Introduction to Fluorescence Techniques

Fluorescent probes enable researchers to detect particular components of complex biomolecular assemblies, including live cells, with exquisite sensitivity and selectivity. The purpose of this introduction is to briefly outline fluorescence techniques for newcomers to the field.

The Fluorescence Process

Fluorescence is the result of a three-stage process that occurs in certain molecules (generally polyaromatic hydrocarbons or heterocycles) called fluorophores or fluorescent dyes. A fluorescent probe is a fluorophore designed to localize within a specific region of a biological specimen or to respond to a specific stimulus. The process responsible for the fluorescence of fluorescent probes and other fluorophores is illustrated by the simple electronic-state diagram (Jablonski diagram) shown in Figure 1.

Stage 1 : Excitation

A photon of energy $h\upsilon_{EX}$ is supplied by an external source such as an incandescent lamp or a laser and absorbed by the fluorophore, creating an excited electronic singlet state (S_1'). This process distinguishes fluorescence from chemiluminescence, in which the excited state is populated by a chemical reaction.

Stage 2 : Excited-State Lifetime

The excited state exists for a finite time (typically 1–10 nanoseconds). During this time, the fluorophore undergoes conformational changes and is also subject to a multitude of possible interactions with its molecular environment. These processes have two important consequences. First, the energy of S_1' is partially dissipated, yielding a relaxed singlet excited state (S_1) from which fluorescence emission originates. Second, not all the molecules initially excited by absorption (Stage 1) return to the ground state (S_0) by fluorescence emission. Other processes such as collisional quenching, fluorescence resonance energy transfer (FRET, see Section 1.3) and intersystem crossing (see below) may also depopulate S_1. The fluorescence quantum yield, which is the ratio of the number of fluorescence photons emitted (Stage 3) to the number of photons absorbed (Stage 1), is a measure of the relative extent to which these processes occur.

Stage 3 : Fluorescence Emission

A photon of energy $h\upsilon_{EM}$ is emitted, returning the fluorophore to its ground state S_0. Due to energy dissipation during the excited-state lifetime, the energy of this photon is lower, and therefore of longer wavelength, than the excitation photon $h\upsilon_{EX}$. The difference in energy or wavelength represented by ($h\upsilon_{EX} - h\upsilon_{EM}$) is called the Stokes shift. The Stokes shift is fundamental to the sensitivity of fluorescence techniques because it allows emission photons to be detected against a low background, isolated from excitation photons. In contrast, absorption spectrophotometry requires measurement of transmitted light relative to high incident light levels at the same wavelength.

Fluorescence Spectra

The entire fluorescence process is cyclical. Unless the fluorophore is irreversibly destroyed in the excited state (an important phenomenon known as photobleaching, see below), the same fluorophore can be repeatedly excited and detected. The fact that a single fluorophore can generate many thousands of detectable photons is fundamental to the high sensitivity of fluorescence detection techniques. For polyatomic molecules in solution, the discrete electronic transitions represented by $h\upsilon_{EX}$ and $h\upsilon_{EM}$ in Figure 1 are replaced by rather broad energy spectra called the fluorescence excitation spectrum and fluorescence emission spectrum, respectively. The bandwidths of these spectra are parameters of particular importance for applications in which two or more different fluorophores are simultaneously detected (see below). With few exceptions, the fluorescence excitation spectrum of a single fluorophore species in dilute solution is identical to its absorption spectrum. Under the same conditions, the fluorescence emission spectrum is independent of the excitation wavelength, due to the partial dissipation of excitation energy during the excited-state lifetime, as illustrated in Figure 1. The emission intensity is proportional to the amplitude of the fluorescence excitation spectrum at the excitation wavelength (Figure 2).

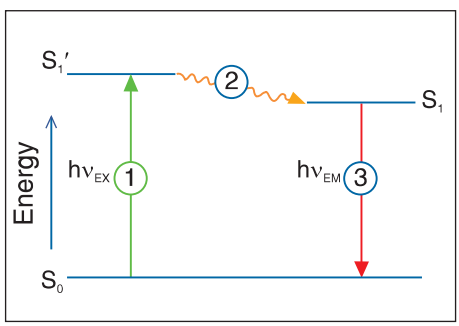

Figure 1 Jablonski diagram illustrating the processes involved in the creation of an excited electronic singlet state by optical absorption and subsequent emission of fluorescence. The labeled stages 1, 2 and 3 are explained in the adjoining text.

Fluorescence Detection

Fluorescence Instrumentation

Four essential elements of fluorescence detection systems can be identified from the preceding discussion: 1) an excitation source, 2) a fluorophore, 3) wavelength filters to isolate emission photons from excitation photons and 4) a detector that registers emission photons and produces a recordable output, usually as an electrical signal or a photographic image. Regardless of the application, compatibility of these four elements is essential for optimizing fluorescence detection.

Fluorescence instruments are primarily of four types, each providing distinctly different information:

- **Spectrofluorometers and microplate readers** measure the *average* properties of bulk (µL to mL) samples.
- **Fluorescence microscopes** resolve fluorescence as a function of spatial coordinates in two or three dimensions for microscopic objects (less than ~0.1 mm diameter).
- **Fluorescence scanners**, including microarray readers, resolve fluorescence as a function of spatial coordinates in two dimensions for macroscopic objects such as electrophoresis gels, blots and chromatograms.
- **Flow cytometers** measure fluorescence per cell in a flowing stream, allowing subpopulations within a large sample to be identified and quantitated.

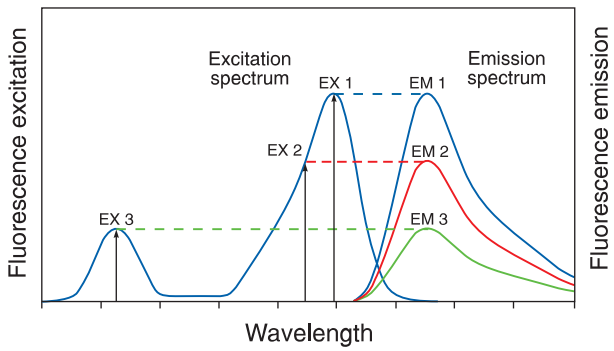

Figure 2 Excitation of a fluorophore at three different wavelengths (EX 1, EX 2, EX 3) does not change the emission profile but does produce variations in fluorescence emission intensity (EM 1, EM 2, EM 3) that correspond to the amplitude of the excitation spectrum.

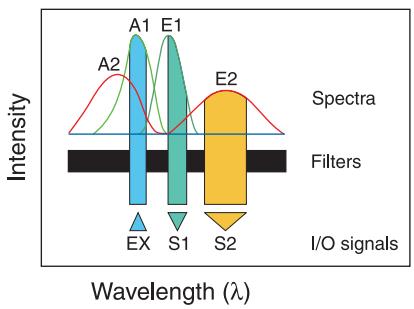

Figure 3 Fluorescence detection of mixed species. Excitation (EX) in overlapping absorption bands A1 and A2 produces two fluorescent species with spectra E1 and E2. Optical filters isolate quantitative emission signals S1 and S2.

Other types of instrumentation that use fluorescence detection include capillary electrophoresis apparatus, DNA sequencers and microfluidic devices. Each type of instrument produces different measurement artifacts and makes different demands on the fluorescent probe. For example, although photobleaching is often a significant problem in fluorescence microscopy, it is not a major impediment in flow cytometry or DNA sequencers because the dwell time of individual cells or DNA molecules in the excitation beam is short.

Fluorescence Signals

Fluorescence intensity is quantitatively dependent on the same parameters as absorbance — defined by the Beer–Lambert law as the product of the molar extinction coefficient, optical pathlength and solute concentration — as well as on the fluorescence quantum yield of the dye and the excitation source intensity and fluorescence collection efficiency of the instrument. In dilute solutions or suspensions, fluorescence intensity is linearly proportional to these parameters. When sample absorbance exceeds about 0.05 in a 1 cm pathlength, the relationship becomes nonlinear and measurements may be distorted by artifacts such as self-absorption and the inner-filter effect.[1] Because fluorescence quantitation is dependent on the instrument, fluorescent reference standards are essential for calibrating measurements made at different times or using different instrument configurations.[2–4] To meet these requirements, Molecular Probes offers high-precision fluorescent microsphere reference standards for fluorescence microscopy and flow cytometry and a set of ready-made fluorescent standard solutions for spectrofluorometry (Section 24.1, Section 24.2).

A spectrofluorometer is extremely flexible, providing continuous ranges of excitation and emission wavelengths. Laser-scanning microscopes and flow cytometers, however, require probes that are excitable at a single fixed wavelength. In contemporary instruments, the excitation source is usually the 488 nm spectral line of the argon-ion laser. As shown in Figure 3, separation of the fluorescence emission signal (S1) from Rayleigh-scattered excitation light (EX) is facilitated by a large fluorescence Stokes shift (i.e., separation of A1 and E1). Biological samples labeled with fluorescent probes typically contain more than one fluorescent species, making signal-isolation issues more complex. Additional optical signals, represented in Figure 3 as S2, may be due to background fluorescence or to a second fluorescent probe.

Background Fluorescence

Fluorescence detection sensitivity is severely compromised by background signals, which may originate from endogenous sample constituents (referred to as autofluorescence) or from unbound or nonspecifically bound probes (referred to as reagent background). Detection of autofluorescence can be minimized either by selecting filters that reduce the transmission of E2 relative to E1 or by selecting probes that absorb and emit at longer wavelengths. Although narrowing the fluorescence detection bandwidth increases the resolution of E1 and E2, it also compro-

mises the overall fluorescence intensity detected. Signal distortion caused by autofluorescence of cells, tissues and biological fluids is most readily minimized by using probes that can be excited at >500 nm. Furthermore, at longer wavelengths, light scattering by dense media such as tissues is much reduced, resulting in greater penetration of the excitation light.[5]

Multicolor Labeling Experiments

A multicolor labeling experiment entails the deliberate introduction of two or more probes to simultaneously monitor different biochemical functions. This technique has major applications in flow cytometry,[6,7] DNA sequencing,[8,9] fluorescence *in situ* hybridization [10,11] and fluorescence microscopy.[12,13] Signal isolation and data analysis are facilitated by maximizing the spectral separation of the multiple emissions (E1 and E2 in Figure 3). Consequently, fluorophores with narrow spectral bandwidths, such as Molecular Probes' Alexa Fluor dyes (Section 1.3) and BODIPY dyes (Section 1.4), are particularly useful in multicolor applications.[8] An ideal combination of dyes for multicolor labeling would exhibit strong absorption at a coincident excitation wavelength and well-separated emission spectra (Figure 3). Unfortunately, it is not easy to find single dyes with the requisite combination of a large extinction coefficient for absorption and a large Stokes shift [14] (see Limitations of Low Molecular Weight Dyes in Section 6.5).

Ratiometric Measurements

In some cases, for example the Ca^{2+} indicators fura-2 and indo-1 (Section 20.2) and the pH indicators BCECF, SNARF and SNAFL (Section 21.2), the free and ion-bound forms of fluorescent ion indicators have different emission or excitation spectra. With this type of indicator, the ratio of the optical signals (S1 and S2 in Figure 3) can be used to monitor the association equilibrium and to calculate ion concentrations. Ratiometric measurements eliminate distortions of data caused by photobleaching and variations in probe loading and retention, as well as by instrumental factors such as illumination stability.[15] For a thorough discussion of ratiometric techniques, see Loading and Calibration of Intracellular Ion Indicators (Section 20.1).

Fluorescence Output of Fluorophores

Comparing Different Dyes

Fluorophores currently used as fluorescent probes offer sufficient permutations of wavelength range, Stokes shift and spectral bandwidth to meet requirements imposed by instrumentation (e.g., 488 nm excitation), while allowing flexibility in the design of multicolor labeling experiments (Figure 4). The fluorescence output of a given dye depends on the efficiency with which it absorbs and emits photons, and its ability to undergo repeated excitation/emission cycles. Absorption and emission efficiencies are most usefully quantified in terms of the molar extinction coefficient (ε) for absorption and the quantum yield (QY) for fluorescence. Both are constants under specific environmental conditions. The value of ε is specified at a single wavelength (usually the absorption maximum), whereas QY is a measure of the total photon emission over the entire fluorescence spectral profile. Fluorescence intensity per dye molecule is proportional to the product of ε and QY. The range of these parameters among fluorophores of current practical importance is approximately 5000 to 200,000 $cm^{-1}M^{-1}$ for ε and 0.05 to 1.0 for QY. Phycobiliproteins such as R-phycoerythrin (Section 6.4) have multiple fluorophores on each protein and consequently have much larger extinction coefficients (on the order of 2×10^6 $cm^{-1}M^{-1}$) than low molecular weight fluorophores.

Photobleaching

Under high-intensity illumination conditions, the irreversible destruction or photobleaching of the excited fluorophore becomes the factor limiting fluorescence detectability. The multiple photochemical reaction pathways responsible for photobleaching of fluorescein have been investigated and described in considerable detail.[16,17] Some pathways include reactions between adjacent dye molecules, making the process considerably more complex in labeled biological specimens than in dilute solutions of free dye. In all cases, photobleaching originates from the triplet excited state, which is created from the singlet state (S_1, Figure 1) via an excited-state process called intersystem crossing.

The most effective remedy for photobleaching is to maximize detection sensitivity, which allows the excitation intensity to be reduced. Detection sensitivity is enhanced by low-light detection devices such as CCD cameras, as well as by high–numerical aperture objectives and the widest emission bandpass filters compatible with satisfactory signal isolation. Alternatively, a less photolabile fluorophore may be substituted in the experiment. Molecular Probes' Alexa Fluor 488 dye is an important fluorescein substitute that provides significantly greater photostability than fluorescein (Figure 1.9, Figure 1.42), yet is compatible with standard fluorescein optical filters. Antifade reagents such as

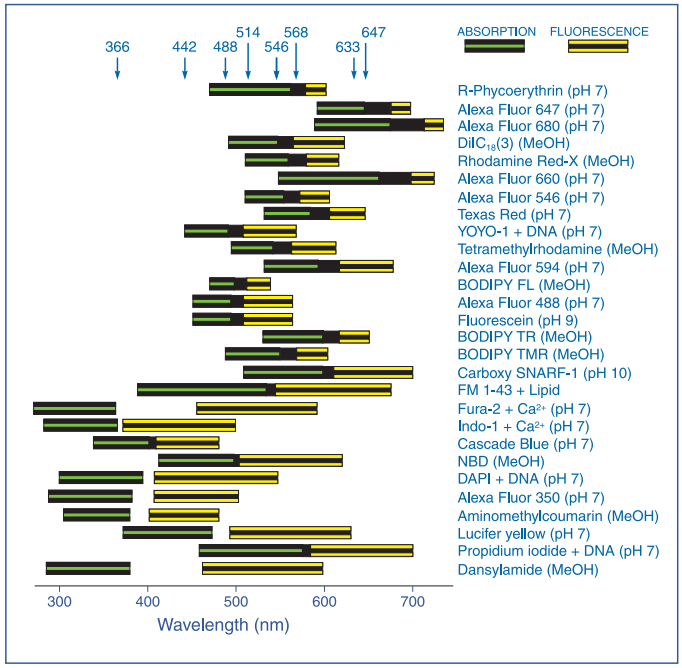

Figure 4 Absorption and fluorescence spectral ranges for 28 fluorophores of current practical importance. The range encompasses only those values of the absorbance or the fluorescence emission that are >25% of the maximum value. Fluorophores are arranged vertically in rank order of the maximum molar extinction coefficient (ε_{max}), in either methanol or aqueous buffer as specified. Some important excitation source lines are indicated on the upper horizontal axis.

Molecular Probes' *SlowFade* and ProLong products (Section 24.1) can also be applied to reduce photobleaching; however, they are usually incompatible with live cells. In general, it is difficult to predict the necessity for and effectiveness of such countermeasures because photobleaching rates are dependent to some extent on the fluorophore's environment.[17–19]

Signal Amplification

The most straightforward way to enhance fluorescence signals is to increase the number of fluorophores available for detection. Fluorescent signals can be amplified using 1) avidin–biotin or antibody–hapten secondary detection techniques, 2) enzyme-labeled secondary detection reagents in conjunction with fluorogenic substrates [20,21] or 3) probes that contain multiple fluorophores such as phycobiliproteins and Molecular Probes' FluoSpheres fluorescent microspheres. Our most sensitive reagents and methods for signal amplification are discussed in Chapter 6.

Simply increasing the probe concentration can be counterproductive and often produces marked changes in the probe's chemical and optical characteristics. It is important to note that the effective intracellular concentration of probes loaded by bulk permeabilization methods (see Loading and Calibration of Intracellular Ion Indicators in Section 20.1) is usually much higher (>tenfold) than the extracellular incubation concentration. Also, increased labeling of proteins or membranes ultimately leads to precipitation of the protein or gross changes in membrane permeability. Antibodies labeled with more than four to six fluorophores per protein may exhibit reduced specificity and reduced binding affinity. Furthermore, at high degrees of substitution, the extra fluorescence obtained per added fluorophore typically decreases due to self-quenching (Figure 1.49).

Environmental Sensitivity of Fluorescence

Fluorescence spectra and quantum yields are generally more dependent on the environment than absorption spectra and extinction coefficients. For example, coupling a single fluorescein label to a protein typically reduces fluorescein's QY ~60% but only decreases its ε by ~10%. Interactions either between two adjacent fluorophores or between a fluorophore and other species in the surrounding environment can produce environment-sensitive fluorescence.

Fluorophore–Fluorophore Interactions

Fluorescence quenching can be defined as a bimolecular process that reduces the fluorescence quantum yield without changing the fluorescence emission spectrum; it can result from transient excited-state interactions (collisional quenching) or from formation of nonfluorescent ground-state species. Self-quenching is the quenching of one fluorophore by another; it therefore tends to occur when high loading concentrations or labeling densities are used (Figure 1.49, Figure 1.71). Molecular Probes' DQ substrates (Section 10.4) are heavily labeled and therefore highly quenched biopolymers that exhibit dramatic fluorescence enhancement upon enzymatic cleavage [22] (Figure 10.47). Studies of the self-quenching of carboxyfluorescein show that the mechanism involves energy transfer to nonfluorescent dimers.[23]

Fluorescence resonance energy transfer (FRET, see Section 1.3) is a strongly distance-dependent excited-state interaction in which emission of one fluorophore is coupled to the excitation of another.

Some excited fluorophores interact to form excimers, which are excited-state dimers that exhibit altered emission spectra. Excimer formation by the polyaromatic hydrocarbon pyrene is described in Section 13.2 (see especially Figure 13.8).

Because they all depend on the interaction of adjacent fluorophores, self-quenching, FRET and excimer formation can be exploited for monitoring a wide array of molecular assembly or fragmentation processes such as membrane fusion (see Assays of Volume Change, Membrane Fusion and Membrane Permeability in Section 14.3), nucleic acid hybridization (Section 8.5), ligand–receptor binding and polypeptide hydrolysis.

Other Environmental Factors

Many other environmental factors exert influences on fluorescence properties. The three most common are:

- Solvent polarity (solvent in this context includes interior regions of cells, proteins, membranes and other biomolecular structures)
- Proximity and concentrations of quenching species
- pH of the aqueous medium

Fluorescence spectra may be strongly dependent on solvent. This characteristic is most often observed with fluorophores that have large excited-state dipole moments, resulting in fluorescence spectral shifts to longer wavelengths in polar solvents. Representative fluorophores include the aminonaphthalenes such as prodan, badan (Figure 2.23) and dansyl, which are effective probes of environmental polarity in, for example, a protein's interior.[24]

Binding of a probe to its target can dramatically affect its fluorescence quantum yield (see Monitoring Protein-Folding Processes with Anilinonaphthalenesulfonate Dyes in Section 13.5). Probes that have a high fluorescence quantum yield when bound to a particular target but are otherwise effectively nonfluorescent yield extremely low reagent background signals (see above). Molecular Probes' ultrasensitive SYBR Green, SYBR Gold, SYTO, PicoGreen, RiboGreen and OliGreen nucleic acid stains (Section 8.3, Section 8.4) are prime examples of this strategy. Similarly, fluorogenic enzyme substrates, which are nonfluorescent or have only short-wavelength emission until they are converted to fluorescent products by enzymatic cleavage (see below), allow sensitive detection of enzymatic activity.

Extrinsic quenchers, the most ubiquitous of which are paramagnetic species such as O_2 and heavy atoms such as iodide, reduce fluorescence quantum yields in a concentration-dependent manner. If quenching is caused by collisional interactions, as is usually the case, information on the proximity of the fluorophore and quencher and their mutual diffusion rate can be derived. This quenching effect has been used productively to measure chloride-ion flux in cells (Section 22.2). Many fluorophores are also quenched by proteins. Examples are NBD, fluorescein and BODIPY dyes, in which the effect is apparently due to charge-transfer interactions with aromatic amino acid residues.[25–27] Consequently, antibodies raised against these fluorophores are effective and highly specific fluorescence quenchers (Section 7.4).

Fluorophores such as BCECF and carboxy SNARF-1 that have strongly pH-dependent absorption and fluorescence characteristics can be used as physiological pH indicators. Fluorescein and hydroxycoumarins (umbelliferones) are further examples of this type of fluorophore. Structurally, pH sensitivity is due to a reconfiguration of the fluorophore's π-electron system that occurs upon protonation. Molecular Probes' BODIPY FL fluorophore and the Alexa Fluor 488 dye, both of which lack protolytically ionizable substituents, provide spectrally equivalent alternatives to fluorescein for applications requiring a pH-*in*sensitive probe (Section 1.3, Section 1.4).

Modifying Environmental Sensitivity of a Fluorophore

The environmental sensitivity of a fluorophore can be transformed by structural modifications to achieve a desired probe specificity. For example, conversion of the prototropic 3′- and 6′-hydroxyl groups of fluorescein to acetate esters yields colorless and nonfluorescent fluorescein diacetate. This derivatization causes fluorescein to adopt the nonfluorescent lactone configuration that is also prevalent at low pH [28] (Figure 21.1); cleavage of the acetates by esterases under appropriate pH conditions releases anionic fluorescein, which is strongly colored and highly fluorescent. Fluorogenic substrates for other hydrolytic enzymes can be created by replacing acetates with other appropriate functional groups such as sugar ethers (glycosides, Section 10.2) or phosphate esters (Section 10.3). Furthermore, unlike fluorescein, fluorescein diacetate is uncharged and therefore somewhat membrane permeant. This property forms the basis of an important noninvasive method for loading polar fluorescent indicators into cells in the form of membrane-permeant precursors that can be activated by intracellular esterases [29] (see Loading and Calibration of Intracellular Ion Indicators in Section 20.1).

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Selected Books and Articles

The preceding discussion has introduced some general principles to consider when selecting a fluorescent probe. Application-specific details are addressed in subsequent chapters of this *Handbook*. For in-depth treatments of fluorescence techniques and their biological applications, the reader is referred to the many excellent books and review articles listed below.

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