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Advanced Fluorescence Reporters in Chemistry and Biology I

Fundamentals and Molecular Design

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Volume Editor
Prof. Dr. Alexander P. Demchenko
Palladin Institute of Biochemistry
National Academy of Sciences of Ukraine
Kyiv 01601
Ukraine
alexdem@ukr.net

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Series Editor

Prof. Dr. Otto S.Wolfbeis

Institute of Analytical Chemistry
Chemo- and Biosensors
University of Regensburg
93040 Regensburg
Germany
otto.wolfbeis@chemie.uni-regensburg.de

Aims and Scope

Fluorescence spectroscopy, fluorescence imaging and fluorescent probes are indispensible tools in numerous fields of modern medicine and science, including molecular biology, biophysics, biochemistry, clinical diagnosis and analytical and environmental chemistry. Applications stretch from spectroscopy and sensor technology to microscopy and imaging, to single molecule detection, to the development of novel fluorescent probes, and to proteomics and genomics. The *Springer Series on Fluorescence* aims at publishing state-of-the-art articles that can serve as invaluable tools for both practitioners and researchers being active in this highly interdisciplinary field. The carefully edited collection of papers in each volume will give continuous inspiration for new research and will point to exciting new trends.

Preface

Fluorescence reporter is the key element of any sensing or imaging technology. Its optimal choice and implementation is very important for increasing the sensitivity, precision, multiplexing power, and also the spectral, temporal, and spatial resolution in different methods of research and practical analysis. Therefore, design of fluorescence reporters with advanced properties is one of the most important problems. In this volume, top experts in this field provide advanced knowledge on the design and properties of fluorescent dyes. Organic dyes were the first fluorescent materials used for analytical purposes, and we observe that they retain their leading positions against strong competition of new materials – conjugated polymers, semiconductor nanocrystals, and metal chelating complexes. Recently, molecular and cellular biology got a valuable tool of organic fluorophores synthesized by cell machinery and incorporated into green fluorescent protein and its analogs.

Demands of various fluorescence techniques operating in spectral, anisotropy, and time domains require focused design of fluorescence reporters well adapted to these techniques. Near-IR spectral range becomes more and more attractive for various applications, and new dyes emitting in this range are strongly requested. Two-photonic fluorescence has become one of the major tools in bioimaging, and fluorescence reporters well adapted to this technique are in urgent need. These problems cannot be solved without the knowledge of fundamental principles of dye design and of physical phenomena behind their fluorescence response. Therefore, this book describes the progress in understanding these phenomena and demonstrates the pathways for improving the response to polarity, viscosity, and electric field in dye environment that can be efficiently used in sensing and imaging. Prospective pathways of synthesis of new dyes, including creation of their combinatorial libraries, and of their incorporation into molecular and supramolecular sensor elements are highlighted in this book.

Demonstrating the progress in an interdisciplinary field of research and development, this book is primarily addressed to specialists with different background – physicists, organic and analytical chemists, and photochemists – to those who develop and apply new fluorescence reporters. It will also be useful to specialists in bioanalysis and biomedical diagnostics – the areas where these techniques are most extensively used.

Kyiv, Ukraine Alexander P. Demchenko
June 2010

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Part I
General Aspects

Comparative Analysis of Fluorescence Reporter Signals Based on Intensity, Anisotropy, Time-Resolution, and Wavelength-Ratiometry

Alexander P. Demchenko

Abstract The response signal of an immense number of fluorescence reporters with a broad variety of structures and properties can be realized through the observation in changes of a very limited number of fluorescence parameters. They are the variations in intensity, anisotropy (or polarization), lifetime, and the spectral changes that allow wavelength-ratiometric detection. Here, these detection methods are overviewed, and specific demands addressed to fluorescence emitters for optimization of their response are discussed.

Keywords Anisotropy · Intensity sensing · Time-resolved fluorimetry · Wavelength ratiometry

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A.P. Demchenko
Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Kyiv 01601, Ukraine
e-mail: alexdem@ukr.net

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1 Why Fluorescence?

Fluorescence is the basic reporting technique in many chemical sensors and biosensors with a broad range of applications in clinical diagnostics, monitoring the environment, agriculture, and in various industrial technologies. Being an efficient method of transforming the act of target binding into readable signal already on molecular level, it puts virtually no limit to target chemical nature and size. The range of its applications extends to imaging the living cells and tissues with the possibility of recording the target spatial distribution. In all these applications, fluorescence competes successfully with other detection methods that are based on electrochemical response or on the change in mass, heat, or refractive index on target binding [1]. There are many reasons for such great popularity:

- Fluorescence techniques are the *most sensitive*. With proper dye selection and proper experimental conditions, the absolute sensitivity may reach the limit of single molecules. This feature is especially needed if the target exists in trace amounts. High sensitivity may allow avoiding time-consuming and costly target-enrichment steps.
- They offer very high *spatial resolution* on the level of hundreds of nanometers, which is achieved by light microscopy. Moreover, with recent developments on overcoming the light diffraction limit, it has reached molecular scale. This allows obtaining detailed cellular images and operating with dense multianalyte sensor arrays.
- Their distinguishing feature is the *high speed* of response. This response develops on the scale of fluorescence lifetime of photophysical or photochemical events that provide the response and can be as short as 10^{-8}–10^{-10} s. Because of that, the fluorescence reporting is never time-limiting, so that this limit comes from other factors, such as the rate of target – sensor mutual diffusion and the establishment of dynamic equilibrium between bound and unbound target.
- They allow sensing *at a distance* from analyzed object. Because the fluorescence reporter and the detecting instrument are connected via emission of light, the sensing may occur in an essentially noninvasive manner and allow formation of images.
- The greatest advantage of fluorescence technique that derives from these features is its *versatility*. Fluorescence sensing can be provided in solid, liquid, and gas media, and at all kinds of interfaces between these phases. It can trace rare events with high spatial and temporal resolution. Fluorescence detection can be equally well-suited for remote industrial control and for sensing different targets within the living cells.

To our benefit, fluorescence is a well-observed phenomenon characteristic for many materials. This allows providing broad selection of fluorescence reporters that have to be chosen according to different criteria: high molar absorbance and fluorescence quantum yield, convenient wavelengths of excitation and emission, high chemical stability, and photostability. They are well-described in other chapters of this book and in other books of these series. As we will see subsequently, they should be adapted to particular technique of target detection and to particular method of observation of fluorescence response, which needs establishing additional criteria for their selection.

In this regard, it has to be stressed that fluorescence reporters have to be divided into two broad categories according to two major trends of technologies in which they are used. This division is necessary because some criteria for the choice of optimal reporters are quite the opposite.

One category is the reporters serving as *labels* and *tags*. Their only response should be based on their presence in particular medium or at particular site. Ideally, the response should be *directly proportional* to reporter concentration and *independent* of any factors that influence fluorescence parameters (quenching or enhancing of emission, wavelength shifting). Such emitting dyes or nanoparticles are extensively applied in imaging techniques based on their affinity to particular components of a complex system (e.g., living cell) and also in sensing different soluble targets that uses separation of bound and unbound labeled components. The most advantageous in these applications are the dyes that are nonfluorescent in a free state but become strongly fluorescent on their binding; this allows avoiding separation of labeled compounds and free reporters. The common observation in the application of labels and tags is the detection of fluorescence intensity, so that high spectral resolution may not be needed.

The second category is the reporters serving as *probes* or that involved in *molecular sensors*. As probes, they should respond to the changes of their molecular environment, and as essential parts of the sensors, they should be coupled to recognition units and respond to target binding by the change of parameters of their fluorescence. Ideally, this response should be *independent* of their concentration, and the valuable information should be derived from the concentration-independent *change* of their fluorescence parameters. Therefore, the reporters should be selected with the properties that provide these changes in the broadest dynamic range.

Accordingly, we have to consider two types of sensitivity in fluorescence reporting. One is the absolute sensitivity, which is the ability to detect fluorescence signal with the necessary level of precision. The other, which should be applied to probes and sensors, is the sensitivity in detecting the difference between the probes interacting differently with their environment or between the sensors with bound and unbound target. This type of sensitivity is determined by dynamic range of variation of the recorded fluorescence parameters. Developing such reporters is a much harder task, and it deserves a more detailed discussion.

Several parameters of fluorescence emission can be used as outputs in fluorescence sensing and imaging. Fluorescence intensity F can be measured at given

wavelengths of excitation and emission (usually, band maxima). Its dependence on emission wavelength, $F(\lambda^{em})$ gives the fluorescence *emission spectrum*. If this intensity is measured over the excitation wavelength, one can get the fluorescence *excitation spectrum* $F(\lambda^{ex})$. Emission *anisotropy*, r (or similar parameter, polarization, P) is a function of the fluorescence intensities obtained at two different polarizations, vertical and horizontal. Finally, emission can be characterized by the *fluorescence lifetime* τ_F, which is the reverse function of the rate of emissive depopulation of the excited state. All these parameters can be determined as a function of excitation and emission wavelengths. They can be used for reporting on sensor-target interactions and a variety of possibilities exist for their employment in sensor constructs. The major concern here is obtaining reproducible analytical information free from interferences and background signals.

2 Sensing Based on Emission Intensity

Emission intensity measurements with low spectral resolution are frequently used in all types of techniques that involve fluorescence labeling and also in different sensing and imaging technologies that use fluorescence quenching as the reporter signal. Fluorescence reporters in the form of molecules or nanoparticles are either covalently conjugated to molecules of interest or used as stains to detect quantitatively the target compounds by noncovalent attachment. In cellular research, they can penetrate spontaneously into the cell and label genetically prepared protein-binding sites.

The change from light to dark (or the reverse) in fluorescence signal is easily observed and recorded as the change of fluorescence intensity at a single wavelength so that high spectral resolution is commonly not needed. For providing the coupling of sensing event with a change in fluorescence intensity from very high values to zero or almost zero values a variety of quenching effects can be used. The quenching may occur due to *conformational flexibility* in reporter molecule [2], intramolecular *photoinduced electron transfer* (PET) between its electron-donor and electron-acceptor fragments [3], on interaction with other chromophores [4], or with heavy [5] and transition metal [6] ions. Formation–disruption of hydrogen bonds with solvent molecules and different solvent-dependent changes of dye geometry can be observed in many organic dyes. Dramatic quenching in water (and to lesser extent in some alcohols) may occur due to formation by these molecules the traps for solvated electrons [7]. In addition, the solvent can influence the dye energetics, particularly the inversion of n^* (non-fluorescent) and π^* (fluorescent) energy levels [8]. Thus, the researcher has a lot of choice for constructing a sensor with response based on the principle of intensity sensing [9, 10].

Connection between the reversible target binding and the change in fluorescence intensity can be easily established based on the mass action law. In the simplest case

of binding with stoichiometry 1:1, the target analyte concentration [A] can be obtained from the measured fluorescence intensity F as:

$$[A] = K_d\left(\frac{F - F_{\min}}{F_{\max} - F}\right) \tag{1}$$

Here $F_{\min}$ is the fluorescence intensity without binding and $F_{\max}$ is the intensity when the sensor molecules are totally occupied. K_d is the dissociation constant. The differences in intensities in the numerator and denominator allow compensating for the background signal, and the obtained ratio can be calibrated in target concentration. But since F, $F_{\min}$ and $F_{\max}$ are expressed in relative units, they have to be determined in the same test and in exactly the same experimental conditions. This requires proper calibration, which is difficult and often not possible.

Calibration in fluorescence sensing means the operation, as a result of which at every sensing element (molecule, nanoparticle, etc.) or at every site of the image the fluorescence signal becomes independent of any other factor except the concentration of bound target. It is needed because the fluorescence intensity is commonly measured in relative units that have no absolute meaning if not compared with some standard measurement, and therefore, the problem of calibration in intensity sensing is very important [11]. Thus, the recorded changes of intensity always vary from instrument to instrument, and the proper reference even for compensating these instrumental effects is difficult to apply. Additional problems appear on obtaining information from cellular images and sensor arrays where the distribution in reporter concentration within the image or between different array spots cannot be easily measured. Moreover, their number can decrease due to chemical degradation and photobleaching. Therefore, internal calibration and photostability become a great concern in these applications. These difficulties justify strong efforts of the researchers to develop fluorescence dyes and sensing methods that allow excluding or compensating these factors. Those are the "intrinsically referenced" fluorescence detection methods [12, 13] that will be considered below.

3 Variation of Emission Anisotropy

Like other methods of fluorescence sensing, the anisotropy sensing is based on the existence of two states of the sensor, so that the switching between them depends on the concentration of bound target. Anisotropy sensing allows providing direct response to target binding that is independent of reporter concentration. This is because the measured anisotropy (or polarization) does not depend on absolute fluorescence intensity.

The measurement of *steady-state anisotropy r* is simple and needs two polarizers, one in excitation and the other in emission beams. When the sample is excited

by vertically polarized light (indexed as $_V$) and the intensity of emission is measured at vertical (F_{VV}) and horizontal (F_{VH}) polarizations, then one can obtain r from the following relation:

$$r = \frac{F_{VV} - G \times F_{VH}}{F_{VV} + G \times 2F_{VH}} = \frac{1 - G \times (F_{VH}/F_{VV})}{1 + G \times 2(F_{VH}/F_{VV})}, \tag{2}$$

where G is an instrumental factor. Anisotropy has substituted *polarization* P, which was also used for characterizing polarized emission, and their relation is $r = 2P/(3 - P)$.

Equation (2) shows why r is in fact a *ratiometric parameter*: this is because the variations of intensity influence proportionally the F_{VV} and F_{VH} values. Therefore, the anisotropy allows obtaining self-referencing information on sensing event from a single reporter dye. This information is independent on reporter concentration.

Anisotropy describes the rotational dynamics of reporter molecules or of any sensor segments to which the reporter is rigidly fixed. In the simplest case when both the rotation and the fluorescence decay can be represented by single-exponential functions, the range of variation of anisotropy (r) is determined by variation of the ratio of fluorescence lifetime (τ_F) and *rotational correlation time* (φ) describing the dye rotation:

$$r = \frac{r_0}{1 + \tau_F/\varphi} \tag{3}$$

Here r_0 is the limiting anisotropy obtained in the absence of rotational motion. The dynamic range of anisotropy sensing is determined by the difference of this parameter observed for free sensor, which is commonly the rapidly rotating unit and the sensor-target complex that exhibits a strongly decreased rate of rotation.

As follows from (3), the variation of anisotropy can be observed if φ and τ_F are of comparable magnitude, and on target binding, there is the variation of *rotational mobility* of fluorophore (the change of φ) or the variation of its *emission lifetime* τ_F. At given τ_F, the rate of molecular motions determines the change of r, so that in the limit of slow molecular motions ($\varphi \gg \tau_F$) r approaches r_0, and in the limit of fast molecular motions ($\varphi \ll \tau_F$) r is close to 0. This determines dynamic range of the assay, which will decrease if φ and τ_F change in the same direction. Thus, there are three possibilities for using the fluorescence anisotropy in sensing:

- When anisotropy increases with the increase of molecular mass of rotating unit. For instance, the sensor segment rotates rapidly and massive target binding decreases this rate. The target binding can also displace small competitor to solution with increase of its rotation rate.
- When anisotropy increases due to increase of local viscosity producing higher friction on rotating unit. This can happen, for instance, in micelles or lipid

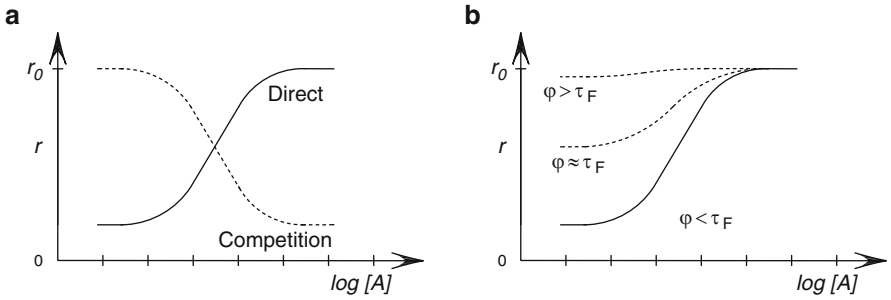

Fig. 1 Dependence of response of anisotropy sensor on analyte concentration in direct and competition assays (**a**) and this dependence for direct assay at different correlations between φ and τ_F (**b**)

vesicles that change their dynamics and order on target binding, and incorporated dye senses that.

- When anisotropy increases due to fluorescence lifetime decrease being coupled to any effect of dynamic quenching.

The differences between two (free and with the bound target) sensor states are detected when they possess different values of anisotropy, r_f of free and r_b of bound state (Fig. 1a). Their fractional contributions depend also on the relative intensities of correspondent forms. Since the additivity law is valid only for the intensities, the parameters derived in anisotropy sensing appear to be weighted by fractional intensities of these forms, F_f and F_b:

$$r = F_f r_f + F_b r_b \tag{4}$$

This means that if the intensity of one of the forms is zero (static quenching), such anisotropy sensor is useless since it will show anisotropy of only one of the forms. The account of fractional intensity factor $R = F_b/F_f$ (the ratio of intensities of bound and free forms) leads to a more complicated function for the fraction of bound target, f:

$$f = \frac{r - r_f}{(r - r_f) + R(r_b - r)} \tag{5}$$

Advantages and disadvantages of sensing technologies based on the measurement of anisotropy were discussed many times [14], and we will address only the questions related to the choice of optimal reporters. The limiting r_0 value 0.4 is theoretically achieved only for fluorophores with collinear absorption and emission transition dipole moments, and this limits the dynamic range of response. But the most important is fitting τ_F to the range of variations of φ (Fig. 1b). The fact is that with typical dyes possessing τ_F of several nanoseconds, the sensors can detect the binding of only small labeled molecules, or labeled receptors should be very flexible without targets. In the case of sensing of high molecular weight targets, τ_F should be

10–100 ns or longer [15]. It should satisfy the best sensing conditions, which correspond to $\varphi < \tau_F$ before the target binding and $\varphi > \tau_F$ after the binding. The possibility to achieve this range with large molecular rotating units is offered only by long-lifetime luminophors and only by those of them, which possess high r_0 values.

The weak point of anisotropy sensing is its great sensitivity to light-scattering effects. This occurs because the scattered light is always 100% polarized, and its contribution can be a problem if there is a spectral overlap between scattered and fluorescent light. For avoiding the light-scattering artifacts, the dyes with large Stokes shifts should be preferably used together with sufficient spectral resolution.

4 Time-Resolved and Time-Gated Detection

Fluorescence decays as a function of time, and the derived lifetimes can be used in fluorescence reporting. In an ideal case, the decay is exponential and it can be described by initial amplitude α and lifetime τ_F for each of the two, free (with index^F) and bound (with index^B), forms. If both of these forms are present in emission, we observe the result of additive contributions of two decays:

$$F(t) = \alpha_F \exp\left(-t/\tau_F^F\right) + \alpha_B \exp\left(-t/\tau_F^B\right) \tag{6}$$

To be detected, the presence of target should provide significant change of τ_F recorded within the time resolution of the method. Application of lifetime detection in sensing is based on several principles:

- Modulation of τ_F by dynamic quencher. Here, the effect of quenching competes with the emission in time and is determined by the diffusion of a quencher in the medium and its collisions with the excited dye. In this case, the relative change of intensity, F_0/F, is strictly proportional to correspondent change of fluorescence lifetime, τ_0/τ_F, where F_0 and τ_0 correspond to conditions without quencher [16]. Successfully this approach was applied only to oxygen sensing using the long-lifetime luminescence emitters [17]. In this case, the decrease of τ_F occurs gradually with oxygen concentration (Fig. 2a).
- The switch between discrete emitter forms with fixed but different lifetimes corresponding to free (F) and bound (B) forms of the sensor. Belonging to the same dye, these two forms can be excited at the same wavelength. When excited, they emit light independently, and the observed nonexponential decay can be deconvolved into two different individual decays with lifetimes τ_F^F and τ_F^B (Fig. 2b). The ratio of preexponential factors α_F and α_B will determine the target concentration [18]:

$$\frac{\alpha_B}{\alpha_F} = \frac{\varepsilon_B}{\varepsilon_F} \frac{\Phi_B \tau_F^F}{\Phi_F \tau_F^B} \frac{[LR]}{[L]} \tag{7}$$

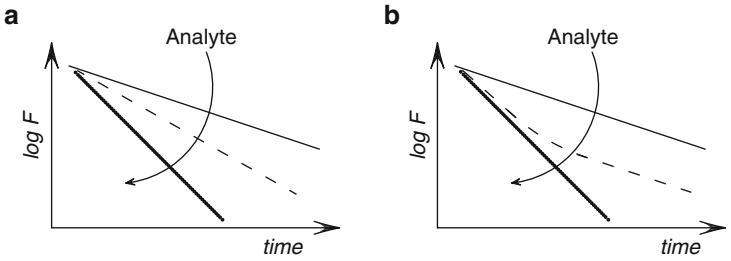

Fig. 2 The changes in fluorescence decay kinetics on binding the analyte. (**a**) The analyte is the dynamic quencher. The decay becomes shorter gradually as a function of its concentration. (**b**) The analyte binding changes the lifetime. Superposition of decay kinetics of bound and unbound forms is observed

It can be seen that the ratio of concentrations of free and occupied receptors is determined not only by α_F and α_B values but also by correspondent lifetimes τ_F^F and τ_F^B and the products of molar absorbances ε_F or ε_B and quantum yields Φ_F or Φ_B.

- Using the long-lifetime emission as a reference in intensity sensing by short-lifetime dye. This approach known as dual luminophore referencing (DLR) will be considered in the next section.

The lifetime detection techniques are self-referenced in a sense that fluorescence decay is one of the characteristics of the emitter and of its environment and does not depend upon its concentration. Moreover, the results are not sensitive to optical parameters of the instrument, so that the attenuation of the signal in the optical path does not distort it. The light scattering produces also much lesser problems, since the scattered light decays on a very fast time scale and does not interfere with fluorescence decay observed at longer times.

Summarizing, we stress that the anisotropy and the fluorescence decay functions change in a complex way as a function of target concentration. Species that fluoresce more intensely contribute disproportionably stronger to the measured parameters. Simultaneous measurements of steady-state intensities allow accounting this effect.

5 Wavelength Ratiometry with Two Emitters

Simultaneous application of two emitting reporters allows providing the self-referenced reporter signal based on simple intensity measurements, without application of anisotropy or lifetime sensing that impose stringent requirements on fluorescence reporters. Usually, the two dyes are excited at a single wavelength with the absence or in the presence of interaction between them.

5.1 Intensity Sensing with the Reference

In intensity sensing, the most efficient and commonly used method of "intrinsic referencing" is the introduction of a *reference dye* into a sensor molecule (or into support layer, the same nanoparticle, etc.) so that it can be excited together with the reporter dye and provide the reference signal [1]. The reference dye should conform to stringent requirements:

- It should absorb at the same wavelength as the reporting dye. The less common is the use of two channels of excitation since this requires more sophisticated instrumentation.
- For recording the intensity ratio at two emission wavelengths, it should possess strongly different emission spectrum but a comparable intensity to that of reporter band.
- In contrast to that of reporting dye, the reference emission should be completely insensitive to the presence of target.
- Direct interactions between the reference and reporter dyes leading to PET or FRET in this approach should be avoided.

If the reference dye is properly selected, then it can provide an additional independent channel of information and two peaks in fluorescence spectrum can be observed – one from the reporter with a maximum at λ_1 and the other from the reference with a maximum at λ_2 (Fig. 3). Their intensity ratio can be calibrated in concentration of the bound target. Thus, if we divide both the numerator and denominator of (1) by $F_{\text{ref}}(\lambda_2)$, the intensity of the reference measured in the same conditions but at different wavelength (λ_2) from that of reporter, we can obtain target concentration from the following equation that contains only the intensity ratios $R = F(\lambda_1)/F_{\text{ref}}(\lambda_2)$, $R_{\min} = F_{\min}(\lambda_1)/F_{\text{ref}}(\lambda_2)$, and $R_{\max} = F_{\max}(\lambda_1)/F_{\text{ref}}(\lambda_2)$:

$$[A] = K_d\left(\frac{R - R_{\min}}{R_{\max} - R}\right) \tag{8}$$

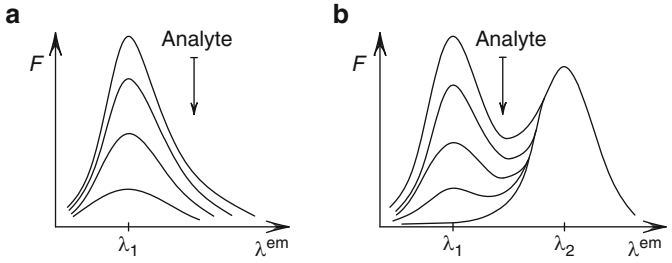

Fig. 3 Intensity sensing (**a**) and this sensing with the reference dye (**b**). The fluorescence intensity with the band maximum at λ_1 decreases as a function of analyte concentration. The reference dye allows providing the ratio of two intensities detected at wavelengths λ_1 and λ_2

Separate detection of these two signals, one from the reporter dye and the other from the reference, can be provided based not only on the difference of their fluorescence band positions but also on the difference in anisotropy [15] or lifetime [15, 19]. The change of these parameters with the variation of intensity of reporter dye is based on the fact that the measured anisotropy or lifetime is a sum of intensity-weighted anisotropies or lifetimes of contributing species. This type of referencing can be used even if the reporter and the reference dyes possess strongly overlapping fluorescence spectra. The intensity calibration in the lifetime domain has an advantage in the studies in highly light scattering media.

An interesting development in this respect is the dual luminophore referencing (DLR) in phase-modulation detection technique [19]. Phosphorescent luminophore with long lifetime serves as the reference producing strong and stable phase shift that can be measured using inexpensive device using LED light source. Reporter dye excited simultaneously with the reference can exhibit short lifetime, but its quenching/dequenching generates the change in phase shift of modulated emission. In this way, the phase angle reflects directly the intensity change of the reporter and consequently the concentration of the target. Here, the two-dye ratiometry combines the advantages of time-resolved detection with simplicity of instrumentation using single filter-detector arrangement and operating at low modulation frequencies. This method was extended recently for detecting two analytes [20].

Summarizing, we outline what is achieved with the introduction of reference dye. The two dyes, responsive and nonresponsive to target binding, can be excited and their fluorescence emission detected simultaneously, which compensates the variability and instability of instrumental factors. In principle, the results should be reproducible on the instruments with a different optical arrangement, light source intensity, slit widths, etc. The two-band ratiometric signal can be calibrated in target concentration. This calibration, in some range of target concentrations, will be insensitive to the concentration of sensor (and reporter dye) molecules.

5.2 Formation of Excimers

When molecule absorbs light, it can make a complex with the ground-state molecule like itself. These excited dimeric complexes are called the excimers. Excimer emission spectrum is very different from that of monomer; it is usually broad, shifted to longer wavelengths, and it does not contain vibrational structure. The double labeling is needed for this technique, which is facilitated by the fact that the dyes are of the same structure. Meantime, a researcher is limited in their selection. Usually pyrene derivatives are used because of unique property of this fluorophore to form stable excimers with fluorescence spectra and lifetimes that are very different from that of monomers. The structured band of monomer is observed at about 400 nm, whereas that of excimer located at 485 nm is broad, structureless, and long-wavelength shifted. Long lifetimes ($\sim$300 ns for monomer and $\sim$40 ns for

excimer) allow easy rejection of background emission and application of lifetime sensing [21].

There are many possibilities to use these complex formations in fluorescence sensing. If the excimer is not formed, we observe emission of the monomer only, and upon its formation there appears characteristic emission of the excimer. We just need to make a sensor, in which its free and target-bound forms differ in the ability of reporter dye to form excimers and the fluorescence spectra will report on the sensing event. Since we will observe transition between two spectroscopic forms, the analyte binding will result in increase in intensity of one of the forms and decrease of the other form with the observation of isoemissive point [22].

Meantime, we have to keep in mind that monomer and excimer are independent emitters possessing different lifetimes and that nonspecific influence of quenchers may be different for these two forms. For instance, dissolved oxygen may quench the long-lifetime emission of monomer but not of the excimer.

5.3 *Förster Resonance Energy Transfer*

Two or more dye molecules or light absorbing particles with similar excited-state energies can exchange their energies due to long-range dipole–dipole resonance interaction between them. One molecule, the *donor*, can absorb light and the other, the *acceptor*, can accept this energy with or without emission. This phenomenon known as Förster resonance energy transfer (FRET) has found many applications in sensing [23, 24]. The FRET sensing usually needs labeling with two dyes serving as donor and acceptor. Only in rare, lucky cases, intrinsic fluorescent group of sensor or target molecules can be used as one of the partners in FRET sensing.

FRET to nonfluorescent acceptor provides a single-channel response in intensity with all disadvantages that were described above. Meantime, there are two merits in this approach. One is over traditional intensity sensing: the quenching can occur at a long distance, which allows exploring conformational changes in large sensor molecules, such as proteins [25] or DNA hairpins [26]. The other is over the FRET techniques using fluorescent acceptor: a direct excitation of the acceptor is not observed in emission.

FRET to fluorescent acceptor is obviously more popular because of its two-channel self-calibrating nature. Sensing may result in switching between two fluorescent states, so that in one of them a predominant emission of the donor can be observed and in the other – of the acceptor. This type of FRET can be extended to time domain with the benefit of using simple instrumentation with the long-lifetime donors [27].

FRET can take place if the emission spectrum of the donor overlaps with the absorption spectrum of the acceptor and they are located at separation distances within 1–10 nm from each other. The efficiency of energy transfer E can be defined

as the number of quanta transferred from the donor to the acceptor divided by all the quanta absorbed by the donor. According to this definition, $E = 1 - F_{DA}/F_D$, where F_{DA} and F_D are the donor intensities in the presence and absence of the acceptor. Both have to be normalized to the same donor concentration. If the time-resolved measurements are used, then the knowledge of donor concentration is not required, and $E = 1 - <\tau_{DA}>/<\tau_D>$, where $<\tau_{DA}>$ and $<\tau_D>$ are the average lifetimes in the presence and absence of the acceptor [28].

The energy transfer efficiency exhibits a very steep dependence on the distance separating two fluorophores, R:

$$E = R_0^6/\left(R_0^6 + R^6\right), \tag{9}$$

here, R_0 is the parameter that corresponds to a distance with 50% transfer efficiency (the Förster radius). Such steep dependence on the nanometer scale allows diversity of possibilities in sensor development. We list several of them:

- FRET sensing based on heterotransfer (the transfer between different molecules or nanoparticles) with reporting to the change of donor–acceptor distance. Since this distance is comparable with the dimensions of many biological macromolecules and of their complexes, many possibilities can be realized for coupling the response with the changes in sensor geometry. The most popular approaches use conformational change in double labeled sensor [29], enzymatic splitting of covalent bond between two labeled units [30] and competitive substitution of labeled competitor in a complex with labeled sensor [31].
- Exploration of collective effects in multiple transfers that appear when the donor and acceptor are the same molecules and display the so-called homotransfer. In this case, the presence of only one molecular quencher can quench fluorescence of the whole ensemble of emitters coupled by homotransfer [32]. The other possibility of using homo-FRET is the detection of intermolecular interactions by the decrease of anisotropy [33].
- FRET modulation by photobleaching. Photobleaching can specifically destroy the acceptor giving rise to fluorescence of the donor. This approach is useful in some sensing technologies and especially in cellular imaging where it is important to compare two signals or images, with and without FRET, with the same composition and configuration in the system [34].
- FRET sensing based on protic equilibrium in the acceptor that changes its absorption spectrum and thus modulates the overlap integral [35]. There are many fluorescent pH indicators that display pH-dependent absorption spectra in the visible with their different positions depending on ionization state. Thus, the change in pH can be translated into the change of FRET efficiency.
- Photochromic FRET using as acceptors the photochromic compounds such as spiropyrans [36]. They have the ability to undergo a reversible transformation between two different structural forms in response to illumination at appropriate wavelengths. These forms may have different absorption (and in some cases,

fluorescence) spectra. Thus, they offer a possibility of reversible switching of FRET effect between "on" and "off" states without any chemical intervention, just by light.

Realization of all these possibilities is traditionally performed with organic dyes [28]. There are many variants in choosing the dye donor–acceptor pair in which two correspondent bands are well separated on the wavelength scale or produce different lifetimes. Meantime, we observe increasing popularity of lanthanide chelates [37] and Quantum Dots [38, 39] as FRET donors, which is mainly because of their increased brightness and longer emission lifetimes [40]. If the acceptor is excited not directly but by the energy transferred from the donor, its lifetime increases to that of the donor [41]. This allows providing many improvements in sensing technologies especially in view that organic dyes are much more "responsive" but are behind these emitters in lifetime and brightness.

Concluding the section on wavelength ratiometry with two emitters, we stress that they provide the two-channel informative signal in sensing, in which these channels are independent or, as in the case of FRET, partially dependent. In the latter case, quenching of fluorescence of the donor quenches also the acceptor emission but the quenching of the acceptor emission does not influence the emission of the donor. Independence of quenching effects may cause a nonspecific and nonaccountable effect on ratiometric reporter signal [42]. It should be also remembered that the reporter molecules can exhibit different degradation and photobleaching as a function of time. These effects may provide the time-dependent but target-independent changes of the measured intensity ratios. In addition, because the sensitivity to quenching (by temperature, ions, etc.) can be different for reporter and reference dyes and they emit independently, every effect of fluorescence quenching unrelated to target binding will interfere with the measured result. This can make the sensor nonreproducible in terms of obtaining precise quantitative data even in serial measurements with the same instrument.

6 Wavelength Ratiometry with Single Emitter

In sensor technologies, the use of a single emitter is more attractive than of two emitters. This is not because of avoiding the necessity of double labeling alone. Chemical degradation and photobleaching producing nonfluorescent products from the reporter dye in this case will not distort its wavelength-ratiometric signal. Meantime, the reporter dyes should conform to stringent requirements: they should possess spectrally recognizable ground-state and/or excited-state forms and the switching between these forms should occur on target binding. Ground-state interactions resulting in differences in excitation energies generate the differences in excitation spectra (Fig. 4a). The excited-state reactions offer additional possibilities

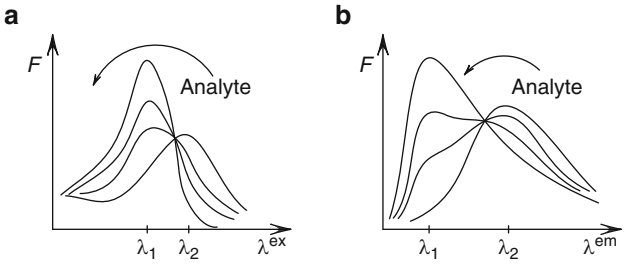

Fig. 4 The changes in excitation (**a**) and emission (**b**) spectra on analyte binding when this binding generates new ground-state or excited-state forms. λ_1 and λ_2 are the positions of the band maxima of the analyte-bound and analyte-free forms

for observing new bands in fluorescence emission spectra belonging to reactant and reaction product forms (Fig. 4b).

In contrast to intensity sensing with the reference, where the reference provides the signal of constant intensity, the two forms in a single reporter molecule interconvert reporting to target binding. We then observe interplay of intensities at two selected wavelengths, λ_1 and λ_2, with their change in converse manner and the generation of isobestic and isoemissive points. If such a point is chosen as the reference, then (8) can be used. In a more general case, when λ_2 is a different wavelength, (e.g., it is the maximum of the second band), the result has to be corrected to include the factor that accounts for this intensity redistribution, which is the ratio of intensities of free and bound forms at wavelength λ_2:

$$[A] = K_d \left(\frac{R - \mathrm{R_{min}}}{\mathrm{R_{max}} - R} \right) \left(\frac{F_\mathrm{F}(\lambda_2)}{F_\mathrm{B}(\lambda_2)} \right) \tag{10}$$

The change in noncovalent intermolecular interactions with the environment changes the energies of electronic transitions resulting in the shifts of electronic absorption and emission bands. If these interactions are stronger in the ground state, then with their increase, the difference in energy between the states increases resulting in the shift of spectra to shorter wavelengths. On the opposite, if the interaction energy is stronger in the excited state, then on increase of this interaction, the spectra shift to longer wavelengths.

6.1 Transitions Between Ground-State Forms

Spectacular differences in absorption/excitation spectra are often observed for the dyes that exist in protonation–deprotonation equilibria. Their straightforward application is for pH sensing and also for designing the reporters, in which the shifting of such equilibrium by external proton donor or acceptor group is involved in sensing event.

Next in importance is the response based on the shifts between H-bond free and H-bonded forms. Formation of intermolecular H-bonds leads to spectral changes in the same direction as the protonation but of smaller magnitude. H-bonding requires steric arrangement between donor and acceptor groups. In certain cases, it is coupled with conformational changes stabilizing one of the conformers. Spectral shifts can be also observed with the formation of ground-state intramolecular charge transfer (ICT) state that originates from polarization of π-electrons and can be stimulated by increased polarity of the medium. The intermolecular H-bonding can be involved also in this case: being formed at an acceptor site of ICT compounds, it causes red shifts in the absorption and emission bands, whereas the interaction at a donor site produces the shift in opposite direction.

One of the applications of these ground-state effects is the sensing of local electric fields with highly polarizable electrochromic dyes [43, 44]. The stilbene-like dyes exhibiting ICT are the popular sensors for Ca^{2+} ions that exhibit interaction of chelated Ca^{2+} ion with the electron-donor nitrogen atom [45]. There are many reports on the construction of chemical sensors for other, beside calcium, ions based on ICT mechanism [8] but those that exhibit ratiometric response are still rare cases. Promising are the systems that use the switching between two ground-state tautomers of the dye [46]. Commonly in all these cases, the wavelength-ratiometric signal is recorded at two excitation wavelengths with the detection of fluorescence at single wavelength (Fig. 4a).

6.2 Transitions Between Excited-State Forms

Being richer in energy than the ground states, the excited states allow broader range of electronic transformations resulting in shifts of fluorescence spectra and in the appearance of new bands that allows ratiometric detection of intensities (Fig. 4b). Unfortunately, many of these reactions result in quenching with the loss of benefits of wavelength-ratiometric recording. Therefore, efforts in sensor design should be directed at achieving the highest brightness of both initially excited and reaction product forms.

Proton dissociation in the excited states commonly occurs much easier than in the ground states, and the great difference in proton dissociation constants by several orders of magnitude is characteristic for 'photoacids' [47]. These dyes exist as neutral molecules and their excited-state deprotonation with the rate faster than the emission results in new red-shifted bands in emission spectra [48]. Such properties can be explored in the same manner as the ground-state deprotonation with the shift of observed spectral effect to more acidic pH values.

Excited-state intramolecular proton transfer (ESIPT) exhibits different regularities [49, 50]. Commonly, this is a very fast and practically irreversible reaction proceeding along the H-bonds preexisting in the ground state. Therefore, only the reaction product band is seen in fluorescence spectra. Such cases are not interesting for designing the fluorescence reporters. The more attractive dual emission is

observed in two cases. (a) When the initially excited state becomes the ICT state stabilized to be of similar energy as the ESIPT product state, then the ESIPT reaction becomes reversible and an equilibrium between two forms can be established on a timescale faster than the emission. This is the case of designed 3-hydroxyflavone derivatives [51]. (b) When the ESIPT reaction exhibits slow kinetics on the timescale of emission. This can be due to intermolecular H-bonding perturbations as observed for parent 3-hydroxyflavone [52] and 3-hydroxyquinolones [53]. In the case of rapidly established equilibrium between two forms, the internally calibrated signal is resistant to any uncontrolled quenching effect produced by collisional quenchers or the temperature. This is because their lifetimes are equal and they are quenched proportionally with the retention of the same intensity ratio [54–56]. Since the ESIPT reaction can provide dramatic shifts in fluorescence spectra (by 100 nm and more), finding new systems exhibiting dual emission based on ESIPT is a great concern.

Usually, the π-electronic system in highly fluorescent organic dyes becomes in the excited state a stronger dipole that interacts stronger with polar environment resulting in long-wavelength shifts [57, 58]. This effect can be enhanced by generating the ICT states by introducing into the π-electronic system the chemical substitutions donating and withdrawing electronic density [50]. Such dyes known as polarity sensors can be used for ratiometric reporting if the difference in polarity of reporter environment can be induced by target binding. If water, protic solvents, or H-bond forming groups of atoms are involved in interactions with reporter dye, then the H-bond formation with its acceptor group (that is usually carbonyl) results in the spectral shifts in the same direction as the increase of polarity [59]. Combination of these effects may result in dramatic spectral shifts that allow ratiometric reporting. The ICT states can be further stabilized with the formation of TICT (twisted intramolecular charge transfer) states that form distinct fluorescence bands [60]. Important point is the finding of such reporters, in which these states are strongly emissive.

6.3 Multiparametric Reporters Combining the Transitions Between Ground-State and Excited-State Forms

From basic photophysics, we may derive that in order to obtain the effects of switching in excitation spectra, we need to operate with two or more ground-state forms of the reporter dyes and to couple the sensing event with the transitions between them. In contrast, for obtaining two or more bands in fluorescence spectra, one ground-state form can be enough (and often preferable), but there should be excited-state reactions generating new species, so that both the reactant and the product in this reaction should emit fluorescence at different wavelengths. The ground-state and excited-state transformations can be governed by different types of molecular forces. Therefore, by proper reporter design, there is a possibility for

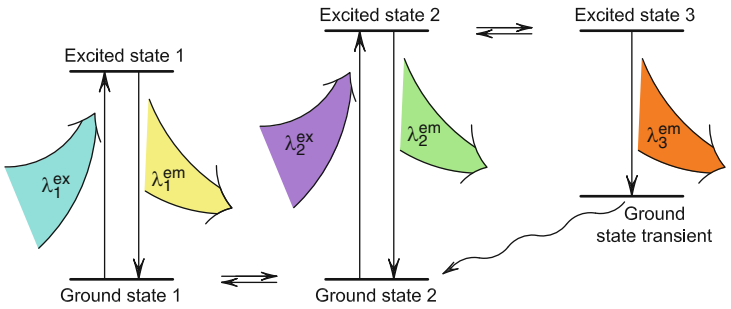

Fig. 5 General scheme of ground-state and excited-state transformations and emissions in the case of a reversible excited-state reaction involving one of the two ground-state species (from Ref. [62], modified)

not only combining these effects but also for obtaining separate information on these interactions. This idea is illustrated in Fig. 5. It was realized with 3-hydroxyflavone dyes exhibiting ground-state equilibrium between the species with and without intermolecular H-bonds [51] and also an excited-state equilibrium between ICT and ESIPT states indicating polarity of the environment [61]. When these dyes are applied to test the unknown properties of their environment, one can observe three partially overlapped emission bands, and their excitation-wavelength-dependent deconvolution allows obtaining independently the polarity and the hydrogen bonding potential [62]. Following this approach and with proper selection of reporter dye, one can design the reporter responding differently to two different properties of their environment and to construct sensors based on this response.

7 Concluding Remarks

From this short survey, one can derive that many possibilities for technology design can be realized based on proper selection of reporters within the limited number of fluorescence detection methods. Each of these methods offers its own advantage in sensing but puts its special demands on photophysical and spectroscopic properties of reporters. Intensity sensing is the simplest technique that is least demanding regarding the dye properties. But it features many disadvantages due to the need for internal calibration of response signal. Such self-referenced signal can be provided by second dye partner serving as the reference or participating in excimer formation and FRET.

The calibration may not be needed in anisotropy and lifetime sensing. In lifetime sensing, the single-channel response allows obtaining the signal that does not need calibration. In anisotropy sensing, the two (vertical and horizontal) polarizations provide the necessary two channels, and in FRET to fluorescent acceptor, these two channels are selected as the intensities at two wavelengths.

Being the most convenient way of providing the self-referenced signal, the two-band wavelength-ratiometric recording can be realized not only by the application of two dyes but also with a single dye exhibiting ground-state or excited-state reaction leading to wavelength-shifting and generation of new bands. In two-band ratiometric sensing because the signal comes from a single type of the dye and the forms emitting at two wavelengths may have the same lifetimes, the internally calibrated signal has the advantage to be resistant to any uncontrolled quenching effect.

Every one of these techniques needs proper selection of reporter dyes. Many requests therefore should be addressed to synthetic chemists and photochemists.

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Part II
Design of Organic Dyes

Optimized UV/Visible Fluorescent Markers

M. Sameiro T. Gonçalves

Abstract Fluorescent molecules have been widely used as biomolecular labels, enzyme substrates, environmental indicators, and cellular stains and thus constitute indispensable tools in chemistry, physics, biology, and medicinal sciences. The large variation in the photophysics of the available fluorophores connected with chemical alterations give fluorescent probe techniques an almost unlimited scope for the detection of specific molecules and the investigation of intermolecular interactions on a molecular scale.

This chapter focuses on recent developments in the design and applications of fluorescent organic markers, such as coumarins, benzoxadiazoles, acridones, acridines, polyaromatics (naphthalene, anthracene, and pyrene), fluorescein, and rhodamine derivatives, which display maximum fluorescence emission in the UV/visible region and have been applied in the labeling of relevant biomolecules, namely DNA, RNA, proteins, peptides, and amino acids, among others.

Keywords Benzoxadiazoles · Coumarins · Fluorescein · Fluorescent probes · Rhodamine

Contents

M.S.T. Gonçalves
Centro de Química, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal
e-mail: msameiro@quimica.uminho.pt

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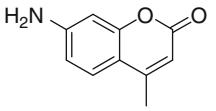
1 Introduction

Over the last years, fluorescent molecules have been widely used as biomolecular labels, enzyme substrates, environmental indicators, and cellular stains, and thus constitute indispensable tools in chemistry, physics, biology, and medicinal sciences [1–10]. Owing to their high sensitivity, the detection of single fluorescent molecules and investigation of the interaction of these molecules with their local environment, the visualization of a biochemical or biological process, have all become routinely possible through the use of appropriate instrumentation, near-field microscopy, or confocal techniques [11]. In addition, the large variation in the photophysics of the available fluorophores connected with chemical modifications give fluorescent probe techniques an almost unlimited scope for the detection of specific molecules and the investigation of intermolecular interactions on a molecular scale.

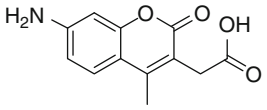
This chapter focuses on recent developments in the design and applications of fluorescent organic markers, such as coumarins, benzoxadiazoles, acridones, acridines, polyaromatics (naphthalene, anthracene, pyrene), fluoresceins, and rhodamines, which display maximum fluorescence emission in the UV/visible and have been applied in the labeling of relevant biomolecules, namely DNA, RNA, proteins, peptides, and amino acids, among others.

2 Coumarin Markers

2-Oxo-2*H*-benzopyrans, trivially designated as coumarins, represent one of the most widespread and interesting class of heteroaromatic reagents for fluorescent labeling. Organic probes built on the coumarin scaffold have been reported in the derivatization of amino acids [12–14], peptides [15], nucleic acids [16, 17], as well as in studies with proteins, namely enzymes [18–22]. Examples include 7-amino-4-methylcoumarin (AMC) **1** derivatives, such as 7-amino-4-methylcoumarin-3-acetic acid (AMCA) **2**, having a carboxylic acid as reactive group for derivatization and wavelength of maximum excitation (λ_{ex}) at 350 nm, which was a widely used UV-excitable probe for the fluorescent labeling of proteins [23]. AMCA **2** and its more recent analogue, Alexa Fluor 350, displayed an intense blue fluorescence with a narrow emission peak between 440 and 460 nm and showed excellent photostability (AMCA **2** is over three times more photostable when compared with fluorescein).

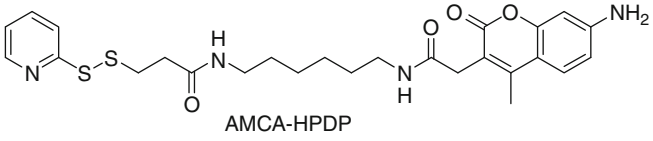


AMC
1



AMCA
2

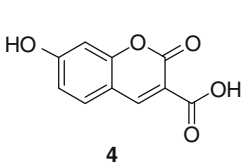
AMCA **1** is still being used. For example, Han and co-workers recently reported a fluorescence-based procedure designated as the "AMCA switch method," in which the S-nitrosylated cysteines are converted into AMCA fluorophore-labeled cysteines [24]. AMCA-HPDP **3** was used in the labeling step. The labeled proteins were then analyzed by nonreducing SDS-PAGE, and the S-nitrosylated molecules could be readily detected as brilliant blue bands under UV light. Furthermore, when combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS), the S-nitrosocysteines can be identified with the recognizable AMC tag in the MS spectra.



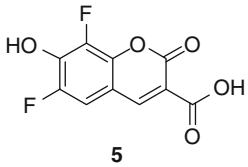
AMCA-HPDP

3

Violet-excitable derivatives of 7-hydroxycoumarin were developed and widely used in the preparation of fluorescent protein conjugates and enzyme substrates [25, 26]. Several 7-hydroxycoumarins conjugated to enzyme substrates were used for assays of phosphatases, β-galactosidases, and β-lactamases [27]. The 3-carboxy-7-hydroxycoumarin **4** has an excitation maximum at 386 nm, and the pK_a of its phenolic hydroxyl group is at about 7.5. In the physiological pH range of 6–8, the dye molecules are not fully deprotonated and, consequently, do not display their maximum fluorescence intensity. Studies of the influence of fluorination in the photochemical properties of 7-hydroxycoumarins demonstrated that fluorinated derivatives, namely 3-carboxy-6,8-difluoro-7-hydroxycoumarin (Pacific Blue **5**, wavelength of maximum absorption (λ_{abs})/wavelength of maximum emission (λ_{em}) 401/452 nm), have higher fluorescent quantum yields [28, 29] and red-shifted in excitation wavelengths (λ_{ex} ~400 nm).



4

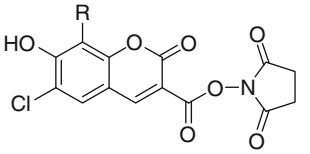


5

More recently, with the aim of searching for new violet-excitable dyes with improved photophysical and photochemical properties, three mono- and bis-halogenated hydroxycoumarins **6a**–**c** were synthesized, conjugated with antibodies, and cell analysis was screened using flow cytometry [30]. The monochlorinated hydroxycoumarin (V450) **6a** ($\lambda_{abs}/\lambda_{em}$ 404/448 nm, after reacting with 1 M glycine at pH 9.6 to stabilize its absorption maxima) was found to have a high fluorescence quantum yield ($\Phi_F \sim 0.98$), and human leucocyte-specific monoclonal antibodies (CD3, CD4 and CD45) conjugated with this dye displayed reliable performance in flow cytometry assays.

The results reported showed that V450 **6a** is as fluorescent as, or more fluorescent than, the existing fluorophores with similar spectral characteristics (e.g., Pacific Blue **5**), whereas two of the three antibody clones tested demonstrated that a 20–30% gain in signal could be obtained by using V450 **6a**.

In addition, comparisons of photostability with conjugates made from existing dyes revealed good results for V450 **6a**. V450–antibody conjugates are also appropriate for use in multicolor immunophenotyping panels. Furthermore, this fluorophore proved to be compatible with protocols employing both BD FACS Lysing Solution and BD PharmLyse, and multicolour reagent mixtures containing V450–antibody conjugates were found to be functional and stable.



6

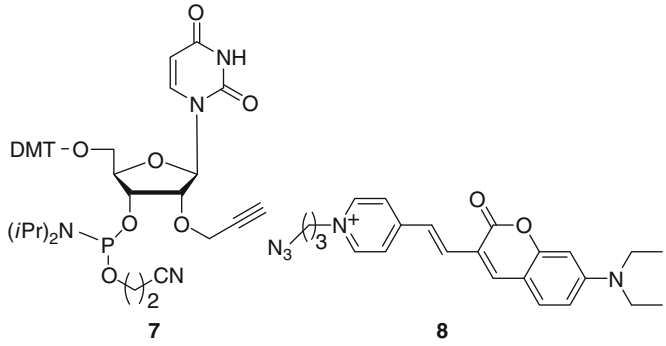
a R = H (V450)
b R = F
c R = Cl

The use of "click" chemistry is a promising strategy as a postsynthetic ligation for nucleic acids in order to circumvent the time-consuming synthesis of phosphoramidites as DNA building blocks [31, 32]. This is particularly relevant for several fluorophores that are unstable under the acidic, oxidative, or basic conditions of automated DNA phosphoramidite chemistry and DNA workup.

Coumarins are an example of brightly emitting organic fluorophores that are unstable under the typically strong basic conditions usually used during DNA cleavage and deprotection. Thus, the incorporation of these labels into oligonucleotides through the conventional phosphoramidite chemistry is difficult or unpraticable. Berndl and co-workers [33] reported the use of postsynthetic "click" chemistry in the modification of presynthesised alkynylated oligonucleotides **7** (bearing the alkyne group at the 2′-position of uridine) with the fluorescent azide **8** to prepare the corresponding modified duplexes **DNA1Y**-**DNA3Y**.

The UV/vis spectra of the modified single strands and duplexes showed an absorption maximum in the range between 515 and 534 nm. Duplexes bearing guanine as the counterbase (**DNA1G**) or as the base adjacent to the coumarin modification site (**DNA3Y**) showed the most red-shifted absorption, particularly significant in **DNA3G** (534 nm). The steady-state fluorescence spectra of the coumarin-modified duplexes displayed maxima in the range 606–637 nm. All modified duplexes exhibited a significant Stokes' shift of approximately 100 nm. The duplexes **DNA1Y** showed quantum yields in the range between 0.30 and 0.35, while Φ_F of the duplexes with adjacent G–C base pairs (**DNA3Y**) were lower (0.20–0.27).

Overall, the significant Stokes' shift of ~100 nm and the good quantum yields make the coumarin dye a powerful fluorescent probe for nucleic acids assays or cell biology. The postsynthetic "click" chemistry makes this fluorophore readily accessible for fluorescent labeling of nucleic acids.



7 **8**

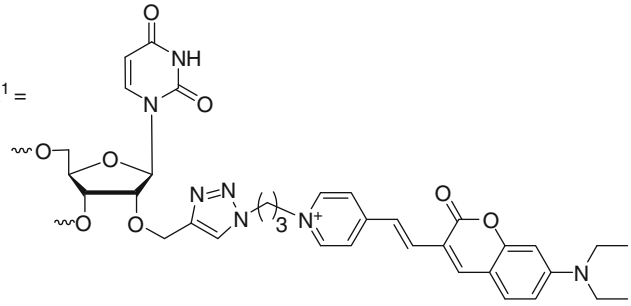
5′ G-C-A-G-T-C-T-T-X- T-T-C-A-C-T-G-A 3′
3′ C-G-T-C-A-G-A-A-Y-A-A-G-T-G-A-C-T 5′

for Y = A X = X^1: **DNA1A**
X = T: **DNA2A**

5′ G-C-A-G-T-C-T-G-X-G-T-C-A-C-T-G-A 3′
3′ C-G-T-C-A-G-A-C-Y-C-A-G-T-G-A-C-T 5′

for Y = A X = X^1: **DNA3A**
X = T: **DNA4A**

X^1 =



Recently, novel polymethine carbonyl-dyes based on coumarin moiety and their boron difluoride complexes **9a–d** and **10a–d** [34–36] were evaluated as fluorescent dyes for the detection of native proteins using bovine serum albumin (BSA) as a model protein, and as probes for the nonspecific detection of proteins using a BSA/sodium dodecyl sulfate (SDS) mixture [37]. Optical properties of these compounds in the absence and presence of BSA, as well as in SDS and BSA/SDS mixture, were measured in Tris–HCl buffer (pH 8.0) (Table 1).