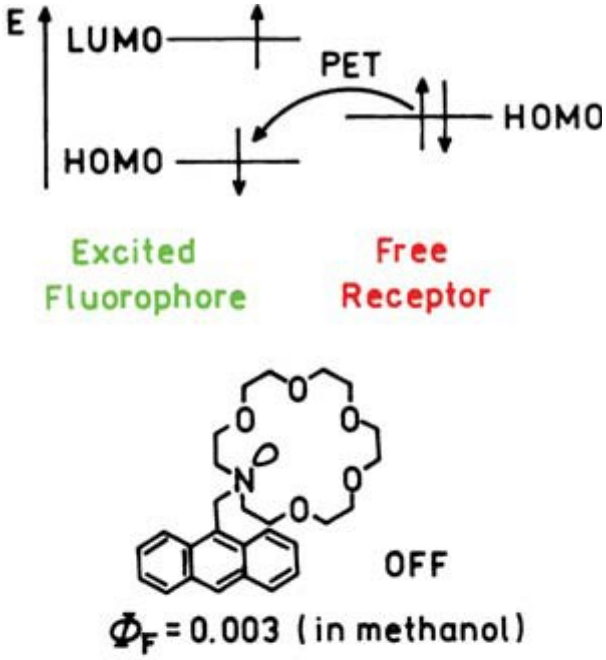


Nonfluorescent



Fluorescent

Figure 6. Chemical sensing based on photo induced electron transfer.



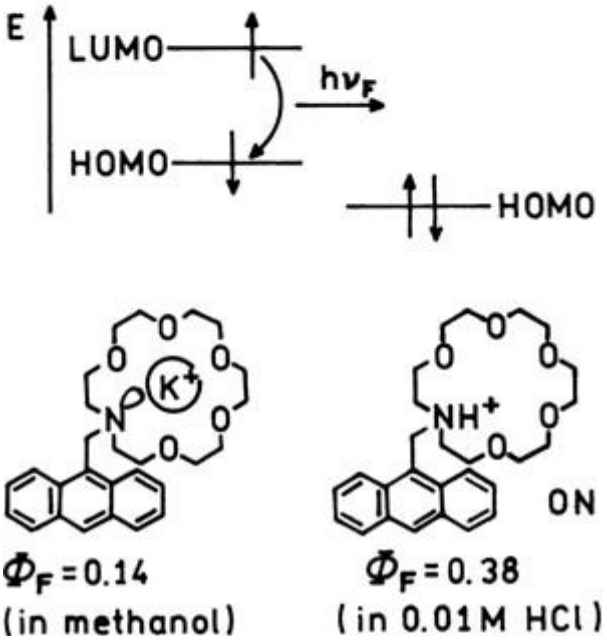


Figure 7. Molecular orbital energy and a typical structure for a PET sensor.

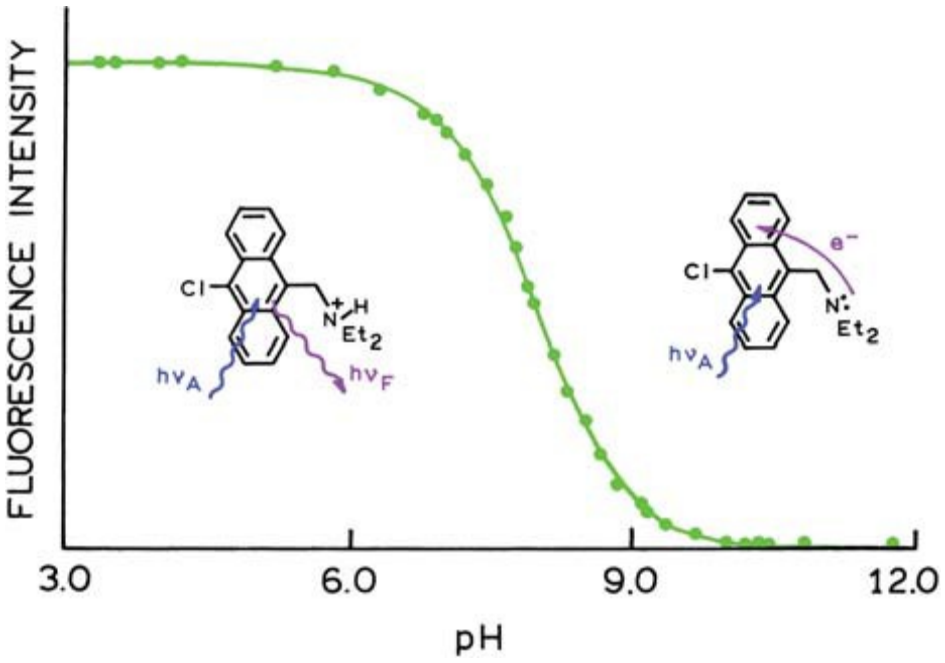


Figure 8. pH-dependent fluorescence of 9-chloro-10-(diethylaminomethyl) anthracene.

PROBES OF ANALYTE RECOGNITION

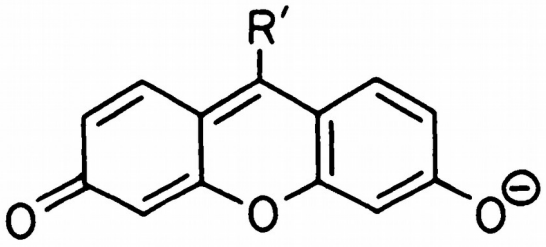
Extensive efforts have been directed toward the design and synthesis of fluorescent probes for cations: Na^+, K^+, Mg^{2+} and especially Ca^{2+}. These efforts can be traced to the discovery of crown ethers and their ability to form complexes with metal ions, and subsequent work to create more complex structures to bind a variety of small molecules. The greatest effort has been in synthesis of probes for calcium, and entire books have been devoted to calcium probes. Much of this work can be traced to the development of intracellular cation probes by Tsien and colleagues. Since these initial publications many additional cation probes have been developed. It is not possible to completely describe this extensive area of research. Instead we describe the most commonly used cation sensors, and the strength and weaknesses of existing probes.

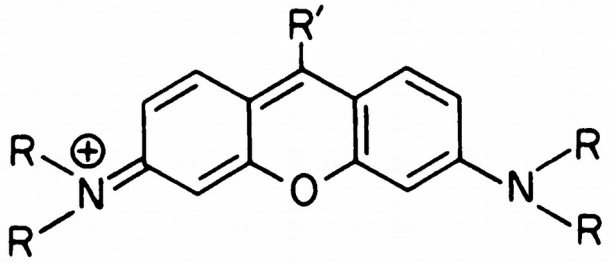
Fluorescence Efficiency of Laser Dyes

The recent development of the dye laser has opened up an important new field of applications for organic dyes. This has led to a renewed interest in the theory of nonradiative transitions in dyes and also to the synthesis of new highly fluorescent dyes. In this article we review briefly the relations between fluorescence and molecular structure in the most important classes of laser dyes: xanthenes, oxazines, and 7-amino coumarins. Following this discussion specific suggestions for improved fluorescence standards are made.

Xanthene Dyes

The chromophore of xanthene dyes has typically the following structures





Depending on the end groups, in particular number and type of the substituents R, the maximum of the main absorption band falls somewhere in the range 480-580 nm. The transition moment is parallel to the long axis of the molecule. With R' = H and amino end groups the dyes are called pyronins. Due to a convenient syntheses with phthalic anhydride many xanthene dyes have R ' = carboxyphenyl and are called fluorescein or rhodamines (fig. 9). The methyl substituents of Rhodamine 6G have practically no influence on the optical properties of the dye except for the dimerization in aqueous solution which we are not concerned with here. The absorption maximum of the main band occurs almost at the same wavelength in pyronins and rhodamines, if the end groups are identical. The absorption band near 350 nm is stronger in rhodamines than in pyronins, and rhodamines show a slightly larger Stokes shift than pyronins. The chemical stability of rhodamines is generally superior, as pyronins in alkaline solution are readily oxidized by dissolved oxygen to form a colorless, blue fluorescing xanthone.

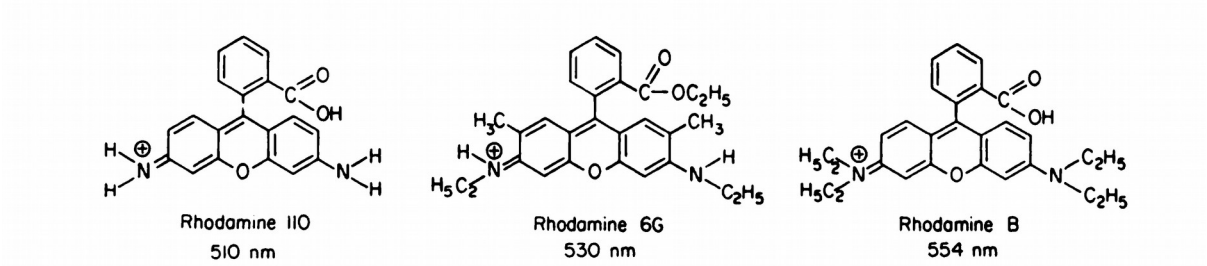


Figure 9. Molecular structure of rhodamine dyes; absorption maximum in ethanol.