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2 Fluorescence-Based Assays

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INTRODUCTION

In recent years, the use of fluorescence techniques for monitoring protein–ligand and protein–protein interactions, or for measuring enzymatic activity, has grown rapidly and has now largely replaced assays using radioactivity. This methodological shift is due to the fact that the sensitivity of fluorescence methods approaches or equals that of radioactive methods, and they avoid the problems of radioactive and scintillation fluid disposal. Also, they do not require separation of bound and free ligand, and so binding measurements are made under true equilibrium conditions (as opposed, for example, to filter binding assays). For kinetic measurements, unlike radioactive assays, a continuous record of the reaction can also be monitored.

The increased use of fluorescence methods is connected with the development of a wide range of instrumentation, including multi-well plate readers, and fluorophores. Many assay kits are also now available which allow novices to readily apply fluorescence-based assays to their particular system. However, knowledge of the fundamentals of fluorescence will always allow the user to optimize the method, avoid pitfalls and recognize artefacts. This chapter aims to discuss the basics of both the fluorescence phenomenon and the instrumentation for solution studies of equilibrium and kinetic measurements.

THE FLUORESCENCE PROCESS

The fluorescence process is best described by reference to the Perrin-Jabłoński diagram shown in Figure 2.1. Upon absorption of light, a fluorophore in the ground state (S_0) is excited into higher energy singlet state levels (the level reached, i.e. S_1, S_2 etc., will depend upon the wavelength of the absorbed light). Rapid thermalization (which occurs in the picosecond timescale) leaves the excited molecule in the lowest vibrational level of the first excited state (S_1). This excited singlet state can persist for a short time, in the order of nanoseconds,

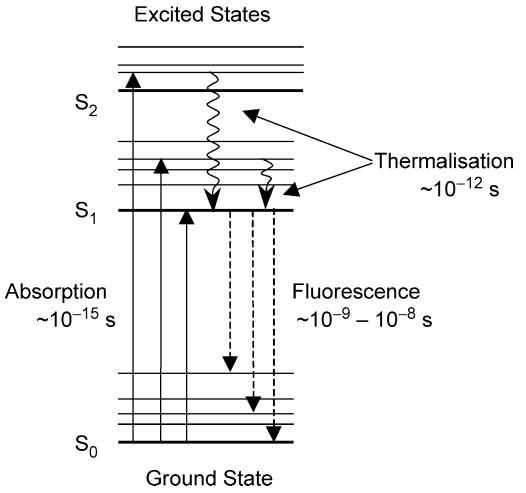


Fig. 2.1 Perrin-Jabłoński diagram. S_0 is the ground state while S_1 and S_2 are electronically excited states.

before the system decays back to the ground state upon emission of a photon. Since this excited to ground state transition is usually of lower energy than the excitation process (as depicted in Figure 2.1), the emission is usually at longer wavelengths than the excitation. This wavelength difference is known as the Stokes Shift, after Sir George Gabriel Stokes, who was also the person who coined the term 'fluorescence'. Exceptions to this basic mechanism, such as emission from the S_2 state, or conversion of the excited state to a triplet state (which can lead to phosphorescence), do occur but are rare and are not discussed here.

Virtually all fluorescence data can be described by one of the following five parameters: the excitation spectrum, the emission spectrum, the quantum yield, the fluorescence lifetime and the anisotropy or polarization of the emission. Before discussing these parameters in detail, the instrumentation typically used for fluorescence measurements is described.

FLUORESCENCE INSTRUMENTATION

A basic fluorimeter consists of a light source, a means of selecting the wavelength of exciting light (monochromator or filter), a sample cell (or sample well in the case of plate readers), a means of selecting the emission wavelength (again monochromator or filter) and a detector. By using a commercial fluorimeter, as compared to homebuilt instrumentation, the user will have only

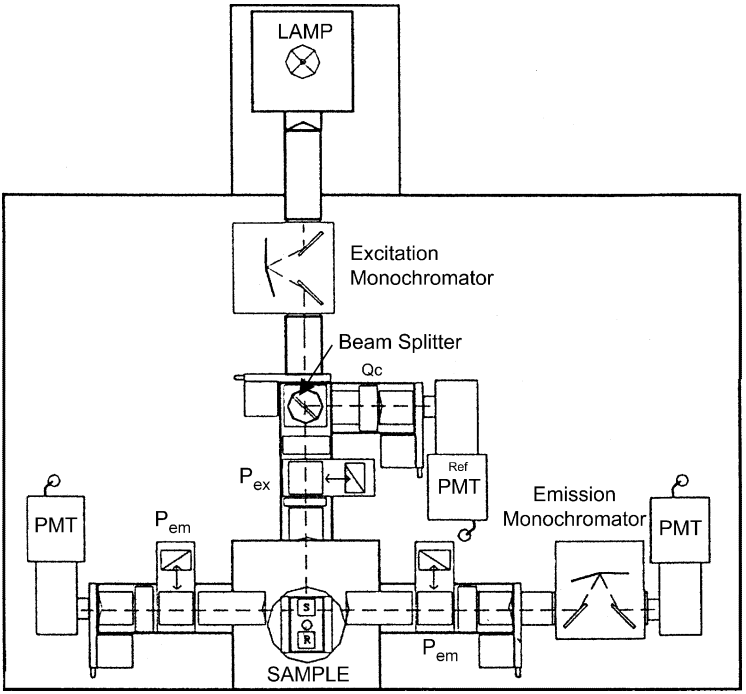


Fig. 2.2 Schematic representation diagram of a spectrofluorimeter indicating key components: revised with permission from commercial literature from ISS, Inc. The principle components include the Xenon arc lamp, the excitation and emission monochromators, a quartz beam splitter, a quantum counting solution (Q_c), three photomultiplier tubes (PMT) and excitation (P_{ex}) and emission (P_{em}) calcite prism polarizers.

marginal control of most of these components but an understanding of component parts will allow the user to optimize the measurement. The design of a basic, modern spectrofluorimeter is shown in Figure 2.2; more details on the instrumentation can be found in several recent references [1–3].

LIGHT SOURCES

Deuterium and tungsten lamps used in absorption spectrophotometry are not often used in general purpose fluorescence instruments since they are relatively weak light sources. The most common light source used in fluorimeters are Xenon or Xenon–Mercury arc lamps. The Xenon arc lamp emits useful light over the range of around 250–2,000 nm (useful light down to 200 nm can be

achieved if the arc lamp is not of the 'ozone-free' variety, which are manufactured using glass that absorbs lower UV wavelengths so as to curtail the production of ozone), although the intensity varies significantly with wavelength. The Xenon–Mercury arc lamp has the same emission but superimposed on it are sharp lines due to mercury transitions. The most intense mercury lines are at 254, 297, 302, 313, 365, 405, 436, 546 and 578 nm. Lasers as light sources are at present typically used only for specialized applications such as fluorescence-activated cell sorters (FACS), lifetime measurements or fluorescence correlation spectroscopy (FCS). The most commonly used lasers include Neodymium-YAG, Argon-ion, Krypton-ion, Helium–Cadmium, Helium–Neon and Titanium–Sapphire lasers. Light emitting diodes (LEDs) and laser diodes, which are becoming increasingly popular for instrumentation dedicated to specific excitation wavelengths, produce nearly monochromatic light and are likely to be the important light sources in the future. The characteristics of these various light sources were recently reviewed [3]. We should note that most of the light sources listed above can be operated in a continuous mode (the intensity is constant as a function of time) or in a pulsed mode (the intensity varies with time) and the choice will depend on the instrument design and function.

EXCITATION MONOCHROMATOR

In a conventional fluorimeter, monochromatic light from the arc lamp is selected using a monochromator (Figure 2.2). This approach has the advantage over optical filters (see below) of offering continuously variable wavelength selectivity. Light from the arc lamp is focussed on the inlet slit and monochromatic light, produced by the dispersion element (typically a diffraction grating) is focussed on the outlet slit. The user generally has control over these slit widths, which may be defined based on either the physical size of the slit (in mm) or the bandwidth of light passed by the monochromator (in nm). The two are related by the dispersion factor of the monochromator in terms of nm/mm. If both inlet and outlet slits of the monochromator are of the same size, the shape of the transmitted light is approximately Gaussian with a width of half-maximum based on the selected wavelength. If the monochromator dispersion is 8 nm/mm, slit widths of 2, 1 and 0.5 mm will give bandwidths of half-maximum of 16, 8 and 4 nm, respectively. It should be noted that a significant fraction of light is transmitted outside the half-maximum bandwidth, a fact that must be taken into account when selecting the observation wavelengths if scattered light is to be avoided. In addition to the selected wavelength light, grating monochromators, which operate using the principle of constructive and

destructive interference, also pass higher order light. For example, a monochromator set at 280 nm will also pass this wavelength when set at 560 nm (referred to as second order light) [3].

SAMPLE CUVETTE

Fluorescence measurements are generally made at 90° to the excitation direction in order to reduce the level of exciting light reaching the detector, and so fluorescence cells are generally polished on all four sides. A wide range of cells is available manufactured from various types of glass. Quartz cells have the advantage of passing light down to below 200 nm, whereas cells made from typical optical glass can only be used at wavelengths above 320–350 nm, depending on the precise glass chemistry.

Most conventional fluorimeters are designed for 4 cm × 1 cm × 1 cm cells and require 2–3 ml of solution to reach the optical path. However, cells of the same external dimensions, which can be used with much less solution volume (e.g. as little as 70 μl), are available. Also micro cells can be accommodated in a home-built metal adapter painted optical black, which allows the exciting light to pass through the sample but without passing through the sides of the cuvette or solution meniscus. If non-standard cells are used, it is important to make sure that the exciting light impinges fully on the sample and not at the meniscus. This criterion can be readily verified visually by exciting with green light (550 nm), measuring the distance from the bottom of the cell holder to this light beam and then making sure that the sample reaches this height from the bottom of the cell. If not, spacers may be used beneath the cell. Cuvettes are available from numerous vendors and information can be found on their web sites (see, e.g. www.starna.com, www.brandtech.com/cuvettes, www.oceanoptics.com, www.optiglass.com, www.scicominc.com/cuvettes.htm.

THE EMISSION MONOCHROMATOR OR FILTER

The emitted light is passed through either a second monochromator or through a filter, which ideally allows isolation of the fluorescence signal from extraneous light. The latter includes Rayleigh scattering (light at the excitation wavelength) as well as Raman scattering. Raman scattering is due to the O–H stretching mode of the water and its position is excitation wavelength dependent and can be approximated from the equation

$$\frac{1}{\lambda_R} = \frac{1}{\lambda_{EX}} - 0.00034 \tag{2.1}$$

where λ_{EX} is the excitation wavelength and λ_R is the Raman wavelength. Therefore, excitation at 350 nm will give, in addition to any fluorescence emission, light at 350 nm (Rayleigh scattering of excitation light) at 397 nm (Raman scattering of water) and a band at 700 nm (the second order of the 350 nm Rayleigh scattering). In cases of excitation at visible wavelength, e.g. 490 nm for fluorescein, the Raman scatter may lie at longer wavelengths ($\sim$588 nm) than the emission.

It is therefore necessary to isolate the fluorescence signal from these other sources of light. This process is most conveniently done by using a second monochromator, which allows for facile selection of the desired wavelength range (Figure 2.2). However, emission can also be viewed through a filter which will pass a higher percentage of emitted light relative to spurious light and hence improve the signal-to-noise ratio. A wide variety of filters is available. These fall into the general categories of (a) interference filters characterized by narrow transmission bands, (b) bandpass filters which are centred at a specific wavelength (like interference filters) but which pass a relatively broad wavelength range and (c) cut-off filters which pass all light above a specific wavelength. This latter filter is often referred to as a cut-on filter depending on the viewpoint of whether the transmission commences sharply at a given wavelength (cuts-on) or equivalently if the optical density decreases sharply at that wavelength (cuts-off). Regardless, the operational principle of these types of filters, which are also known as longpass filters, is that they can be used to block any excitation light scattered towards the emission direction and then collect a large percentage of the total emission. Transmission data of a wide variety of filters can be found on web sites such as www.mellesgriot.com, www.spectra-physics.com and www.oceanoptics.com.

SIGNAL DETECTION

Most modern fluorimeters use photomultiplier tubes for measuring the emitted light. In analogue systems, the output signal is dependent on the high voltage applied to the photomultiplier tube. However, the most sensitive instruments use photon counting methods which have several advantages over conventional methods [3]. The use of charge-coupled devices (CCDs) has become more common, though, especially in fluorescence microscopy, as the sensitivity of these devices has improved.

OTHER COMPONENTS OF A FLUORIMETER

The components described above are the basic requirements of a fluorimeter. However, many commercial instruments have additional features (Figure 2.2).

For example, a quartz beam splitter, which diverts a small percentage of the excitation light to a quantum counter (typically a concentrated solution of rhodamine B in ethanol) is often used to monitor the excitation intensity. The light from the beam splitter is directed onto the quantum counter (typically placed in a triangular cuvette) and the emission is observed through a cut-off filter. By recording the ratio of the sample signal to this reference signal, one can significantly reduce any time-dependent variations in lamp intensity. The reference signal is also useful for measuring corrected excitation spectra as briefly mentioned later.

Other useful accessories are polarizers. If the excitation light is polarized and the emitted light is viewed through a second polarizer, one can calculate the fluorescence polarization or anisotropy of the emission – a parameter discussed in more detail later.

In the above instrumental set-ups, the emitted light is viewed at 90° by a single photomultiplier. This arrangement is conventionally described as an 'L' format. However, if a second monochromator/filter and photomultiplier is also at 90° to the excitation light, this gives a 'T' format. The T format is mainly of use for polarization measurements, but for some specialized applications it allows emitted light at two different wavelengths to be detected simultaneously.

FLUORESCENCE PARAMETERS

As mentioned above, all fluorescence data can be described by five parameters.

EXCITATION SPECTRA

The wavelength of the emitted light is fixed and the wavelength of the exciting light is varied. Generally, the excitation spectrum is the same as the absorption spectrum since the number of emitted photons depends upon the number of photons absorbed (assuming that the efficiency of the emission process is invariant with excitation wavelength, which is almost always the case). However, the arc lamp light source does not give constant light intensity at different excitation wavelengths and the efficiency of the monochromator is wavelength dependent; hence, the technical or uncorrected excitation spectrum will be distorted by these factors. Uncorrected spectra can be converted to true molecular spectra by taking into account the wavelength dependence of the excitation system. This correction procedure has been described in detail elsewhere [3] and will not be discussed further because here we are generally concerned with fixed excitation and emission values.

EMISSION SPECTRA

Here the wavelength of the exciting light is fixed and the wavelength of the emission monochromator is varied. The emission maximum is virtually always at longer wavelengths than the excitation maximum (the Stokes Shift). Again, the emission spectrum usually measured is termed a technical spectrum because both the monochromator and photomultiplier have wavelength-dependent responses, which can be corrected if necessary [3].

QUANTUM YIELD

The quantum yield (Q) is defined as the fraction of light absorbed by the fluorophore that is emitted as fluorescence ($Q =$ number of photons emitted/number of photons absorbed) and so can vary between 0 and 1. Q may also be defined as

$$Q = \frac{k_r}{k_r + k_{nr}} \tag{2.2}$$

where k_r and k_{nr} are the rate constants of the radiative and non-radiative decay processes from the first excited state. Direct or absolute measurement of the quantum yield of a fluorophore is complicated. Hence, it is more common to measure the quantum yield relative to a known standard. In this case, a fluorescent standard with an excitation and emission spectrum similar to the unknown is used. Ideally, the optical density of both samples will be matched at the excitation wavelength and then the relative emission intensities (corrected for instrument response functions) can be determined. These instrument correction factors are typically provided by the manufacturer of the instrument. As discussed elsewhere [3], these correction factors can be very much dependent upon the polarization of the emission.

FLUORESCENCE LIFETIME

The typical reader of this chapter may not have occasion to make lifetime measurements, as these, like quantum yield measurements, are very specialized. However, knowledge of the fluorescence lifetime may be important to the particular fluorescence assay being considered (especially polarization or anisotropy-based assays) and so some understanding is useful. Basically, the fluorescence lifetime (τ) is the time that the fluorophore remains in the excited state before decaying to the ground state and is defined as $1/k_r$ where k_r is the rate constant of the decay process. The reader should remember, however, that excited state lifetimes – like radioactive decay half-lives – are parameters

applied to populations of molecules, i.e. the lifetime of any particular excited fluorophore cannot be predicted but rather the average behaviour of a large population of molecules can be determined (the specialized topic of single-molecule measurements is beyond the scope of this article). More precisely, the lifetime of a fluorophore population is the time it takes for the intensity (excited by a very short light pulse) to decay to $1/e$ of its original intensity. Typically, fluorescence lifetimes are in the region of 0.1–30 ns – although exceptions occur (e.g. pyrene and ruthenium derivatives can have lifetimes in the range of hundreds of nanoseconds).

Fluorescence lifetimes are usually measured in one of two ways. First, with the pulse method, the fluorophore is excited with a very short pulse of light (modern lasers allow this to be in the picosecond to femtosecond range) and the time dependence of the emission process is measured. Second, in the traditional phase-modulation method, the fluorophore is continuously excited with light, which is sinusoidally modulated. Because of the finite lifetime of the excited state, the emitted light has a phase delay and is demodulated with respect to the excitation light. These two measurements allow lifetimes to be calculated. For a detailed description of these methods, the reader is referred to Refs. [1–4]. It should be noted that most fluorophores in biological systems do not decay with a single lifetime but show multi-exponential behaviour, which often reflects on the diverse molecular environments present.

ANISOTROPY/POLARIZATION

Fluorescence anisotropy and polarization measurements are made in the same way and contain the same information, they are merely different ways of defining the measurement. The excitation light is polarized in a direction vertical to the laboratory axis and the emitted light is measured through a polarizer parallel and then perpendicular to the exciting light. (By use of a 'T' format the parallel and perpendicular intensities can be measured simultaneously whereas with an 'L' format, they are measured consecutively.) The two measurements are then used to calculate polarization (P) or anisotropy (r) by the following equations:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \tag{2.3}$$

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \tag{2.4}$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of the parallel and perpendicular polarized emission. We should note that even though these two functions differ slightly their information content is the same and the use of one or the other term is

usually a matter of convenience. From these definitions one can demonstrate that: $r = 2P/(3 - P)$.

In practice, the measurement is complicated by the fact that the detection system (monochromator/filter and photomultiplier) may have different responses to light of the same intensity polarized in the parallel and perpendicular planes and so a correction must be applied. This correction is done by first exciting the fluorophore with light polarized in the direction horizontal to the laboratory axis. In this case, due to symmetry, there exists an equal chance of the emitted light being polarized in the parallel or perpendicular planes and any deviation from unity thus gives the normalization instrumental factor (usually known as the 'G' factor) required to obtain the correct values.

The maximum values of anisotropy and polarization for fluorophores randomly oriented in solution are 0.4 and 0.5, respectively (for the derivation of these limits the reader is referred to Weber's classic article [5]). The measured anisotropy or polarization is dependent on the ratio of the fluorescent lifetime of the fluorophore, τ and its rotational rate, which can be expressed either as the Debye rotational relaxation time (ρ) or the rotational correlation time (τ_c) (we note that $\rho = 3\tau_c$).

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho}\right) \tag{2.5}$$

$$\frac{r_0}{r} = \left(1 + \frac{\tau}{\tau_c}\right) \tag{2.6}$$

In these equations, r_0 and P_0 are the limiting anisotropy or polarization of the fluorophore and τ is the lifetime. The limiting anisotropy or polarization values are those observed when the fluorophore is immobile and can be made by placing the fluorophore in anhydrous glycerol and making the measurement at a low temperature (typically below 0 °C). Although values of 0.4 and 0.5 are typically assumed for r_0 and P_0, respectively, care should be taken since there are many exceptions and also the limiting values are usually wavelength dependent [1–3, 5, 6]. From these equations it can be seen that for any given lifetime, the anisotropy/polarization of the fluorophore in solution depends on its rotational diffusion. For small fluorophores, with typical lifetimes in the nanosecond range, rapid rotation gives a low anisotropy/polarization whereas if the fluorophore is bound to a macromolecule with slow rotation the anisotropy/polarization will be higher.

To convert the observed anisotropy/polarization from a mixture of free and bound fluorophore into the fraction of bound ligand, we must understand the additivity properties of these functions. Weber explicitly derived the relationship

governing additivity of polarizations from different species, namely ref. [7]:

$$\left(\frac{1}{\langle P\rangle} - \frac{1}{3}\right)^{-1} = \sum f_i \left(\frac{1}{P_i} - \frac{1}{3}\right)^{-1} \tag{2.7}$$

where P is the actual polarization observed arising from i components, f_i represents the fractional contribution of the ith component to the total emission intensity and P_i is the polarization of the ith component. This additivity principle was later expressed in terms of anisotropy (r) by Jabłoński [8] as

$$r_0 = \sum f_i r_i \tag{2.8}$$

We note that the anisotropy formulation appears to be the simpler function, but clearly the information content of the two approaches is identical and given the present day computer-assisted data analysis the difference is often moot. Perhaps, the most important consideration in such studies is the relative quantum yields of the free and bound probe. Specifically, if the quantum yield of the fluorophore changes upon binding, the fractional intensity terms in the additivity equations will alter. Although many probes (such as fluorescein) do not significantly alter their quantum yield upon interaction with proteins, others, such as methylanthraniloyl derivatives [9] can demonstrate significant enhancement of signal upon binding and one would be well advised to ascertain if such alterations occur. If the quantum yield does in fact change, one can correct the fitting equation to take this yield change into account. In terms of anisotropy the correct expression relating observed anisotropy (r) to the fraction of bound ligand (x) (where fraction bound plus fraction free equals 1), the anisotropy of bound ligand (r_b), the anisotropy of free ligand (r_f), and the quantum yield enhancement factor (g) is [10]

$$x = \frac{r - r_f}{r_b - r_f + (g-1)(r_b - r)} \tag{2.9}$$

A rigorous propagation of error treatment has explored the effect of uncertainties in various experimental parameters upon the calculated value of fraction bound and dissociation constant [11].

FLUORESCENCE INTENSITIES

Although all fluorescence data can be described by the above five measurements, most readers will be more interested in fluorescence intensity measurements. It is important to realize that unlike absorbance measurements, fluorescence intensity measurements are not absolute. The absorbance (A), or optical density (OD) of a solution at a particular wavelength will be the same regardless of the spectrophotometer used since it is defined by Beer's Law: $A = \varepsilon cl$,

where ε is the molar extinction coefficient, c is the concentration and l is the path length.

The fluorescence intensity of a fluorophore will depend on its intrinsic probability of absorbing light at the excitation wavelength (determined by its extinction coefficient) and the percentage of this light that is emitted as fluorescence (determined by its quantum yield). However, it also depends on the intensity of the light source, the efficiency of the optical system to transmit light, the slit widths of the monochromators, the geometry of the light collection (e.g. the apertures of the lenses), the efficiency of the photomultiplier tube (as well as the applied voltage in the case of analogue measurements) and the amplifier gain. For these reasons fluorescence intensity measurements can only be expressed in terms of arbitrary units or relative measurements.

FLUORESCENCE RESONANCE ENERGY TRANSFER

In the description of the fluorescence process above, it was stated that the excited state could decay with the emission of fluorescence, a radiative process, or by a non-radiative process. However, if a chromophore is in the proximity of the excited state fluorophore radiationless energy transfer may occur between the excited state fluorophore (donor) and this second chromophore (acceptor). If the acceptor is itself fluorescent, it may then emit light from its excited state and hence the observed emission will correspond to this secondary fluorophore. The efficiency of this energy transfer process can be measured by either the decrease in intensity of the donor fluorophore or the increase in intensity of the acceptor.

The mechanism of fluorescence energy transfer was first proposed in 1918 by Jean Perrin and quantitative theories were developed over the next few decades. Between 1946 and 1949, T. Förster developed the most complete quantitative theory of molecular resonance energy transfer, and hence the phenomenon is often now referred to as Förster resonance energy transfer (FRET). The efficiency of transfer is given by the equation

$$E = \frac{R_0^6}{R_0^6 + R^6} \tag{2.10}$$

where R is the distance between the donor and acceptor and R_0 is the distance between the donor and acceptor when transfer efficiency is 50%. The sixth power dependence results in a very large dependence of efficiency with distance, as shown in Figure 2.3, which depicts transfer efficiency *versus* R for a donor/acceptor pair with an R_0 of 40 Å. R_0 can be calculated from the equation

$$R_0^6 = (8.79 \times 10^{-25}) Q_D \kappa^2 n^{-4} J \tag{2.11}$$

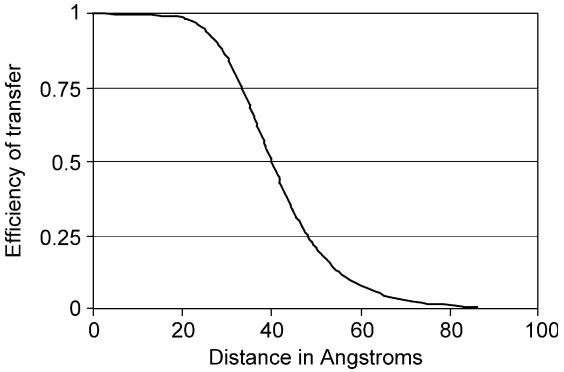


Fig. 2.3 Depiction of the inverse sixth-power distance dependence of FRET. The transfer efficiency versus donor/acceptor distance shown is calculated for a donor/acceptor pair with an R_0 of 40 Å.

where Q_D is the quantum yield of the donor, κ^2 is the orientation factor (related to the relative orientation of the donor and acceptor dipoles; κ can vary between 0 and 4 but is usually assumed to be 2/3, the value corresponding to the case of rapid reorientation between donor and acceptor dipoles, n is the refractive index of the intervening solvent and J is the overlap integral between the emission spectra of the donor and the absorption spectrum of the acceptor. For a detailed account of this equation, see refs. [1–3, 12, 13].

For any pair of donor and acceptor, these parameters can be measured although some assumptions may need to be made for κ^2. Thus, R_0 can be calculated. However, a list for values of R_0 for a range of donors and acceptors is given in ref. [13].

It can be seen from the above equations that energy transfer can be used to measure distances between donor and acceptor. However, the main use of FRET in the context of this chapter is merely to see whether or not donor and acceptor are in close proximity and hence provide a signal for binding or kinetic experiments. Choosing a pair of donor and acceptor for this purpose may be a matter of trial and error, although obviously the emission spectrum must overlap to some extent the absorption spectrum of the acceptor. However, if structural information is available, for example, of a protein–ligand complex, then the above equations can be used to choose a suitable donor/acceptor pair to optimize measurements on the protein–ligand complex.

FLUOROPHORES AND LABELLING STRATEGIES

Fluorophores used for fluorescence assays can be divided into intrinsic probes, which occur naturally in the system, and extrinsic probes, which need to be introduced into the system. Intrinsic probes can be part of a protein's covalent

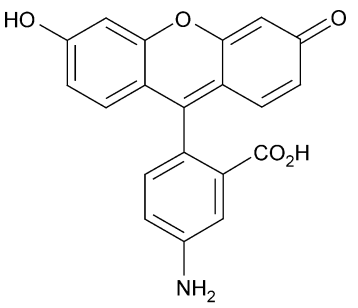
structure, a co-factor or a ligand. Examples include the aromatic amino acids tryptophan and tyrosine (although the latter has relatively weak fluorescence), NADH, FAD, FMN, some porphyrins, some fatty acids and lipids, some modified nucleic acids, pyridoxal phosphate, chlorophylls and pteridines. Although intrinsic fluorophores find utility in mechanistic experiments and low throughput assays, their typically short excitation wavelengths (in the UV or mid-UV) make them less suitable for assays used in high throughput screening.

Extrinsic probes have the advantage of being chosen for a particular purpose but the disadvantage is that their introduction may perturb the system. In the absence of structural information on the system of interest, their introduction is often an empirical process. The choice of which component in a system to label is determined by the nature of the process to be monitored by the assay, as discussed later. A vast variety of probes is now available with different reactive groups and lengths of spacer between the fluorophore and the reactive moiety (see, e.g. www.probes.com, www.evidenttech.com, www.intracellular.com, www.perkinelmer.com and www.amershambiosciences.com). Amino and thiol groups are those most commonly modified in biological systems, and the majority of probes are available as *N*-hydroxysuccinimide esters or derivatized with maleimides or iodoacetamides for this purpose.

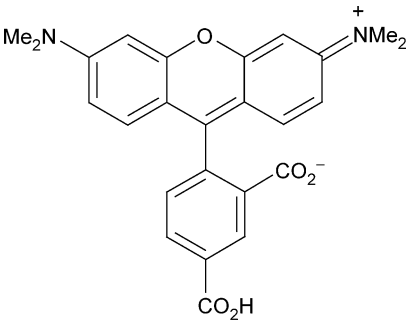
The choice of probe depends on the fluorescence parameter being measured and the intended use of the assay. Factors which should be considered include the excitation wavelength, excited state lifetime, environmental sensitivity, pH dependence of the fluorescence emission and susceptibility of the probe to photobleaching. Photobleaching is caused by irreversible chemical reaction of the excited triplet state (often via reaction with oxygen) and is a particular problem if high intensity light sources such as lasers are to be used.

The fluorescence emission intensity of UV-wavelength excitable probes such as dansyl and coumarin derivatives is often highly sensitive to the polarity of their environment and can be used to monitor the binding of labelled ligands to a protein. However, these probes are generally only suitable for mechanistic evaluations and low throughput assays, since their relatively short excitation wavelength makes the measurement susceptible to test compound interference.

As discussed later, interference from compounds and biological components is minimized substantially by using probes with excitation wavelengths above $\sim$500 nm. The majority of longer wavelength probes also have much higher extinction coefficients which greatly improves sensitivity. Fluorescein (1) ($\lambda_{ex} = 493$ nm) has good aqueous solubility and remains a popular probe for fluorescence polarization high throughput screening assays but is quite sensitive to photobleaching and is non-fluorescent in its protonated state ($pK_a \sim 6.4$). Tetramethylrhodamine (2) ($\lambda_{ex} = 550$ nm) is a better choice for its longer wavelength fluorescence, photostability and pH insensitivity. The CyDye™ fluorophores (Amersham Biosciences), for example, Cy3 (3) ($\lambda_{ex} = 550$ nm)

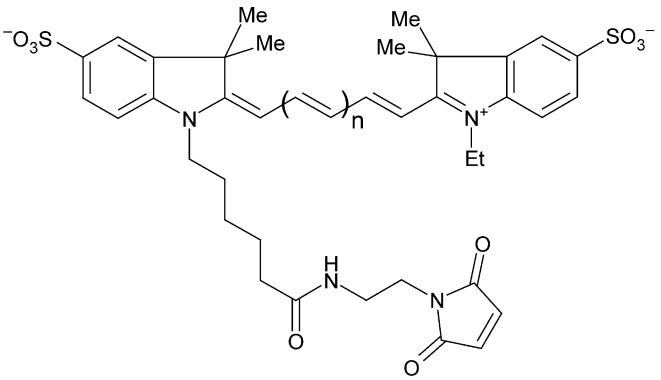


(1) 5-aminofluorescein

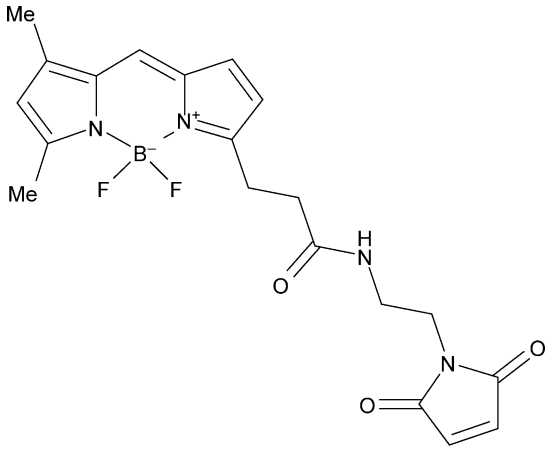


(2) 5-carboxytetramethylrhodamine

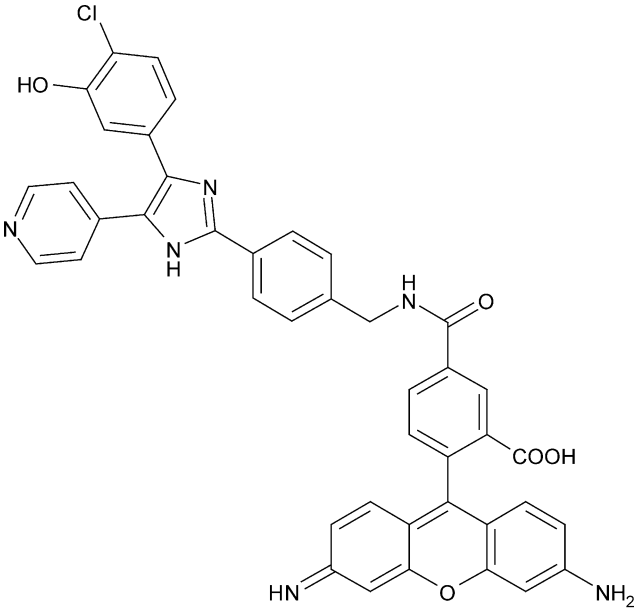
and Cy5 (4) ($\lambda_{ex} = 649$ nm), are versatile long wavelength probes which are photostable and insensitive to pH between 3 and 10. Two other classes of probe which are good for high throughput screening applications are the AlexaFluor™ and Bodipy™ (5) dyes (both from Molecular Probes). The AlexaFluors are available in a wide range of excitation wavelengths from UV to the furthest visible. They are robustly photostable, pH insensitive across a broad range, show good aqueous solubility and a high FRET efficiency. Bodipy dyes are available in a range of visible excitation wavelengths and are particularly suitable for fluorescence polarization applications because of their longer lifetimes. They have high extinction coefficients and quantum yields, and are polarity and pH insensitive. Their compact, neutral and non-polar structure is potentially less disruptive to the interaction with the binding partner.



(3) n = 0: Cy3 maleimide
(4) n = 1: Cy5 maleimide



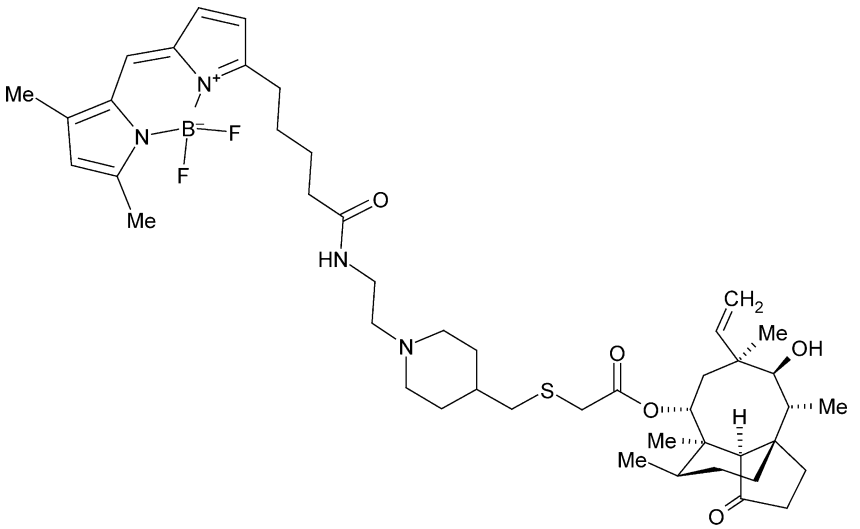
(5) BODIPY FL *N*-(2-aminoethyl)maleimide



(6) Rhodamine green labelled kinase ligand

Labelling of small molecule ligands with the chosen probe is relatively straightforward since there may only be one reactive group present; if not, protecting group strategies can be used. If structural information is available on the interaction of interest, this can be used to guide the introduction of a probe, for example, via a group which protrudes from the binding site, and the choice of linker length. Often, however, the points available for covalent attachment of labels are limited and the process is somewhat empirical. Purification of the derivatized product is typically performed by HPLC. For example, many fluorescent nucleotide analogues have been synthesized by reacting the amino-group of 2′-amino-2′ deoxy nucleotides or 2′(3′)-*O*-carboxyethyl nucleotides [9].

There has been considerable success generating fluorescent ligands for active sites in proteins by labelling characterized inhibitors. These inhibitors can then be used to configure fluorescence polarization assays, which are sensitive to test compounds which compete with the probe for binding to that site. Two recent examples where known inhibitors were labelled to generate fluorescence polarization ligands, which were used for high throughput screening, are (6) and (7). (6) shows a ligand for the ATP-binding site of B-Raf kinase generated by labelling an ATP-competitive kinase inhibitor, which bears a unique amino group, with activated rhodamine green [14]. The labelled inhibitor is bound tightly by the kinase and with about a 10-fold higher affinity than the unlabelled inhibitor. (7) shows a semi-synthetic analogue of a pleuromutilin antibiotic,



(7) BODIPY-FL labelled pleuromutilin

which was labelled via a spacer with the Bodipy-FL probe [15]. This fluorescent derivative binds tightly to ribosomes from a number of bacterial species (*Escherichia coli* $K_d = 7$ nM) and was used in a fluorescence polarization assay to screen for compounds interacting at the pleuromutilin binding site. The configuration of these assays is discussed in more detail in a later section.

For the labelling of peptides, a fluorescent amino acid can be incorporated into the synthetic procedure or fluorescent probes can be introduced at the N-terminal amino group or the ε-amino group of lysine residues within the sequence. Since the C-terminal amino acid is bound to the resin in solid phase synthesis procedures, selective N-terminal labelling is readily performed before cleavage of the chain from the resin and side chain deprotection.

Labelling of proteins is more difficult because of the possibility of many different functional groups being modified. It may be that labelling of more than one functional group is not a problem. However, it is preferable to attempt to label at a single site in order to reduce the possibility that the labelling affects the biological function of the protein and increases reproducibility between preparations of a labelled protein.

There are several approaches to labelling a protein with a fluorophore at a specific site. First, one may be lucky that the protein contains only one reactive cysteine residue. If this is not the case, then site-directed mutagenesis can be used to introduce a cysteine in a conserved manner. If a structure of the protein is available, then this can be used to select a residue which is on the outside of the protein and is likely to report on protein–protein or protein–ligand interactions. For example, a single cysteine was introduced into a phosphate binding protein by site-directed mutagenesis, which was then labelled with a thiol reactive coumarin derivative [16]. On binding phosphate the labelled protein shows a large enhancement of the coumarin fluorescence, and can be used as a sensor for changes in phosphate concentration.

The second group often modified is the amino group. Most proteins contain many reactive lysine groups in addition to the N-terminal group. However, as the reactive species with reagents such as *N*-hydroxysuccinimide esters is $-NH_2$ rather than NH_3^+ selective, use is made of the differing p*K*a's of lysine ($pK_a \sim 9$) and the N-terminal group ($pK_a \sim 7.9$). Labelling near neutrality favours N-terminal labelling over lysine labelling. This approach also reduces the loss of the reagent by hydrolysis of the *N*-hydroxysuccinimide ester.

Despite the widespread use of chemical modification of proteins, the problems of selective labelling and possible loss of function have led to the search for alternative methods of introducing fluorophores into proteins. The first and best known of these is to generate a fusion with one of the green fluorescent proteins [17]. However, this is a large protein (wildtype GFP from *Aequorea* has 238 residues) and so may affect the function of the fusion

protein. A second approach is that of expressed protein ligation [18]. This approach uses the inducible self-cleavage activity of a protein-splicing element (an intein). The gene of the target protein is fused to a tag consisting of the intein and also a binding domain allowing affinity purification of the fusion protein, such as a chitin-binding domain. In the presence of a thiol (including fluorescent thiols) the intein undergoes specific self-cleavage which releases the fluorescently labelled target protein from the chitin bound intein tag. This method allows the introduction of one or more fluorophores at either the N-terminal or C-terminal ends of the protein or within the sequence of the protein.

Another alternative to chemical modification is the C-terminal labelling of an expressed protein with a fluorescent derivative of the antibiotic puromycin [19]. Puromycin inhibits protein synthesis by competing with aminoacyl tRNA for incorporation into the growing chain. However, low concentrations of puromycin tend to incorporate only at the C-terminus of a full-length chain. Thus, the fluorescently modified group can be covalently attached to the C-terminus when mRNA lacking a stop codon is synthesized in a cell-free translation system in the presence of puromycin.

EXAMPLES OF FLUORESCENCE ASSAYS

BINDING ASSAYS

As discussed previously, the anisotropy/polarization of the fluorescence emission of a probe is sensitive to its excited state lifetime and its rotational correlation time. Since the rotational motion is influenced by the size of the molecule or complex to which the probe is bound, this fluorescence parameter is particularly suitable for following binding reactions between a small labelled ligand and a macromolecular partner. Small molecules labelled with probes with lifetimes in the nanosecond range display a low anisotropy due to relatively rapid rotational motion, whereas the labelled ligand bound to a macromolecule displays a high anisotropy because of the reduced global motion. Local motion of the probe about rotatable bonds will, however, reduce the anisotropy of the bound state. The application of this method to the study of biomolecular interactions has received recent review [6, 11]. Figure 2.4 illustrates the specific use of fluorescence polarization for the mechanistic study of a protein–ligand interaction. In this experiment, 1 μM mant-GTPγS (a fluorescent, slowly hydrolysable GTP analogue) was titrated with increasing concentrations of the GTP-binding protein dynamin. The resulting binding curve was fitted with the binding Equation (1.9), which takes account of the differing quantum yields of the free and bound states of the probe, to yield the dissociation constant for

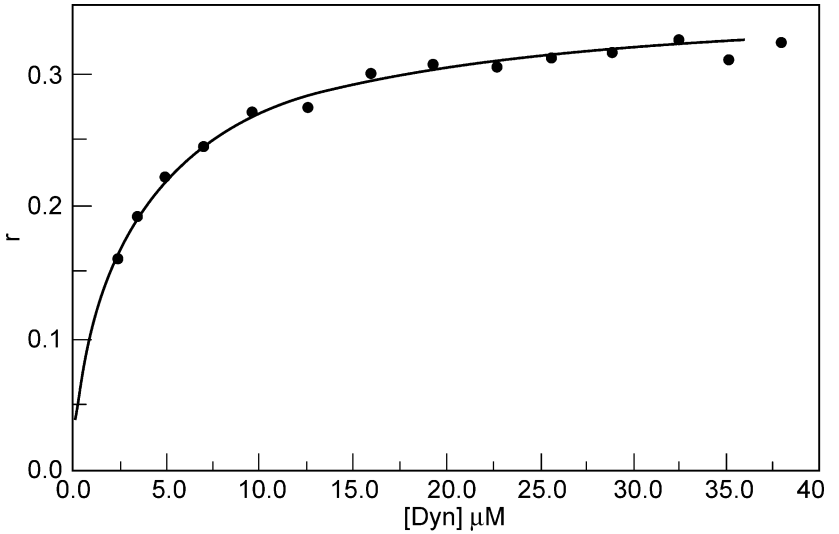


Fig. 2.4 Example of ligand–protein binding followed by fluorescence anisotropy. 1 μM mant-GTPγS (a fluorescent, slowly hydrolysable GTP analogue) was present and the concentration of the GTP-binding protein, dynamin, was varied by starting at high concentrations followed by dilution. The binding curve was fit to the anisotropy equation (in this case the yield of the fluorophore increased about 2-fold upon binding). A K_d of 8.3 μM was found. These data were obtained by Dr. Derk Binns and Dr. Joseph P. Albanesi.

the interaction ($K_d = 8.3$ μM). More details regarding the interaction of this nucleotide with dynamin can be found in ref. [20].

Fluorescence polarization is particularly suitable as a readout in high throughput screening assays which are sensitive to antagonism of a small molecule–macromolecule interaction at equilibrium. An example was presented previously of a labelled inhibitor which is tightly bound by the B-Raf kinase. Such assays are usually configured with a macromolecule concentration similar to the dissociation constant for its interaction with the ligand (i.e. halfway up the binding curve), which gives a large polarization change but retains sensitivity to competitively binding test compounds, which cause a reduction in the observed polarization. The lower limit with which this type of assay can determine IC_{50} values depends on the affinity with which the labelled ligand is bound – ideally this should be <100 nM. Further examples of this type of assay are given in ref. [21].

In clinical studies, fluorescence polarization-based immunoassays (FPIA) have become widespread since the introduction of the Abbott TDx instrument in the early 1980s [22]. In this method, a fluorescent derivative of a target molecule (e.g. a drug or metabolite) is bound to an antibody against the target molecule, which gives rise to a high polarization, and then introduction of a sample

containing the unlabelled target molecule (e.g. from bodily fluids) results in competitive release of the fluorophore-labelled target molecule and a reduction in the polarization. Recent examples of this specialized application are given in refs. [23, 24].

Fluorescence polarization can also be used to monitor protein–protein interactions, but in this case care must be taken to ensure that the lifetime of the fluorophore is sufficiently long so that a measurable change in polarization occurs upon dissociation of the protein oligomer. In some cases, even if the lifetime of the probe is not intrinsically long enough to expect significant changes in polarization upon dissociation of the protein complex, changes in local mobility of the fluorophore upon dissociation may occur such that significant changes in polarization are detected [25].

It is possible that a fluorophore attached nearer to the binding interface will display an environmentally induced intensity change, but such labelling attempts would require knowledge of the complex structure and risk perturbing the interaction. Another strategy is to label each component with an appropriately chosen pair of probes and use FRET measurement to detect the complex. The detection of a FRET signal is critically dependent on the distance between the probes in the complex, as discussed previously. These distance constraints can in part be relaxed by using a modification of the technique called time-resolved energy transfer (TRET). Here, long-lifetime donors based on lanthanide ion complexes transfer energy to long wavelength acceptors (e.g. allophycocyanins). The distance at which this energy transfer is effective is extended compared to conventional fluorophore pairs. This approach also allows indirect labelling strategies to be used, for example, via antibodies to a specific sequence or tag engineered into the protein. The use of pulsed excitation and time-gated detection allows short-lived background (e.g. scatter and autofluorescence) to decay before the longer lifetime emission of the probe is measured, improving the assay performance and sensitivity. TRET and allied methods are discussed in more detail in ref. [26].

KINETIC ASSAYS

Fluorescence methods are widely used for the determination of the kinetic mechanism of enzyme reactions, and are also used to configure kinetically read assays for high throughput screening. Here, we are concerned with kinetic measurements where the enzyme is at catalytic concentrations with a large excess of substrate and the steady state rate of the reaction is measured. Studies of enzyme mechanisms where the concentration of the enzyme is higher than that of the substrate resulting in the so-called single turnover conditions in which

the formation and decay of intermediates of the mechanism are observed require rapid reaction equipment and are beyond the scope of this chapter.

At the simplest level, for a fluorescence-based assay the reaction needs to proceed with either the formation of a fluorescent product from a non-fluorescent substrate or *vice versa*. An example of the first of these is the use of the non-fluorescent butyl ester of resorufin as a substrate in a hydrolase assay, where enzymatic cleavage of the ester bond yields highly fluorescent resorufin. An example of the latter is any enzyme reaction involving the oxidation of NAD(P)H to the non-fluorescent $NAD(P)^+$ [27]. FRET may also be used for enzyme assays where the enzymatic reaction changes the distance or orientation of the two fluorophores in the substrate, thus changing the observed fluorescence intensity of the donor or acceptor. Using a protease reaction as an example, doubly labelled peptide substrates where the labels span the cleavage site, such as DABCYL-$(Xaa)_n$-EDANS and Abz-$(Xaa)_n$-3-nitrotyrosine where $(Xaa)_n$ is any aminoacid sequence, have been used [28,29]. The degree of resonance energy transfer is highly dependent on the distance between donor and acceptor and this method is generally applicable for peptides of 11–12 amino acids or less.

The major aim of steady-state mechanistic measurements is to study the effect of substrate concentration on the steady-state rate of the enzymic reaction and so derive the v_{max} for the reaction (the rate at saturating substrate concentration) and the K_m of the reaction (the substrate concentration giving half-maximum rate). Studies can then be made in the presence of potential inhibitors of the reaction in order to determine the mechanism of inhibition such as competitive, non-competitive or mixed inhibition. Analysis of such data is common to any assay method and the reader is referred to standard text books on enzymology.

Before making fluorescence-based enzyme assays, it is extremely useful to record the excitation and emission spectra at the start and at the end of the reaction using the instrument conditions to be used for the kinetic measurement. Overlaying these spectra will show the optimal excitation and emission wavelengths for the kinetic measurements, i.e. the wavelengths that give the largest change in intensity and hence greatest sensitivity of the method. Such measurements will also show the presence of other components of the system, which may contribute to the fluorescence signal. For example, in the work described below with a protease from the malarial parasite, it was shown that the detergent, NP-40, contained a weakly fluorescent impurity [30].

It is then necessary to show that the intensity change is proportional to the extent of the reaction. In the case where only the substrate or product is fluorescent, this can be done by simply making a set of dilutions of the fluorophore and determining the linearity of the fluorescence intensity. In the case of energy transfer measurements, appropriate mixtures of substrate and

product can be made to check the linearity of response. There are two reasons why the response may not be linear. The first is that at high concentrations of fluorophore, the detector becomes saturated. This problem can be easily solved by reducing the amount of light reaching the detector by either reducing the size of the slits on the excitation or emission monochromators or by using a neutral density filter. A second reason is that inner filter effects may affect the measurement if the absorbance of the solution at the excitation wavelength becomes excessive during the reaction. This problem is well illustrated in the following assay for a malarial parasite protease. A decapeptide containing the target sequence with N- and C-terminals replaced by cysteine residues was synthesized [30]. This peptide was doubly labelled by reaction with iodoacetamidotetramethylrhodamine. Normally, the intermolecular K_d for rhodamine dimerization is $\sim$1 mM.

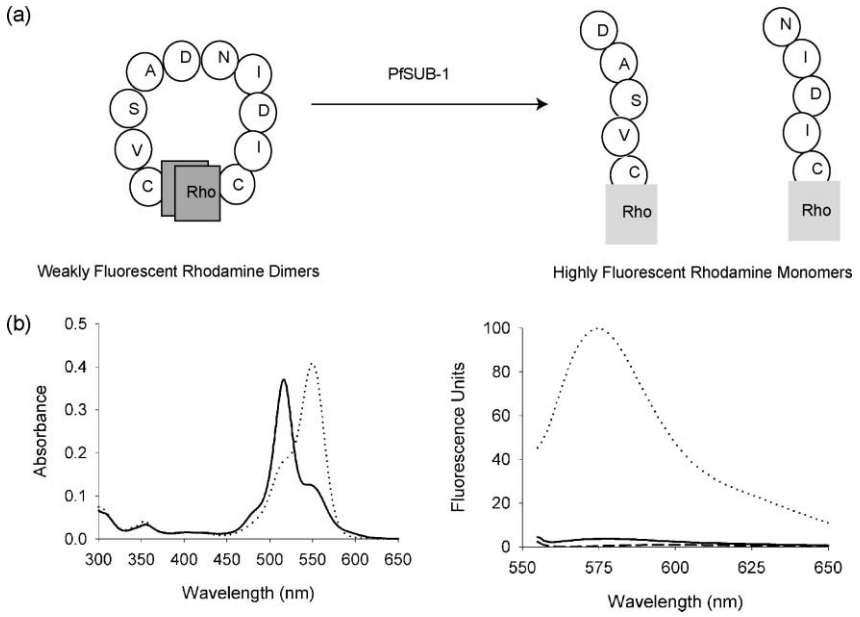


Fig. 2.5 (a) Sketch depicting a decapeptide labelled at both N- and C-termini with rhodamine. Enhancement of the fluorescence occurs upon proteolytic cleavage with PfSUB-1 (a subtilisin-like serine protease expressed in the merozoite stage of the human malaria parasite Plasmodium falciparum) and subsequent disruption of the weakly fluorescent ground-state rhodamine dimer. (b) Left. Absorption spectra of peptide (part **A***) before (solid line) and after (dotted line) proteolysis. Right: Emission spectra of peptide (part* **A***) before (solid line) and after (dotted line) proteolysis. Dashed line shows buffer background.*

However, because of the high local concentration of rhodamine in the doubly labelled peptide, it exists as a dimer in the μM concentration range. The rhodamine dimer has very low fluorescence compared with the monomer so on cleavage of the peptide by the protease, there is a 15-fold increase in the fluorescence intensity which allows the reaction to be monitored. This scenario is depicted in Figure 2.5. However, there is a large difference in the extinction coefficient at the excitation wavelength (550 nm) between the rhodamine monomer (98,600 M^{-1} cm^{-1}) and dimer (29,900 M^{-1} cm^{-1}). Therefore, when a solution of 2 μM peptide (4 μM rhodamine) is cleaved by the protease, the absorbance, in a 1-cm pathlength cell, at 550 nm changes from 0.120 to 0.394. In this case, the excitation light transmitted through the 1-cm cell falls from 76 to 40% and hence the light available in the centre of the cuvette – the region typically monitored by the collection optics – to excite the fluorophore in the monomeric state is less than that in the dimer state, resulting in less than expected emitted light. This phenomenon is termed the inner filter effect and can be important when large changes in absorbance at the excitation wavelength occur during the reaction. To minimize such effects, and to conserve sample volume, these types of measurements are often carried out using smaller cuvettes, e.g. 3 mm pathlength.

APPLICATION OF FLUORESCENCE TO HIGH THROUGHPUT SCREENING

High throughput screening is now almost universally practised by the pharmaceutical industry in the search for small molecule leads against drug targets. Many components contribute to make a successful high throughput screening campaign – a suitable assay which tests the activity of the target being screened, a chemically diverse collection of test compounds, liquid handling equipment to transfer test compounds and assay reagents into the screening plates, a plate reader to measure the assay signal coming from each well and software to deal with the considerable amount of data generated. In the early years, assays based on radiochemical detection were typically used, but more recently the emphasis has shifted to designing assays based on fluorescence, which offers a potentially higher quality and more discriminating output, is more amenable to miniaturization and avoids the logistical problems associated with using radioactivity.

DETECTION PLATFORMS

The majority of high throughput screening campaigns are run in plates of either 96, 384 or 1,536 wells. The detectors (plate readers) used to measure

fluorescence in high throughput screens can be considered in two categories – point readers and imagers. The different detection modes (i.e. intensity, polarization, lifetime) and the information obtained from them have been discussed earlier. Point readers are photomultipier-based detectors, which measure the fluorescence signal from each well of the plate in turn, typically by moving the plate but in some cases by moving the detection head. The optical set up of a typical reader is slightly different to that of the cuvette-based fluorimeter described earlier, since the geometry of plates requires that the emitted light is monitored along the same path as the excitation light, usually from above the plate. In some instances, the detection is through the underside of glass-bottomed plates but the principles are the same. Figure 2.6 shows a schematic diagram of a typical point reader. The light source is usually a Xenon arc lamp but can be a laser or pulsed source for more sophisticated measurements. The desired excitation wavelength is usually selected using a bandpass filter rather than a monochromator. The excitation light is reflected into the sample in

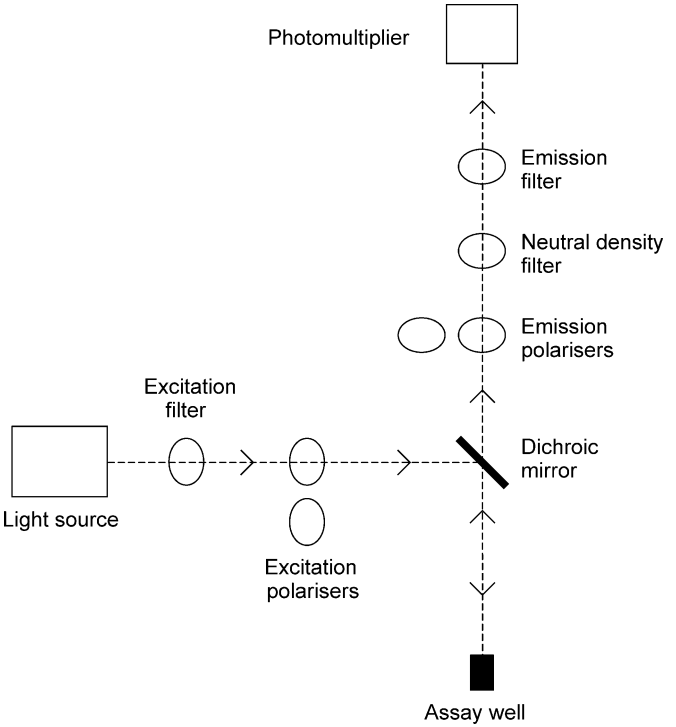


Fig. 2.6 Schematic diagram of a fluorimetric plate reader designed for high throughput screening.

the plate by a dichroic mirror which is chosen to reflect light of the excitation wavelength. The longer wavelength emitted light, however, passes through the dichroic mirror thus separating it from the excitation path. A second bandpass filter appropriate to the emission wavelength of the fluorophore reduces scattered light reaching the photomultiplier which generates the signal. For the detection of fluorescence polarization, additional polarizing filters are used on the excitation and emission paths. The parallel and perpendicular components of the emission are measured in turn by exchanging the two emission polarizers (T-format measurements as discussed earlier are not possible because of the geometry of the system). For time-resolved measurements (e.g. TRET) a pulsed light source is used together with electronically time-gated detection of the emitted light. The pulsed source is obtained either using a flash lamp or a rapidly spinning disc called a chopper.

Imaging readers have gained popularity in recent years, where the emitted light from the whole plate is captured on a CCD chip using a sophisticated lens array. The optical path is similar to that shown in Figure 2.6, where the CCD camera replaces the photomultiplier. Computational analysis then imposes the well structure of the plate on the image and evaluates an intensity value for each well. Polarization and time-resolved measurements are also possible with these devices. No mechanical movement of the plate is required during the measurement and the overall reading time can be greatly reduced.

INTERFERENCE IN FLUORESCENCE MEASUREMENTS

All types of assay are prone to interference – much of which originates from the test compounds themselves. In fluorescence-based assays, autofluorescence, quenching, inner-filter effects and test compound insolubility, can all perturb the measurement and an understanding of these in the context of the physical principles of fluorescence is valuable. Autofluorescence is a slightly misleading term, which refers to either a specific fluorescence signal from the test compound under the particular detection conditions or more likely a spurious signal originating from some contaminant in the sample. Quenching, which can occur by more than one mechanism, refers to a reduction in the probe's emission intensity caused by an interaction between the quencher and the probe. The inner-filter effect is an absorbance-based phenomenon where either the excitation or emission light is attenuated by test compound or contaminant absorption (as discussed earlier). Test compound insolubility can give rise to excessive light scattering. Although this will be at the same wavelength as the excitation light, if the effect is large enough it can distort the measurement. Depending on the direction of the signal change in an assay based on fluorescence intensity, all of these effects can generate either false

positives or false negatives. The frequency of false negatives is statistically low, since it depends on the concurrence of two low probability events (a hit and an interference phenomenon in the same well). However, the likelihood of generating false positives is very real and it is essential to bear this in mind at the analysis stage and have mechanisms in place to reject these in favour of the real hits. Furthermore, the assay should be designed so as to minimize this. The effect of compound interference on some readout modes is more specific. For example, consider a fluorescence polarization assay measuring the displacement of a small labelled ligand from a macromolecular binding partner, as discussed previously. Compound autofluorescence is likely to be of low polarization and can thus exaggerate the apparent level of ligand displacement in that well, hence generating a false positive. Conversely, the formation of aggregates between test compounds and a small labelled ligand will give rise to a high polarization state.

STRATEGIES TO DEAL WITH INTERFERENCE

A general strategy to mitigate optical interference, which should be followed whenever possible, is to use long wavelength probes. The four sources of test compound interference described above are all observed to decrease quite sharply as the excitation wavelength is increased. A striking reduction in interference is typically seen when moving from UV wavelength probes (e.g. coumarins) to visible wavelength probes. Some readout modes are more robust to interference. For example, in TRET measurements, long-lifetime donors (e.g. lanthanide ion complexes) are used in conjunction with time-gated detection of the acceptor emission. Compound autofluorescence, which is typically of very short lifetime, is allowed to decay before the longer lifetime emission from the probe is measured. A downside to this technique is that the donors used are of UV wavelength and prone to quenching. However, by monitoring the emission from both the donor and the acceptor, a correction for quench can be introduced. The direct measurement of a probe's lifetime, which is currently in its infancy and requires sophisticated instrumentation, is another strategy to minimize interference from autofluorescence, but these measurements do suffer from quenching effects. A particular advantage of using polarization is that the total intensity (which, upon excitation with polarized light is equal to the sum of the parallel emission intensity plus twice the perpendicular emission intensity) is available as a secondary parameter, and this should be used to highlight wells displaying anomalously high or low intensity values indicative of autofluorescence or quenching respectively.

CONCLUSION

Fluorescence techniques occupy a central position in the study of protein–ligand and protein–protein interactions. Knowledge of the origins of the various fluorescence parameters described here allows sensitive and informative assays to be designed, which can be used in detailed mechanistic evaluations of interactions at equilibrium as well as the kinetics of enzyme–catalysed processes. The development of sensitive and accurate multi-well plate readers and a diversity of long wavelength fluorophores have revolutionized the use of fluorescence-based assays in miniaturized high throughput screening, and these methods now underpin the majority of early stage drug discovery programmes.

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