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REVIEW ARTICLE

The Concept of λ-Ratiometry in Fluorescence Sensing and Imaging

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Abstract Very limited number of parameters is available for fluorescence sensing and imaging. The changes of intensity are of low analytical value due to the absence of internal reference. Anisotropy and lifetime sensing have their own limitations. In this respect the λ-ratiometric (based on intensity ratios at two or more wavelengths) recording of spectral changes becomes more popular. Because the spectral changes are connected directly with the variations of interaction energies this approach is seen as the most universal method to study intermolecular interactions. It is applicable for different sensor formats and for obtaining analytical information from cell images. Here we critically analyze different approaches in λ-ratiometric sensing that use single and double fluorescence emitters and are based on different mechanisms producing spectroscopic change. Very promising is the exploration of mechanisms that allow obtaining ratiometric response from a single dye.

Keywords Intensity sensing · Anisotropy · Time-resolved fluorimetry · Wavelength ratiometry · Excited-state reactions

Dedicated to Professor Michael Kasha on the occasion of his 90-th birthday.

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Abbreviations

3HC	3-hydroxychromone
DLR	Dual luminophore referencing
ESIPT	Excited-state intramolecular proton transfer
FRET	Förster resonance energy transfer
ICT	Intramolecular charge transfer
LE	Locally excited
PET	Photoinduced electron transfer
TICT	Twisted intramolecular charge transfer

Introduction

Fluorescence techniques occupy unique position in biophysical research and in various applications in sensing and imaging due to their important distinguishing features [1–3]. One is the ultimate absolute sensitivity that in special experiments reached the limit of single molecules. But even in common analyses the picomolar detection range can be frequently achieved. This is especially needed if the analyte exists in trace amounts, and the time-consuming and costly enrichment steps have to be avoided. A very high spatial resolution allows obtaining images with fine detail. In common microscopy it is limited to a fraction of wavelength of light and with overcoming the light diffraction limit has reached molecular scale. These techniques allow obtaining the fastest response that develops on the scale of fluorescence lifetime and can detect the events on a scale as short as 10^{-8}–10^{-12} s. The non-destructive and non-invasive character of fluorescence techniques may be beneficial primarily for many biological and medical applications. Due to these features, universality is seen as the great advantage of these techniques. Fluorescence response can be obtained in solid, liquid and gas media

and at all kinds of interfaces between these phases. It is because the fluorescence reporter and the detecting instrument are connected via emission of light, the fluorescence measurements can be equally well suited for remote industrial control and for sensing different molecular targets within the intact living cells.

Several parameters of fluorescence emission can be used as outputs in fluorescence sensing and imaging (Fig. 1):

(1). *Fluorescence intensity F* measured at given wavelengths of excitation and emission (usually, band maxima). With proper calibration it can be related to fluorescence quantum yield that is the measure of relative rates of emissive and non-emissive transitions to the ground state and therefore of different quenching-dequenching effects produced by molecuiar surrounding in the excited states. Meantime such calibration is a special task that cannot be easily provided. Therefore F is commonly expressed in arbitrary units that makes sense only for comparative experiments and analyses under the same instrumental and environment conditions.

(2). *Anisotropy r* of fluorescence emission that allows estimating the rates of molecular rotations (rotational diffusion) during the fluorescence lifetime τ_F. It is a function of the fluorescence intensities obtained at two orthogonal polarizations, vertical and horizontal. In ideal conditions, it is the robust characteristics of studied system that does not depend on instrumental factors.

(3). *Fluorescence lifetime* τ_F, which is the reverse function of the rate of emissive depopulation of the excited state. Its measurement allows not only expanding these quenching-dequenching effects into time domain but also determining the rates of different excited-state reactions. The lifetimes can be obtained from decay curves that are measured directly using pulsed or periodically modulated excitation. These decays are characteristic for fluorophore in particular environment and do not depend on instrumental factors.

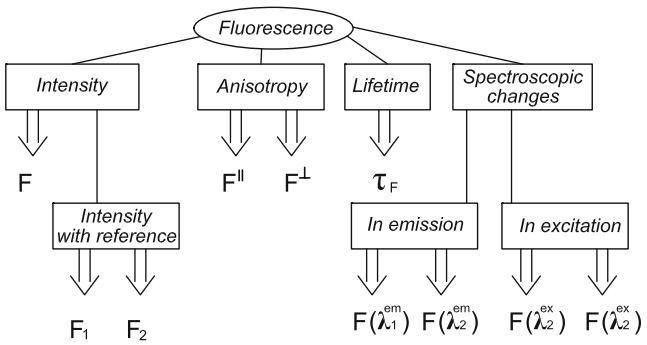

Fig. 1 Parameters used in fluorescence sensing and imaging. *Double arrows* indicate the techniques, in which the self-referencing is provided by simultaneous recording of two parameters

(4). The dependence of intensity F on emission wavelength, $F(\lambda^{em})$, that gives the fluorescence *emission spectrum*. If this intensity is measured over the excitation wavelengths, one can get the fluorescence *excitation spectrum* $F(\lambda^{ex})$. The positions of fluorescence excitation and emission spectral bands are determined by the energies of correspondent electronic transitions, so that the transitions between different ground-state forms are reflected in $F(\lambda^{ex})$. In addition, the shifts in emission spectra, $F(\lambda^{em})$, reflect different intramolecular and intermolecular relaxations that can be produced by weak noncovalent intermolecular interactions. The excited-state reactions can generate very strong changes in $F(\lambda^{em})$.

Thus, the number of parameters that can be derived from the measurements of fluorescence is very limited. It can be increased by applying their combinations (such as time-resolved fluorescence spectral shifts, spectrally-resolved or time-resolved anisotropy). Such combinations can provide additional useful information on the behavior of studied system. Meantime, due to complexity of instrumentation and analysis these techniques are available to only a limited number of laboratories and did not appear in large-scale practice. In view of these complications and limitations there is a necessity to look for simple but informative and reliable methods for research and practical use.

The methods of *λ-ratiometry* (ratiometric recording of intensity at two or more wavelengths) offer such promise mainly due to simplicity of detection and potentially rich content of output information. Conventional microscopes, flow cytometers and plate readers can be applied for two-wavelength detection. Moreover, these methods offer extreme sensitivity and correction for interfering factors even at low level of absolute intensities [4, 5]. The key point is therefore the design of fluorescence reporters that can produce the requested spectral changes. Different possibilities exist for that, and they will be analyzed in this review. The advantages of λ-ratiometry will be discussed and the range of its applications outlined. There is always possibility for improvement, and some directions for future developments will be discussed.

Survey of the methods that do not use λ-ratiometry

There are fluorescence techniques that do not use λ-ratiometry and, even more, for many practical tasks they can be applied with low wavelength resolution. This can be done in emission intensity measurements and the measurements of anisotropy on a condition of efficient elimination of scattered light. Lifetime measurements are even more

advantageous in this respect, since the scattered light can be rejected in time-resolved decays. Low spectral resolution is commonly applied in microscopy, which allows obtaining more intensive response signals forming the image. These techniques are frequently used in different sensing and imaging technologies, and we will outline only some of their characteristic features needed for objective comparison.

Emission intensity

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. Since the fluorescence spectra of organic dyes are rather broad (commonly with half-widths of 50–70 nm), then high spectral resolution in these measurements may not be needed. Fluorescence quenching/enhancement is commonly used as the reporter signal in different sensing and imaging technologies. The 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.

Fluorescence quenching can be provided in different ways, and a variety of photophysical mechanisms can be used for generating the channels for relaxation to the ground state without emission [6]. They involve induction of photoinduced electron transfer (PET) between the electron-donor and electron-acceptor fragments of the same molecule that can be coupled with its conformational flexibility. The quenching by heavy atoms or spin labels can also be efficiently used as well as the Förster resonance energy transfer (FRET) to nonfluorescent acceptor. The dye environment can strongly modulate fluorescence intensity by formation-disruption of intermolecular hydrogen bonds and inducing the changes in dye geometry. The solvent can also influence the dye energy states, particularly producing the inversion of n (non-fluorescent) and π (fluorescent) energy levels. In the course of quenching the remaining emission does not change its spectral properties. Thus, the researcher has a lot of choice for constructing a sensor with response based on the principle of intensity sensing [7].

Meantime, in common intensity measurements the observed changes cannot be quantified on an absolute scale. Their recorded magnitudes can vary in broad ranges depending on the intensity of light source, sensitivity of the detector or the size of illuminated volume. That is why the intensity is commonly expressed in relative units, and even if the output is presented as the number of emitted quanta, it has no absolute value. Only when measured in exactly the same experimental conditions and with permanent number of fluorescence emitters, intensity F can be used in sensor technologies for determining the analyte $[A]$ concentration according to popular formula that is based on the mass action law applied for the simplest case of stoichiometry 1:1:

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

Here F_{max} is the intensity when the sensor molecules are totally occupied and K_d is the dissociation constant. Since the background emission is always present and the quenching may not be total, F_{min} is measured as the fluorescence intensity without analyte binding. It should be stressed that 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 requirement reduces dramatically the range of applications of intensity sensing. The calibration of the output signal, especially in the case of sensor arrays or microscopic images, is difficult and often not possible [8]. The recorded changes of intensity always vary from instrument to instrument, and the proper reference even for compensating these effects is difficult to apply. Moreover, in many practical cases, such as analysis of cellular images and sensor arrays, the distribution in reporter concentration within the image or between different array spots cannot be easily quantified. It can decrease due to chemical degradation and photobleaching. Since intensity is proportional to dye concentration that can be variable, the precise intensity based quantitative measurements are not possible. In all these cases an "external" calibration that allows accounting for the instrumental factors is not sufficient, so an "internal" calibration on molecular and nanoscopic level is needed. It can be provided by additional channel of information beside just an one-wavelength steady-state intensity [9, 10].

Emission anisotropy

Like other detection methods based on fluorescence, the anisotropy sensing should distinguish the two states of the sensor, with and without bound target or the two states of fluorescent ligand, free and adsorbed to a particular site. These states should possess comparable levels of fluorescence intensity but differ strongly in recorded anisotropy. The measurement of steady-state anisotropy r is simple and needs two polarizers, one in the 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. Emission anisotropy r substitutes the previously used parameter, polarization, P, and their relation is $r=2P/(3-P)$. For obtaining r (or P) the two readings of fluorescence intensity are needed, which makes this parameter ratiometric. Since any variation of light source intensity, detector response, illuminated volume or even the dye concentration influence proportionally the F_{VV} and F_{VH} values (their ratio is present in Eq. 2), the anisotropy allows obtaining self-referencing information on sensing event from a single reporter dye.

If two or more forms with different r values are present in the system (for instance, for sensor with free, r_f, and bound, r_b, analyte), then 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:

$$r = F_f r_f + F_b r_b \tag{3}$$

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.

Anisotropy describes the rotational dynamics of dipole of the excited dye molecule. 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:

$$r = \frac{r_0}{1 + \tau_F/\varphi} \tag{4}$$

Here r_0 is the limiting anisotropy obtained in the absence of rotational motion. Excitation by polarized light selects from the randomly oriented dye molecules those oriented in particular direction (usually, vertical). In rigid environments these oriented molecules emit polarized light. If they are able to rotate during lifetime of emission, their emission becomes depolarized. The rotating unit can be the dye molecule itself or a larger unit (macromolecule or its segment) to which the dye is rigidly fixed. The sensing event reflects the change of this mobility.

As follows from Eq. 4, the variation of anisotropy can be observed if φ and τ_F are of comparable magnitude and there is a variation of *rotational mobility* of fluorophore (the change of φ) or the variation of *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 can be achieved with the selection of proper φ and τ_F. It decreases if φ and τ_F change in the same direction.

Fitting τ_F to the range of variations of φ is often a difficult problem. Common for organic dyes, τ_F of several nanoseconds allows detecting the change in mobility of only small molecules. In the case of anisotropy sensing of high molecular weight targets, τ_F should be 10–100 ns or longer. The possibility to increase this time window is offered by long-lifetime luminophors [11], and both homogeneous and heterogeneous polarization immunoassays can be developed based on decreased rotational mobility of target-bound antibodies. An alternative solution is the application of low molecular weight competitors, the displacement of which on binding the target can be detected as the decrease of anisotropy [12, 13]. With these developments, fluorescence anisotropy has become a popular test method in single nucleotide polymorphism genotyping [14], protein kinase assays [15] and drug discovery [16].

The weak point of anisotropy as the detection technique is not only in the limiting choice of reporters with proper r_0 and τ_F values. This is also its great sensitivity to light-scattering effects. This occurs because the scattered light is always 100% polarized, and for providing efficient spectral rejection of scattered light the dyes with strong absorption and emission band separation (Stokes shift) are needed. The advantage of anisotropy sensing is the absence of necessity for the reporter dye to participate directly in sensing event. Because of that the dye can be attached to any site of the sensor, target or competitor that changes its rotational mobility on intermolecular interaction that is necessary for providing the sensor signal.

In comparison of anisotropy sensing with intensity sensing we have to consider that r, in contrast to F, is an intensity-independent parameter. Therefore the response in r is self-referenced. This means that, ideally, it can be reproduced on any instrument and will not depend on illuminated volume or dye concentration (only in high dye concentrations or high-density labeling the concentration depolarization can be observed). In this case in addition to robustness in analysis we get new possibilities for reading microarrays and for cell imaging [17], where the spatial distribution of reporter dye can be heterogeneous but the response is concentration-independent providing the pattern of desired properties in the studied system.

Time-resolved fluorimetry

Time-resolved fluorimetry presents a different example of obtaining the parameters that do not depend on intensity. In an ideal case the decay is exponential and is characteristic for the fluorescent emitter in particular system and does depend neither on instrumental factors nor on dye concentration. The deviation from exponential law can witness for heterogeneity of emitters or for the presence of excited-state reactions generating emissive products.

If collisional quenching occurs during fluorescence lifetime and competes with the emission, then the relative change of intensity, F_0/F, is strictly proportional to the change of fluorescence lifetime, τ_0/τ_F, where F_0 and τ_0 correspond to conditions without the quencher [2]. In practice, this effect can be observed only with long-lifetime luminescence emitters and efficient diffusional quenchers, such as oxygen [18]. In this case the decrease of τ_F occurs gradually with increasing oxygen concentration. The most commonly observed thermal quenching allows the emission to remain exponential.

The other possibility for using the lifetime detection in sensing is to observe discrete forms of emitters belonging to the states of the sensor that differ in lifetime values. If two such forms, free (with index ^{F}) and bound (with index ^{B}), are present in emission, then the total decay of intensity in time t can be described by additive contributions of these two decays that are characterized by initial amplitude α and lifetime τ_F:

$$F(t) = \alpha_F \exp\left(-t/{\tau_F}^F\right) + \alpha_B \exp\left(-t/{\tau_F}^B\right) \quad (5)$$

In sensing and imaging technologies these two forms commonly belong to the same dye and they can be excited at the same wavelength. The ratio of pre-exponential factors α_F and α_B will determine the target concentration with the account of molar absorbances, quantum yields and lifetimes of contributing forms [19]. It has to be noted that if one of the forms is statically quenched, then the switching between two forms will not influence the decay and sensing will not be possible.

The limited number of available fluorescent dyes that are able to change the lifetimes in controlled manner in different environments discourages researchers from active use of response in lifetimes in sensing technologies. Meantime, the utility of several selected dyes for this application in microwell formats used in high-throughput screening was demonstrated [20] and recent developments in squaraine dyes show their usefulness for excitation with cheap red diode lasers that allows attractive extension to immunoassays [21].

When fluorescent ligand binds to two types of sites with different lifetimes, this binding can be quantitatively characterized by the ratio of fractional contributions in saturation of these sites. This was shown in binding of a cyanine dye to serum albumin, in which the differential binding to these sites indicated conformational changes [22].

The lifetime detection techniques are self-referenced in a sense that fluorescence decay does not depend upon concentration of the emitter and is the sole characteristics of this emission in particular environment. The results of decay measurements 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 commonly produces no problems, since the scattered light decays on a very fast time scale and does not interfere with fluorescence decay observed at longer times. In many practical cases this technique does not require high spectral resolution because within homogeneous distribution of emitters in the absence of excited-state reactions the lifetime should not vary over the spectrum. These features can explain why lifetime detection has become popular in live cell imaging [17]. The time-resolved anisotropy measurements are especially attractive for cellular imaging since they allow obtaining extended information on local viscosity and intermolecular interactions [23]. The limited choice of proper emitters and complexity of instrumentation are limiting in extending this research.

Important positive feature of any detection method is the insensitivity to interferences of different origin or the ability for their compensation. In this respect, the time-resolved fluorescence shows its disadvantages. It is sensitive to variations of temperature, pressure and composition of dye environment producing dynamic quenching.

Wavelength-ratiometry with two partners

The λ-ratiometry suggests an alternative to above mentioned methods. Ratiometic detection of intensities at fixed wavelengths is a simple technique that can use common spectroscopic and microscopic instrumentation. Selection of these wavelengths can be provided by monochromators or filters. Often these wavelengths are well separated, so high spectroscopic resolution may not always be needed. Detection at two or more emission wavelengths allows establishing additional channels of information and thus avoiding many problems existing in simple intensity sensing. The λ-ratiometry is reasonable when not only the intensity but the strong spectral shifts are recorded or the new bands appear with the variation of their relative intensities. This provides not only robustness and scaling of output information [9] but also expands the possibilities in addressing new tasks, such as the evaluation of viscosity, detection of intermolecular interactions and proximity between macromolecules, etc. The simplest way to produce the λ-ratiometric signal is to use second emitter interacting or not interacting with the primary one.

Whereas for responding dye F can change between zero and some limiting high values, the intensity of the reference dye F_{ref} can remain constant. Thus, by simple division of the numerator and denominator of Eq. 1 by $F_{ref}(\lambda_2)$, the intensity of the reference measured in the same conditions but at different wavelength (λ_2) from that of reporter, the analyte concentration [A] can be obtained from the following equation that contains only the intensity ratios

$R=F\ (\lambda_1)/\ F_{ref}(\lambda_2)$, $R_{min}=F_{min}(\lambda_1)/\ F_{ref}(\lambda_2)$, and $R_{max}=F_{max}(\lambda_1)/\ F_{ref}(\lambda_2)$ [24]:

$$[A] = K_d\left(\frac{R - \mathrm{R_{min}}}{\mathrm{R_{max}} - R}\right) \tag{6}$$

Figure 2 illustrates the advantages of applying the second emitter in the case of inert emitting reference. Meantime, a different case can be realized, in which the second emission band can appear as a result of intramolecular or intermolecular reaction occurring with the reporter dye. In this case two emissions can interconvert, one band decrease, and the other—increase as a result of this reaction. 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 (Fig. 3).

If such point is chosen as the reference, then Eq. 6 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 ratiometric signal 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 [24]:

$$[A] = K_d\left(\frac{R - \mathrm{R_{min}}}{\mathrm{R_{max}} - R}\right)\left(\frac{F_F(\lambda_2)}{F_B(\lambda_2)}\right) \tag{7}$$

Intensity measurement with molecular or nano-scale reference

When only an internal reference is needed in intensity measurements, then the simple solution can be the introduction of a reference dye into a sensor molecule or nanoparticle. This dye should be excited at the same wavelength as the reporting dye. For optimal recording of reference signal, it should possess strongly different emission spectrum but of comparable intensity to that of reporter band. In contrast to that of reporting dye, the reference emission should be insensitive to the presence of analyzed target or to other perturbation producing the

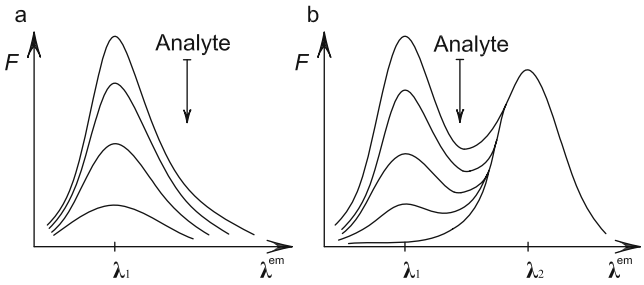

Fig. 2 The effects of quenching produced by sensor-analyte interaction on fluorescence spectra in the case of intensity sensing (**a**) and intensity sensing with the reference (**b**). The reference dye allows providing the ratio of two intensities detected at wavelengths λ_1 and λ_2

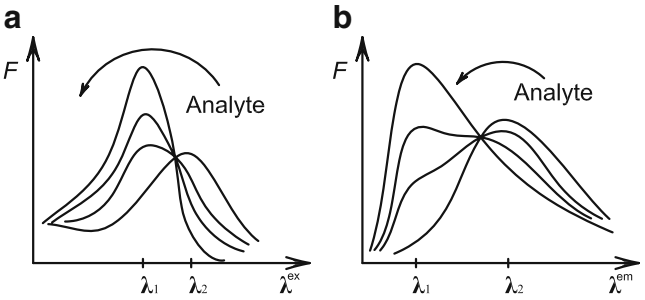

Fig. 3 Transition between two emissive forms possessing different excitation (**a**) and emission (**b**) spectra. Isobestic in (**a**) and isoemissive in (**b**) are the points, at which the intensity does not change on transition between the two forms of emiter

analyzed signal. For optimal operation of such system, 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 (see Fig. 2b). Their intensity ratio can be calibrated in concentration of the bound target.

When the spectral resolution between reporter and reference intensities is not sufficient, the possibility exists to distinguish their signals based on the difference in anisotropy [11] or lifetime [11, 25]. 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 (see above). Intensity calibration in the lifetime domain has an advantage in the studies in highly light scattering media and when the reference has easily recorded long lifetime that can be perturbed by short lifetime of the reporter.

The latter principle is behind the dual luminophore referencing (DLR) in phase-modulation detection technique [25]. 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 with short lifetime can be excited simultaneously with the reference, but its quenching/dequenching in a sensor response generates the change in phase shift of modulated emission that reflects directly the intensity change of the reporter and consequently the concentration of the target.

The approach to quantitative target assay based on introduction of emissive reference into hybrid sensor molecules or nanocomposites has found many applications. As an example, it was used in the construction of double-labeled molecular biosensors based on glutamine and

glucose binding proteins [26, 27]. In other research, for the design of ratiometric Cl^- sensor a molecular hybrid was synthesized that contained two covalently linked dyes [28]. They can be excited simultaneously but exhibit separate emission spectra. One of them is strongly quenched by Cl^- ion, while the other does not interact with Cl^- and serves a reference for the sensor concentration. The sensor designs with the introduction of additional dyes as the references are also used in cation and pH sensing [29] and also in probing microviscosity [30].

The nanoparticles that incorporate two dyes, one responsive and the other insensitive to target binding, are used in cellular research. This approach has been suggested for the measurement of pH [31], oxygen [27] and ion concentrations [32] in different test media and also within the living cells.

There were also attempts to apply two dyes as a mixture in solution or in cellular milieu, as a 'cocktail', in which the optimal intensity ratios can be selected by cocktail composition. Both the combination of responsive and reference dye [33] and of two responsive dyes changing their intensities in opposite directions [34] were suggested to apply for this purpose. In both cited papers the sensors were developed for the detection of Zn^{++} ions. The intensity-responding probes are also very popular in intracellular measurements of Ca^{++} ions [35], and for obtaining the more accurate representation of ion concentrations, the simultaneous injection into the cell of Ca^{++}-responsive and Ca^{++}-insensitive dyes is used [36]. Of course, this is not the best solution, since the intracellular distribution of the two dyes can be different.

This problem can be avoided if 'cocktails' are made of the complexes containing the same antenna chromophore coordinating different luminescent lanthanide ions, Tb^{3+} and Eu^{3+}. In this case the intermolecular interactions that determine the probe solubility and localization in heterogeneous media are determined by the antenna chromophore. Generation of ratiometric response will come from the antenna (by their differential quenching) resulting in the difference of relative intensities of the sharp spectra of Tb^{3+} and Eu^{3+} luminescence emissions [37].

Summarizing, we outline the strong and weak points of intensity sensing with molecular or nanoscale reference. The arrangement, in which two dyes, responsive and non-responsive to target binding, can be excited and their fluorescence emission detected simultaneously, allows compensating for the variability and instability of instrumental factors. Since variation in these factors changes proportionally the signal in two channels, the intensity ratio should be reproducible on the instruments with a different optical arrangement, light source intensity, slit widths, etc. In both channels the intensities are proportional to concentrations of emitters or to their densities on the surface, but their ratio will remain constant. The two-band ratiometric signal can be easily calibrated in target concentration or in the parameter to be measured. Meantime, the two dyes behave quite independently in response to any perturbations causing their spectroscopic changes or quenching. Chemical degradation and photobleaching may be different for two emitters and this can produce strong, unaccountable and irreversible changes in intensity ratios.

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 easy to observe because it is very different from that of monomer. It is usually broad, strongly shifted to longer wavelengths and it does not contain vibrational structure. If the excimer is not formed, we observe in fluorescence spectra the emission of the monomer and upon its formation there appears characteristic emission of the excimer. Therefore the sensor has to be designed in such a way that during the lifetime of the excited fluorophore the excited dimer has to be formed providing the reporting signal. The λ-ratiometric signal obtained on switching from monomer to excimer is very convenient for observation. For application in sensor technologies, the double labeling is needed, which is facilitated by the fact that the two partners are of the same structure.

Selection of excimer formers is limited to aromatic hydrocarbons. Anthracene excimers demonstrate broad structureless emission bands with the maxima at 495 nm, whereas monomers exhibit well-resolved structure with the main band at 414 nm. The composites with two bound anthracenes that are able to provide switching between monomer and excimer emissions were reported for sensing ions [38] and pH [39]. Pyrene derivatives are frequently used as excimer formers also because their stable excimers possess rather long fluorescence lifetimes (~40 ns), though they are much shorter than that of monomers (~300 ns). The long lifetimes allow easy rejection of background emission and application of lifetime sensing [40]. In some simple constructions, two types of excimers, long-lived and short-lived, can be distinguished [41]. The coupling of monomer-excimer transition with the target recognition and binding is in the background of many sensor technologies.

By definition, the excimers are the complexes formed in the excited states, so that the only initially excited form is that of monomer. Practically this means that both the monomer and the excimer emissions are observed with the excitation at the same wavelength. The formation of excimers requires close location and proper orientation between the partners, specifically, the formation of cofacial sandwich between two heterocycles rich in π-electrons.

Therefore, target recognition should be coupled either with molecular assembly of sensor units, each of them containing the monomer, or with the conformational change in the double-labeled flexible sensor molecule that brings together or force apart the monomers. Weak ground-state complexes can be formed between proximate monomers that become excimers upon excitation. In the case of 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 [42], (see Fig. 3b).

This ability to form excimers coupled with the conformational change in pyrene double-labeled flexible oligo- or polynucleotides is actively explored in DNA hybridization techniques and recognition of specific mRNA sequences. In molecular beacons, pyrene fluorophores can be connected both at 3′ and 5′ ends of a single-stranded sensor oligonucleotide. In ligand-free form these ends form the segment of double helix so that the pyrenes display excimer emission. When such beacons hybridize with the targets, these internal bonds are disrupted and the duplex structure is formed with target DNA, which separates the monomers and brings up their emission. Such beacons can detect selectively the target DNAs and can discriminate the targets from their single-nucleotide mismatches [43, 44]. It was reported also that the labeling with two pyrene residues at the 5′-phosphate end of oligonucleotide displays excimer fluorescence intensity that is highly sensitive to duplex formation with DNA and RNA targets. This allows quantification of these molecules in solution [45]. Pyrene dimers with flexible linkers were suggested for double-stranded DNA detection [46]. For the detection of specific mRNA a construction was developed, in which pyrene excimers are formed on target binding [47]. A photostable pyrene derivative 1-phenylethynylpyrene that exhibits red-shifted fluorescence with excimer emission at 500–510 nm was recently suggested for nucleic acid labeling [48]. And even without covalent labeling, the positively charged pyrene derivatives can assemble on DNA structure indicating its presence [49].

The lipid-induced helical rearrangement of apolipophorin III can be followed based on excimer emission [50]. Pyrene groups were attached to two engineered Cys residues resulting in observation of strong excimer fluorescence. Excimer band disappeared upon lipid binding. This result demonstrates good prospects for the design of protein sensors with monomer-excimer λ-ratiometric response.

Nucleic acid aptamers have found important application for detecting specific protein targets. The necessary response units are formed by pyrene labels appended to their chain ends. In the open structure of aptamer they are separated from each other. After binding the target (in this case the platelet-derived growth factor) the aptamer adopts a closed conformation, bringing two pyrene molecules close to each other that results in the appearance of excimer band [51].

Prospective is the application of excimer reporting in sensors designed on the basis of cyclodextrin and calixarene sensor scaffolds. A γ-cyclodextrin dimer modified with two pyrene moieties was synthesized for sensing bile acids and endocrine disruptors [52]. Calix[4]arene platform allows more flexibility by allowing the conformational changes on analyte binding [53]. Based on it, the chloride selective sensor was developed that efficiently uses the reporting based on excimer formation [54]. Cation selection was demonstrated on their binding to calix[4]arene modified with pyrene groups at their lower rim [55]. Flexible oligomeric units with attachment of pyrene groups at two terminals can also be used for ion sensing. Thus, synthetic sensor molecules were suggested for selective detection of Hg^{++} ions in water [56]. In designed sensor for magnesium ions based on tetra-azacrown derivative the conformational change produces the switching of emission towards the monomeric form on ion binding [57].

Attempts to use more complex self-assembled structures were also successful. The sensor tailored for the recognition and signaling of hydrophobic guest molecules was made of three functional moieties, DNA, per-O-methylated β-cyclodextrin and pyrene. Addition of a porphyrin derivative as a hydrophobic guest molecule induced the self-assembly of dimeric structure. It is promoted by the host-guest binding DNA hybridization resulting in the switching of pyrene fluorescence from monomer to excimer [58].

In the design of sensors for cations the idea was to change on target binding the ground-state configuration of recognition units in such a way that on excitation they can become excimers. Thus for obtaining the sensor for sodium ions a pair of dioxyanthracene molecules was included into a polyether ring. On their binding the configuration of molecule changes and the excimer emission starts to be observed [59]. Similar ideas were realized in construction of sensors for other cations [60, 61]. It was reported about synthesis of sensor for cations, which distinguishes the binding of Cu^{++} ions by a blue shifted excimer emission [60].

Cation binding can trigger dimerization of designed sensor molecules with attached excimer-forming groups [62]. Based on this principle, an efficient ratiometric fluorescent sensor for silver ions was suggested [42]. The complex formed via silver ion-induced self-assembly demonstrates a dramatic increase in fluorescence intensity of the excimer and a simultaneous decrease of monomer fluorescence. The intensity ratio of excimer and monomer emissions (at 462 and 378 nm) was shown to be an ideal measure of Ag^{+} ion concentrations [53].

Development of sensors for neutral molecules can be based on the mechanism of condensation of labeled

synthetic polymer under their influence. In this respect, interesting is an attempt for developing a λ-ratiometric glucose probe. Glucose binding to boronic acid functional groups attached to a synthetic polymer in an aqueous solution effectively turns the polymer into a polyanion, which induces the aggregation of the positively charged trimethylpentylammonium pyrene derivative, leading to a strong excimer emission [63].

Application of pyrene-based fluorescence sensors and probes to the studies of live cells and cellular extracts is complicated by the presence of cellular pigments emitting in the same wavelength region. Such background fluorescence decays with short lifetimes (<5–7 ns), so it can be rejected in pyrene time-resolved measurements [47, 64].

In all these applications we observe that the strong direct interaction between aromatic heterocycles change dramatically their spectra and allow λ-ratiometric recording of interacting pairs. The strong difference in lifetimes between monomers and excimers allows analysis of these interactions in nanosecond time domain. There are many possibilities for using these properties in sensing technologies by coupling the formation-dissociation of these pairs with conformational changes in molecular sensors or with assembly of their subunits. Meantime, because of different lifetimes, the non-specific influence of dynamic quenchers may be different for these two forms. Particularly, the undesired oxygen sensitivity of the long-lifetime emission intensity of pyrene monomer cannot be easily excluded or well-controlled.

Less common is the application of exciplexes, the excited-state complexes that some fluorophores can form with different molecules, particularly, with amines. Emission of such complexes is also broad, red-shifted and structureless. Switching between normal and exciplex emissions offers new possibility for generating the ratiometric signal in sensing. Since the exciplex formation requires very stringent requirements towards location and orientation of partners, such response provides the means for specific DNA detection in situ [65].

Resonance energy transfer to fluorescent acceptor

The techniques based on Förster resonance energy transfer (FRET) are very popular in sensor design [66–68]. Two or more dye molecules or light absorbing particles with similar excited-state energies can exchange these energies due to long-range dipole-dipole resonance interaction. One molecule, the *donor*, absorbs light and the other, the *acceptor*, accepts this energy and then relaxes to the ground state with or without emission.

FRET to nonfluorescent acceptor will not be discussed here. It provides a single-channel response in intensity with all disadvantages that were described above. The only its merit is the absence of directly excited emission of the acceptor in the cases when the excitation spectra of donor and acceptor overlap and the donor excitation is not selective. Thus, the acceptor operates as a common 'static' quencher with a special feature that this quenching effect can be seen at a distance. FRET to fluorescent acceptor is obviously more popular because of its two-channel self-calibrating nature.

For the observation of FRET the direct contact between donor and acceptor is not needed, they can interact through space and can be located at separation distances within 1–10 nm from each other. FRET can take place if the emission spectrum of the donor at least partially overlaps with the absorption spectrum of the acceptor. 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 $E=1-\langle\tau_{DA}\rangle/\langle\tau_D\rangle$, where $\langle\tau_{DA}\rangle$ and $\langle\tau_D\rangle$ are the average lifetimes in the presence and absence of the acceptor [69]. This is because the energy transfer is a stochastic process that develops during fluorescence lifetime of the donor. Therefore the variations of fluorescence lifetime of the donor can be the tool for characterizing the FRET efficiency [68]. In steady-state experiments the E determinations are difficult when the donor-acceptor stoichiometry is not fixed, and for this case the analysis based on acquisition of both excitation and emission spectra [70] can be used.

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

$$E = R_0^6/\left(R_0^6 + R^6\right), \tag{8}$$

Here R_0 is the parameter that corresponds to a distance with 50% transfer efficiency (the Förster radius). It is characteristic for a given donor-acceptor pair in a particular medium and depends strongly on the quantum yield of the donor in the absence of acceptor Φ^D. The overlap of fluorescence spectrum of the donor with absorption spectrum of the acceptor is expressed as normalized spectral overlap integral J:

$$R_0^6 = 9.78 \cdot 10^3\left(\kappa^2 n^{-4}\Phi^D J\right) \tag{9}$$

In Eq. 9 n is the refractive index of the medium between donor and acceptor and κ^2 is the orientation factor that depends on relative orientation of the donor and the acceptor dipoles; it can assume the values from 0 to 4. If the lifetime is long and rotations of segments containing the reporter dyes are fast, then the approximation $\kappa^2=2/3$ is used.

Donor and acceptor can be the same molecules and then in the case of small Stokes shift the energy transfer efficiency will be high. This case is called '*homo-FRET*'. It can be detected in rigid and highly viscous environments by the loss of fluorescence anisotropy, since orientations of donor and acceptor dipoles are generally different. High anisotropy can be retained only on excitation at the red edge of absorption band, where the homo-FRET is suppressed (the so-called Red-Edge effect [71]). If the donor and acceptor dyes are different (the case of '*hetero-FRET*'), then the energy will flow from the dyes with short-wavelength absorption and emission to the dyes, absorption and emission of which occurs at longer wavelengths. This allows observing a decrease or even disappearance of fluorescence band of the donor together with an appearance and increase of the band of acceptor.

The fact that the fluorescence response depends strongly on the properties of both donor and acceptor and on their distance and orientation allows many possibilities for its modulation in generating the sensor response. In all these developments the switching between two fluorescent states (of donor and acceptor) can be realized with the generation of λ-ratiometric signal. In addition, the detection of FRET can be extended to time domain with the benefit of using the long-lifetime donors that allows to apply simple instrumentation [72]. We will consider several important cases below.

The sensing based on hetero-FRET

The hetero-FRET is the transfer between structurally and spectroscopically different dye molecules. This type of using FRET for reporting is the most popular. The switching between donor and acceptor emissions occurs due 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 [73], enzymatic splitting of covalent bond between two labeled units [74, 75] and competitive substitution of labeled competitor in a complex with labeled sensor [76] (Fig. 4).

Very typical is attenuation of relative ratio of the two emission band intensities in the covalently coupled dye dyads (FRET cassettes) [77]. The synthesis of such donor-acceptor dimers with variable distances between them [78] allows obtaining λ-ratiometric chemosensors in different configurations, including those, in which the target binding at the donor site [79] modulates the response.

Extended range of FRET-based technologies are explored in DNA assays [80]. An already classical example in these applications is the 'molecular beacon' that can be used to determine the concentration of single-stranded DNA in solution by a ratiometric fluorescence measurement [81]. Its folded stem-loop conformation in non-hybridized state allows FRET to occur between two dyes. Upon hybridization, the probe opens up resulting in a disappearance of FRET with the fluorescence intensity increase of the donor and a decrease of the acceptor. The same principle is behind the proposed hybrid structures that contain DNA segments connected by a flexible polymer linker [82]. They are designed in such a way that upon the hybridization the labeled DNA segments appear in close proximity resulting in the generation of FRET.

Cascade transfer and FRET-gating

FRET can occur in cascade manner in a sequence of dyes, in which an energy acceptor can serve as the donor to another dye with lower excitation and emission energy. The energy transfer, in which several different dyes provide the chain of transfer events for achieving a very significant shift in emission wavelength, is called a '*cascade transfer*' [83]. Cascade FRET between three or more dyes can be also used as a technical tool to shift the acceptor excitation spectrum and thus to avoid its direct excitation and also for providing the strong separation of the donor and acceptor emission bands making λ-ratiometry very efficient.

A cascade triple transfer [84] that got the name "*FRET-gating*" [85] has already found application in sensor technologies [86]. When the donor and the acceptor are in proximity but their spectral overlap is insufficiently small for the transfer, then an introduction of the third partner that can serve an acceptor to primary donor and a donor to terminal acceptor results in the appearance of efficient transfer. Including/removing of this third component can generate a sensing signal producing a strong separation of donor and acceptor emissions. Based on this approach, the medical diagnostics using the sequence detection of DNA of the infected persons were developed. A peptide nucleic acid (PNA) probe labeled with a fluorescent dye was hybridized with DNA having a sequence complementary to that of PNA, this negatively charged complex is electro-

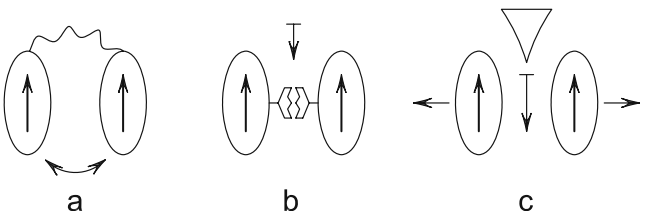

Fig. 4 Generation of sensor response by modulation of donor-acceptor distances: by conformational change in the sensor (**a**), by splitting the bonds between the structures containing donor and acceptor moieties (**b**) and by introduction of competitor substituting their binding sites (**c**)

statically attracted toward the cationic conjugated polymer that can serve as FRET donor. There may be no FRET until the third component appears within the critical transfer distance and FRET proceeds as a two-step process.

Multiple transfers in sensing

Most of the constructions designed for fluorescence sensing are based on strict stoichiometry between FRET donor and acceptor groups that are provided by either covalent linkage between them, often on a protein or oligonucleotide matrix, or by formation of intermolecular complexes. The rapid development of techniques for obtaining the dye-doped nanoparticles made of silica [87], organic polymers [88] and dendrimers [89] offer new possibilities for improvement of sensor properties. If many dye molecules are located within critical transfer distance, new collective effects appear. One is the multiple homo-FRET, which with the introduction of fluorescence quencher results in *super-quenching*, the quenching of the whole ensemble of emitters [90]. Essential is the fact that the bright emission can be provided by the whole ensemble of dye molecules and that a single additional molecule can induce the whole effect of quenching [91].

Of great importance can be the *light-harvesting* 'antenna' effect that can result in a strong amplification of light absorption ability of the donors increasing the sensitivity of FRET assays. Here, many donor molecules donate their excitation energy to a single acceptor increasing dramatically its fluorescence [92]. In this way, not only the intensity can be dramatically increased, but also the lifetime prolonged. With the efficient FRET it reaches the lifetime of the donor [93]. This allows many possibilities in the sensor design [94]. Regarding λ-ratiometric sensing, the efficiency of two strategies can be dramatically increased. One is based on switching the emission between donors and acceptors, and the other—on focusing the excitation energy on the acceptor and exploring its reporting properties on a greatly enhanced level.

FRET modulated by light

Selective photobleaching that specifically destroys the acceptor giving rise to fluorescence of the donor can be used as the measure of FRET efficiency. 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 [95]. The problems complicating this approach, such as insufficient illumination intensities in conventional wide-field instruments, autofluorescence and donor photobleaching, can be resolved with continuous monitoring of donor and acceptor intensities during the bleaching process [96]. Due to the fact that the measurements before and after acceptor bleaching are not simultaneous and cannot be repeated with the same image, this approach has got a limited application.

It is not only the photobleaching but any change in absorption spectrum of the acceptor can produce the modulation of FRET efficiency since it induces the changes of the overlap integral J (Fig. 5). In some photochromic compounds such as spiropyrans these changes can be reversible and controllable [97]. The photochromes 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, even if they are non-fluorescent they offer a possibility of reversible switching of FRET effect between "on" and "off" states without any chemical intervention, just by light [98, 99].

The spiropyran molecules exist in closed, nonpolar and colorless spiro forms absorbing at wavelengths shorter than 400 nm. They undergo a light-driven rearrangement to an open merocyanine form with the appearance of purple color and absorbance at 500–700 nm [100]. Their previously reported applications in FRET involved controlled light-induced quenching of FRET donors such as organic dyes or fluorescent nanocrystals (Quantum Dots) [101]. This approach demonstrated its advantages when applied in live cell imaging, where the FRET switching on and off allows obtaining the necessary controls on the distribution of donor molecules and on their fluorescence parameters that provide response on cellular events [95]. The prospect to generate and monitor λ-ratiometric detection also exists, since in some media (ionic liquids) spiropyrans in their merocyanine form exhibit a strongly increased fluorescence [102].

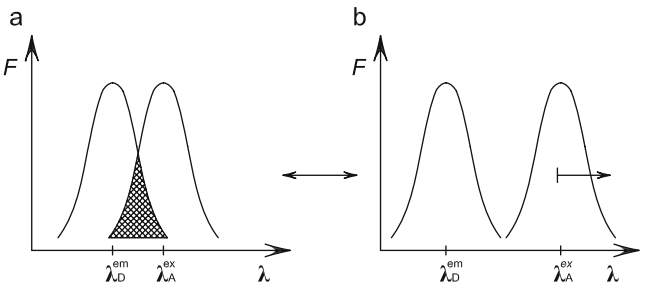

Fig. 5 Interplay between two reporter signals, with FRET (**a**) and without FRET (**b**) by changing the spectral overlap integral (shown as cross-hatched area). This change can be produced by the shift in absorption spectrum of the acceptor or by changing its absorbance. The ratiometric signal can be generated by switching the emission from that of the acceptor, in case (**a**), to that of the donor, in case (**b**)

Modulation of FRET by ground-state or excited-state reaction of the acceptor

According to FRET mechanism, both ground-state and excited-state reactions in the acceptor influence its emission, but in different ways. The ground-state transformations changing the absorption spectra change the transfer efficiency directly by modulation of overlap integral (see Fig. 5). In contrast, the excited-state reactions of the acceptor leading to new emissive states do not depend on the conditions of excitation (directly or via FRET), but their observed spectroscopic effect may depend on these conditions, particularly on the fluorescence lifetime of the donor that modulates the acceptor lifetime [72].

The application of FRET allows designing highly efficient λ-ratiometric pH sensors. There are many fluorescent pH indicators that display the pH-dependent change in absorption spectra due to transition between protonated and deprotonated forms [103, 104]. The large spectral separation and high emission intensities of these forms in the visible wavelength range allow modulating on switching between them the overlap integral [105]. The pairs of Quantum Dot (donor) and organic dye (acceptor) are optimal in this application [106]. There are many possibilities to couple this transition with different sensing events and therefore there are many ways for achieving ratiometric sensing. Different sensing bi-chromophoric constructions can be devised based on this principle [107]. Some of them use cassettes with intramolecular energy transfer for pH detection in living cells [108].

The absorption spectrum of the acceptor can be modulated in different ways, particularly, by the action of hydrolytic enzyme [105]. A variety of bi-chromophoric cassettes can be constructed, in which the target binding changes the overlap integral [109].

If the acceptor exhibits an excited-state reaction that changes its fluorescence, then by the variation of emission lifetime of the donor one can change the time window for this reaction adding new possibilities for generation and modulation of the sensor response. Since the FRET reaction proceeds during the lifetime of the donor, the proper donor choice (e.g. as lanthanide complexes) allows to dramatically increase the acceptor lifetime compared to lifetime observed on direct excitation. In addition, semiconductor Quantum Dots or conjugated polymers with very high molar absorbance are used as the donors, then the brightness of acceptor emission may increase enormously with the retention of its responsive ability. Photodegradation of such dye occurs also at much faster rate, in accordance with the frequency of the acts of excitation [110].

Such improvement of the reporter properties by increasing its brightness and lifetime was demonstrated by many researchers. If the receptor is the polarity-sensitive dye responding to dielectric relaxations in its environment (such as Nile Red), then by modulation of donor lifetime one can change the time window for observing these relaxations [111]. The other example is the use as FRET acceptors the molecular rotors that change their quantum yield as a function of viscosity [30]. If the fluorescence response is observed from both donor and acceptor and the emission of acceptor is quenched as a result of photochemical reaction, then the λ-ratiometric signal can be seen in emission. Fluorescence enhancement on target binding together with the spectral shifts of donor and acceptor emissions can provide strong signal enhancement [112].

The optimal choice of FRET donors and acceptors

Traditionally, organic dyes are used as both FRET donors and acceptors [69]. They are still attractive as the acceptors because of their small size and of the well developed chemistry of their attachment to any molecular unit. Then the change of its location with response to the donor unit generates the response in FRET [113]. In addition, the variation in FRET signal due to modulation of fluorescence intensity (quenching) or of spectroscopic changes (excited-state reactions) of the acceptor can be most easily provided with them. The popular DNA hybridization assays [81] and the sensitive indicators of membrane potential [114] are based on these dyes. These attractive features are shared by fluorescent proteins [115–117] that can be synthesized in living cells and contain organic fluorophore protected inside protein matrix allowing efficient λ-ratiometry [118, 119].

The requirements towards FRET donors are different. They should provide optimal lifetime and brightness, and here the organic dyes are not ideal. Most of them are easily photobleached, their molar absorbance is not very high and their fluorescence lifetimes are typically no longer than several nanoseconds. Therefore we observe an increasing popularity of lanthanide chelates [120], conjugated polymers [121] and Quantum Dots [122, 123] that, being used as FRET donors, offer advanced properties. With their choice the FRET efficiency can be optimized in different directions.

(a). Increase of molar absorbance and fluorescence quantum yield. Conjugated polymers have the advantage of efficient light harvesting together with collective optical response. Their use in combination with fluorescent dye provides very sensitive tools for DNA detection [124, 125].

(b). Increase of fluorescence lifetime [72]. If the acceptor is excited not directly but by the energy transferred from the donor, its lifetime increases to that of the donor [93]. Practically this means not only the ability to observe processes occurring on extended time scale

but also to discriminate the direct excitation of short-living emission of the acceptor in time domain [126]. Luminescent lanthanide complexes (of europium and terbium) with chelating ligands, are particularly interesting for these FRET applications owing to their lifetimes extending to milliseconds [127]. Among other advantages, this allows easy rejection of short-living background fluorescence.

(c). Adjustment to any desired excitation wavelength. Here Quantum Dots are unique, since they allow broad-scale variation of emission wavelengths based on the same materials due to variation of size of their nanoparticles [123]. The other very attractive property is an extremely broad range of wavelengths that can be chosen for their excitation.

(d). Applicability for two-photon excitation. The two-photon excitation allows obtaining sharp fluorescence images and rejecting the background in light-scattering media. Organic dyes exhibit strong variation of two-photonic absorbance [128], and only some of them can compete with conjugated polymers [129] or Quantum Dots [130]. Lanthanide complexes are also prospective for two-photon excited donors [131]. Particularly, it was shown that when the conjugated polymer is used as the donor, the response of intercalator dye (acceptor) bound to DNA increases manifold [132]. The techniques based on two-photonic excitation of fluorescent proteins were developed for cellular imaging [133].

Combination of organic dyes with other luminescent materials allows providing many improvements in sensing technologies especially in view that organic dyes are much more ‘responsive’ but are behind these emitters in photostability, lifetime and brightness. Particularly interesting in applications are the films made of conjugated polymers with covalent attachment of organic dyes [134].

Critical evaluation of FRET technique

The most attractive feature of FRET that is frequently used in different sensing and imaging technologies is the long-range but steep distance dependence of the effect. In addition, as it was shown above, collective effects in energy transfer as well as the variations of spectroscopic properties of donor and acceptor can be efficiently used. The weak point is, of course, the necessity of double labeling that sometimes produces more than double difficulty. Only in rare lucky cases intrinsic fluorescent group of sensor or target molecules can be used as one of the partners in FRET sensing.

Collective effects in FRET, such as homo-transfer and antenna effect, are powerful tools in the design of advanced sensors. But their presence raises the necessity of stringent control on the donor/acceptor stoichiometry, especially when the donor and the acceptor are on different interacting partners. And even in the absence of collective effects, the absence of control on stoichiometry may lead to errors, so that if one of the partners is in excess and does not participate in FRET, its signal could interfere with the informative response. This problem can be avoided in the design of intramolecular FRET sensors and by excluding their aggregation.

The possibility of interference of different factors influencing the light absorption and emission of both donors and acceptors should always be considered. These two emitters respond independently and, in a general case, differently to such factors as photobleaching and quenching of fluorescence. Since the two partners are coupled in FRET event, there may appear a strong variation of fluorescence spectra unrelated to informative response. For instance, the 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. Because of that, just the variation of temperature may change the ratiometric signal.

The overlap of absorption spectra of donor and acceptor may cause significant problems due to direct excitation of the acceptor. Control experiments of samples containing only the donor or the acceptor are not always possible. In addition, variation of noncovalent intermolecular interactions may shift the absorption and fluorescence spectra, influencing the overlap integral. To record these changes, a good spectral resolution between donor and acceptor emissions is needed.

Concluding the section on λ-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. They must have different excitation and/or emission spectra and, therefore, possess the difference in other properties that determine the effects of non-specific quenching (by temperature, ions, etc.). In addition, the two partner emitters can exhibit different degradation and photobleaching as a function of time. This may cause a nonspecific and non-accountable effect on ratiometric reporter signal and can make the sensor non-reproducible in terms of obtaining precise quantitative data even in serial measurements with the same instrument [135].

Mechanisms and applications of λ-ratiometry with single emitter

Starting this section, some introductory comments should be made. In many respects, single emitter is better than two

or more emitters in the sensor element. This is not only because of avoidance of double labeling. In this case there is no need to care of stoichiometry in reporter composition. Being concentration-independent, ratiometric response from a single dye will not change if a part of these dyes will be degraded or photobleached. One can easily record the excitation spectra at single emission wavelength and emission spectra at single excitation wavelength selecting them for maximal response in intensity and without care on photoselection between multiple types of dyes. In sensing with a single dye one can observe not only the shifts of excitation or emission spectra, but also the changes of relative intensities of two or more bands (Fig. 6). But the latter type of λ-ratiometry is not possible without existence of spectrally recognizable ground-state and/or excited-state forms and the switching of excitation or emission spectra between these forms.

Ground-state interactions generate the differences in excitation spectra. Since the ground state is commonly the state of equilibrium, the species displaying multiple light-absorbing states, if they interconvert, should be in equilibrium. The transitions between them may not occur in the excited state and in this case they can be considered as distinct species with their own characteristic excitation and emission spectra (Fig. 7a). There may be different reactions in the excited state that shift the equilibrium to one of these forms or generate new emissive or nonemissive states. The excited-state reactions offer additional possibilities for observing new bands in fluorescence emission spectra belonging to reactant and reaction product forms (Fig. 7b). These reactions develop during the excited-state lifetime adding kinetic component to the observed effects. This makes the design of single-molecular λ-ratiometric reporters a difficult task but also offers additional possibilities. A decade ago only two types of fluorescence sensors with these properties were known and actively used—the probes for pH [136] and the Ca^{++}-sensitive probes introduced by Roger Tsien [24]. This situation has changed,

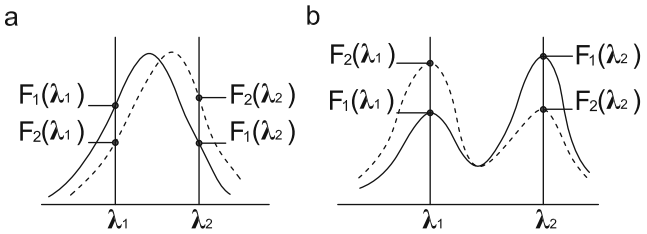

Fig. 6 Two typical ways of observing the spectral changes generated by a single dye. (**a**). The shifts in excitation or emission spectra. Intensities F_1 and F_2 are recorded at the wavelengths of the most significant change of spectra, at their wings. (**b**). The reciprocal changes of two band intensities. If the bands are well resolved, the intensities are recorded at the band maxima

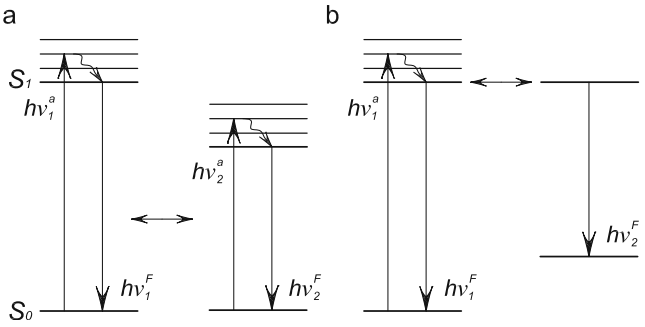

Fig. 7 The energy diagrams showing the spectroscopic effects in the cases of ground-state (**a**) and excited-state (**b**) equilibrium. In the case of ground-state equilibrium the two species behave as independent absorbers and emitters, so both their excitation ($h\nu_1{}^a$ and $h\nu_2{}^a$) and emission ($h\nu_1{}^F$ and $h\nu_2{}^F$) energies commonly differ. In the case of excited-state reaction the excitation energy $h\nu_1{}^a$ is the same but the emission energies ($h\nu_1{}^F$ and $h\nu_2{}^F$) differ. The excited state with the emission energy $h\nu_2{}^F$ is populated kinetically and the equilibrium may not be achieved during the lifetime τ_F

and it is high time to provide systematic analysis of these developments.

Switching between fluorescent and phosphorescent emissions

The two emissive excited states of the same dye could be the singlet (fluorescent) state and the triplet state producing phosphorescence emission. Phosphorescence is usually observed with a strong Stokes shift, long emission lifetimes (up to milliseconds and seconds) and strong temperature-dependent quenching. In λ-ratiometric sensing technologies only those phosphorescent dyes can be used that display strong room-temperature phosphorescence with the steady-state intensity comparable to that of fluorescence. A limited number of dyes demonstrate these properties. They include platinum and palladium ions incorporated into porphyrins [137] and chelating complexes of ruthenium [138, 139] that can be enhanced by dye incorporation into solid polymer matrices.

Due to long lifetimes, phosphorescence is dramatically quenched by the oxygen molecules present in the medium, whereas the short-living fluorescence is not affected by the presence of oxygen and may serve as the reference. Since phosphorescence band is strongly shifted to longer wavelengths, a very precise, convenient and self-calibrating detection can be achieved in a steady-state ratiometric recording [140]. With the aid of fiber-optic probes, oxygen can be analyzed in different media, including the cell culture bioreactors [139].

Since the thermal quenching for fluorescence and phosphorescence is different, the ratio of their intensities is temperature-dependent and can be calibrated in temperature units. The complexes of rare earth ions incorporated

into different glasses and crystals were reported to be efficient thermometers operating on the basis of this principle [141].

Presently the development of fluorescent-phosphorescent ratiometric sensors follows two routes. One is to improve phosphorescent metal complexes by incorporating them into conjugated polymer nanoparticles [142]. The other is to use the most efficient organic dyes and induce in them a phosphorescent emission [143]. Since it is known that heavy atoms such as iodine increase the rate of transition to triplet, the dyes can be incorporated into nanoparticles formed of polymer in which the heavy atom substitutions are made. A strong amplification in phosphorescence intensity that is sufficient for ratiometric measurements can be observed.

The dyes in protic equilibrium

There are many dye molecules in which the ionization of attached groups changes dramatically the spectroscopic properties leading to the appearance of new bands in absorption and in emission spectra. For λ-ratiometric sensing only those of them are interesting that display these bands of high and comparable intensities.

The ranges of pH-dependent transitions in absorption and in emission may not correspond and since the acidity of dissociating groups is commonly much higher in the excited than in the ground state, the pH range of sensitivity in emission can be dramatically shifted to lower pH [144, 145]. This allows providing the wavelength-ratiometric recording in an extended pH range. Presently the most extensively used dyes are the benzo[c]xanthene derivatives, such as C-SNAFL-1 [136, 146] The variation of their excitation spectra reflects the ground-state proton dissociation, and in emission spectra the pH-dependent appearance of strongly red-shifted second band is due to deprotonation of hydroxy group in the excited state. This property is observed also in the new water-soluble 3-hydroxychromone dye, 3,4′-dihydroxy-3′,5′-bis(dimethylaminomethyl)flavone [147]. A remarkable band separation (391 and 560 nm with isoemissive point at 493 nm), dynamic range at neutral pH and high two-photonic absorbance of novel fluorine derivative [103] makes it very attractive for cellular imaging. In contrast, a new squaraine dye Square-650-pH exhibits a dramatic pH-dependent change in excitation spectrum (the shift on deprotonation from 535 to 653 nm) but not in emission spectrum. It is available in the form ready for protein conjugation [104]. Extremely broad pH range of response (1.5–9.0) is achieved when two different pH-sensitive dyes are fused into one molecule [148].

Many processes in the living cell depend on pH, and therefore its values (pH_i) are strongly regulated and kept constant near the neutral values, and their change indicates some pathological conditions. To address the need of researchers, the pH-sensitive fluorescent proteins were developed and they are applicable for λ-ratiometric detection [149, 150]. A variety of cell-permeable fluorescence sensitive dyes were also suggested. Particularly it was shown that the emission spectrum of a cell-permeable macrocyclic Eu (III) complex incorporating an N-methylsulfonamide moiety changes with pH, allowing ratiometric pH measurements in the range from 6 to 8 [151].

Dissociation of substituent groups participating as proton donors in the excited-state intramolecular proton transfer (ESIPT) reactions interrupts these reactions with the appearance of new fluorescence bands, as it was shown for 2-(2′-arylsulfonamidophenyl)-benzimidazole [152] and 3-hydroxychromone [153] derivatives. Such effects open new possibilities for the design of ratiometric sensors tackling different targets.

The λ-ratiometric fluorescence reporters based on response in pH can be used as transducers in the sensors for different analytes, such as urea, penicillin, acetylcholine and organophosphorous pesticides [88]. These analytical tools benefit from the possibility of simple wavelength-ratiometric measurements. Some of them demonstrate dramatic effects. Thus, deprotonation of secondary amines in a Ca^{++} binding probe results in the change of emission color from green to red [154].

Hydrogen bonding in the ground and excited states

The presence of H-bond donor and acceptor groups coupled to aromatic structures possessing π-electrons offers new possibilities for λ-ratiometric sensing based on formation/breaking of intermolecular H-bonds. These bonds may influence the intramolecular charge-transfer (ICT) character of the emission with correspondent spectral shifts. These effects are usually smaller than that on protonation but occur in the same direction [155]. In excitation spectra on interaction with H-bond donor at the acceptor site one can observe the shift to longer wavelengths and on interaction with proton acceptor at the donor site to shorter wavelengths.

The excited-state behavior that determines the shifts in emission spectra is more complicated. The H-bond proton-acceptor group (e.g. carbonyl C=O group) included into aromatic ring being an acceptor for π-electrons on electronic excitation increases its electron acceptor power. This leads to strengthening in the excited state of H-bonds formed by this proton acceptor with proton donor groups [156]. Accordingly, the interaction of aromatic proton acceptors with different external proton donors produces the opposite effect. But this effect is usually small or even unnoticed [157]. What is important is that the shifts in π-electronic density may result in reorganizations of H-bonds and to the formation of new bonds in the excited state.

Many researchers are familiar with the so-called "polarity-sensitive" probes that respond to an increase of polarity by dramatic shifts of fluorescence spectra to longer wavelengths in highly polar media [3, 158]. This effect is the higher the stronger is the increase of their dipole moment on electronic excitation, and such increase is commonly achieved by inclusion into aromatic ring in opposite positions of electron donor and acceptor groups. Since these groups are also the H-bond formers, the bond formation in the excited state produces the shifts in fluorescence spectra comparable or even stronger in magnitude than the effects of polarity [159–161]. Practically this means that if the testing of any compound is performed in water, and its binding to receptor results in screening and dehydration of fluorescence reporter, a great λ-ratiometric signal can be detected.

Carbonyls that are often incorporated into aromatic heterocycles can form with proton acceptors the H-bonds of two types: strong and weak. Both of them can dramatically transform the spectra and the latter are observed when the strong binding sites are already saturated [162]. Reorganization and new formation of H-bonds can be observed in time-resolved spectra [163]. In the presence of strong intramolecular bond that couples the closely located donor and acceptor groups, the additional intermolecular bonds are only weak, and they do not reorganize in the excited state. Such systems offer the pathway to achieve ideal H-bonding sensors [164].

The chromophores are known, in which the H-bonding to proton acceptor and ICT produce the shifts in opposite directions, and this effect can be used to identify protic from aprotic environments [165]. The H-bonding can also interfere with excited-state intramolecular proton transfer (ESIPT) providing switching between reactant and product fluorescence bands (see below).

The H-bond sensitive organic dyes can be suggested for direct determination of protic impurities (water, alcohols) in neutral and aprotic solvents [166–168]. They can perform a more difficult task, such as providing direct response to the binding of anions. Thus, detection of phosphate ion can be based on the H-bonding perturbation of ICT reaction [169]. Fluorescence modulation in anion sensing can be achieved by introducing intramolecular H-bonding interactions in host-guest adducts [170]. It is important that in this approach the receptors for anions can be the neutral derivatives (such as urea or thiourea) interacting with analyte via the H-bonding [171]. This produces fluorescence quenching by ICT reaction [172], and for the detection of these ions not always the optimized wavelength-shift mechanism is explored. Sometimes the researchers are satisfied by the changes of intensity [173] and a great reserve of possibilities remains to be explored.

Photoinduced electron transfer (PET) and intramolecular charge transfer (ICT)

Electron transfer reactions are fundamental in chemistry and biology. They can be realized in different fluorescence emitters that can be coupled with a variety of sensing events. Researchers may be confused sometimes by the existence of two different mechanisms of electron transfer that may occur in the excited states and can dramatically influence the emission—nonadiabatic and adiabatic. *Non-adiabatic* transfer occurs at week electronic coupling between electron donor (D) and acceptor (A) sites. It can be not only intermolecular but also intramolecular when D and A sites are separated by a spacer. Whereas initially excited (locally excited, LE) state can be strongly fluorescent, the product of this PET reaction is commonly nonfluorescent. Different molecular sensors were suggested based on PET mechanism [3, 6], but since their response is based on quenching/enhancement of emission without spectral effects, they will not be discussed here.

When the interaction between the D and A potential energy surfaces is strong, due to the mixing between these surfaces the electron can transfer along connecting energy profile almost without potential barrier [174]. This is the case of *adiabatic* transfer, and since in fluorescent dyes it can be realized only intramolecularly, this mechanism is called intramolecular charge transfer (ICT). The product state of this reaction can be (but not always is) strongly fluorescent. Moreover, the ICT reaction leads to redistribution of electronic charge that often creates (or destroys) strong dipoles. This drives interactions with the environment out of equilibrium and activates the dielectric relaxation processes in surrounding molecules and groups of atoms. The ICT process is coupled with the relaxation and results in the shifts of fluorescence emission bands. It can be suppressed when the environment is rigid and proceed when the surrounding dipoles adopt optimal configuration [175, 176]. This allows many possibilities in coupling the LE—ICT transitions in organic dyes with sensing events employing λ-ratiometric detection. Moreover, in rigid environments the ICT reaction can be modulated by variation of excitation wavelength.

The difference between PET and ICT is illustrated in Fig. 8.

The *charge transfer* states conform to general spectroscopic regularity: if they are formed in the excited states, then they influence the position of emission spectra only, but if they exist already in the ground state, then the positions of absorption and excitation bands should also change. The direction of spectral shifts depends on relative magnitude of ground-state and excited-state interactions: if they are greater in the ground state, the shift should be to shorter wavelengths, and if they are greater in the excited

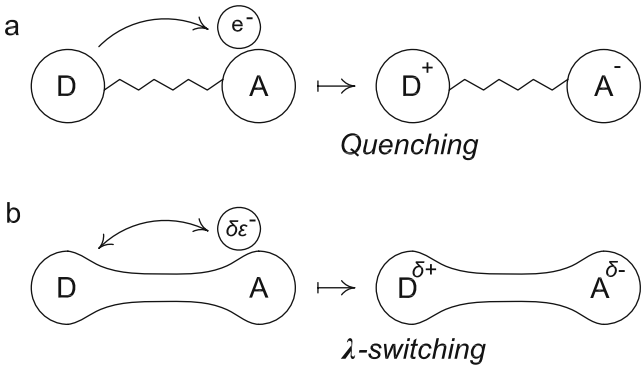

Fig. 8 The schematic illustration of the difference between photoinduced electron transfer (PET) and intramolecular charge transfer (ICT). In PET (**a**) the donor (*D*) and the acceptor (*A*) are interacting weakly and commonly the full transfer of electronic charge results in quenching. In ICT mechanism (**b**) the electronic coupling between donor and acceptor is significant, the electron transfer can be partial and reversible, and this reaction can easily proceed with the generation of new fluorescence band

state, the spectra shift to longer wavelengths. If the dipole moment of dye molecule increases on excitation (as in coumarins and Nile Red) then the dielectric stabilization develops in time. It decreases the excited-state energy level causing the long-wavelength shift of emission. If the dipole moment decreases (such as in betaine dyes), then the ground-state interactions, strong in the ground state, become weaker in the excited state. Formation of H-bond by proton acceptors shifts the electron density along this bond resulting in an additional increase of the fluorophore dipole moment μ_e and therefore the polarity-dependent shifts [177].

For obtaining efficient ICT in the excited state the organic dye molecule should contains an electron donating group (often a dialkylamino group) and an electron-withdrawing group (often, carbonyl) located at opposite sides. A created large dipole moment interacts with the medium dipoles resulting in strong Stokes shifts. Since the ICT state can be stabilized only in polar media, one can produce the LE-ICT switching with ratiometric response by changing the polarity of local environment. This can be done by incorporating the dye into cyclodextrins possessing molecular-size hydrophobic cavities [178].

The binding of charged target to coupled chelating group can produce switching between LE and ICT states with strong spectroscopic effects [3, 179]. The cation bound at the side of electron-donor group reduces the electron-donating character of this group resulting in a blue shift in the absorption spectra. Conversely, a cation interacting with the acceptor group enhances the electron-withdrawing character of this group and the absorption spectrum is thus red-shifted. The fluorescence spectra are commonly shifted in the same direction as the absorption spectra. An anion binding produces the opposite effect.

Very popular λ-ratiometric Ca^{++}-chelating dyes Fura 2 and Indo-1 were first examples for ratiometric imaging of cellular function. They operate on the background of ICT mechanism. In these stilbene-like dyes the bound Ca^{++} ions interact with the electron-donor nitrogen atom incorporated into fluorophore generating the new excitation band shifted to shorter wavelengths [24]. In the excited state this nitrogen atom becomes positively charged and in Fura 2 its interaction with the cation ejects the cation from the binding site. That is why the emission spectra of Fura 2 are insensitive to ion binding. This does not happen with Indo-1, for which the two-band ratiometric response is observed also in fluorescence. In Indo-1 the electrostatic repulsion in the excited state between nitrogen atom and the bound ion is not sufficient for its ejection from the binding site, and this allows generating a new band in emission.

There are many reports on the construction of chemical sensors for other, beside calcium, ions based on ICT mechanism [6] but those that exhibit ratiometric response are still rare cases [180]. In some of their designs the cation binding site is located at electron acceptor and in this way the wavelength ratiometry in emission is achieved [181]. Ratiometric reporting was realized in sensing Cu^{++} [182] and Hg^{++} [183] ions. Recently such sensors were developed for Zn^{++} ion [180, 184, 185], which opens their very important application in intracellular imaging [186, 187]. A novel Cd^{++} sensor that is selective over Zn^{++} ions can be also used in ratiometric fluorescence microscopy [188]. Application of amine substituted tricarbocyanine dyes allows shifting the ion sensing based on ICT mechanism to near-IR wavelength region [189]. The other possibility of shifting to this region is using the distyryl-substituted boradiazaindacene (BODIPY) dyes [190].

Binding of anions to electron donating or withdrawing sites can also change the *ground-state* and *excited-state energies* of fluorophore generating strong spectral changes [191]. The ICT-based λ-ratiometric anion sensors were designed and synthesized [192, 193]. As a recent example, the dye exhibiting the ICT reaction can produce dramatic spectral changes on binding of fluoride ion [165]. The ion binds to electron-acceptor group and switches its emission from that at 500 nm to that at 380 nm. For detecting the cyanine anion (CN^-) the sensor was designed that incorporates the boronic acid group [194]. The binding of cyanide transforms this group from neutral to anionic that becomes an electron donating group providing strong spectroscopic changes.

There is a possibility to apply ICT mechanism for detecting zwitterionic and neutral compounds. It was reported on the synthesis of ICT sensor that on binding of cysteine and homocysteine shifts the fluorescence spectrum to shorter wavelengths by 125 nm [195]. Such drastic spectral change was achieved by applying the aldehyde

group not only as electron acceptor but also as target binder that disrupts the ICT, allowing to observe the LE emission. Development of efficient λ-ratiometric sensor for hypochlorite (ClO^-) [196] also uses ICT as a signaling mechanism. Glucose sensing that is very important for clinical diagnostics employs boronic acid as a receptor that can be conjugated with ICT reporting dyes [197]. A number of such glucose sensors were described. They use boronic acid as electron-withdrawing group attached to the fluorophore [198, 199].

The ratiometric response in ICT dyes to binding neutral compounds can be also generated in a different way. The target binding can influence the *local polarity* in the vicinity of reporter dye if this binding screens the reporting dye from the solvent or produces a conformational change that changes the dye environment. Although some spectroscopic effects have been achieved on this trend in application to proteins [200], they were relatively small and not systematic. Meantime, labeling of oligonucleotides by attaching to their deoxyuridine base of N,N-dimethylaminophenyl-substituted pyrene allowed observing significant ICT-LE transformations on their formation of duplex structure with target DNA [201]. Novel dyes and novel strategies for inducing polarity-changing effects need to be developed.

And finally, the reporting signal based on ICT generation can be modulated by the change in *electronic conjugation* within the fluorophore system. This can be done by changing the planarity between electron-donor and acceptor fragments, since the planar structure produces the best π-π electronic coupling. Such disruption of planarity with the appearance of localized ICT emission is well studied in many systems, and correspondent states got the special name twisted intramolecular charge-transfer (TICT) states [202]. Planarity can be modulated by covalent modifications of the dye and by changing their configurations, so that in planar structures the LE-ICT equilibrium is modulated by polarity of the medium stabilizing the ICT state [156]. Such flexibility suggests many possibilities to generate the sensor response. For instance, the LE-ICT equilibrium can be modulated dramatically by the dye incorporation into cyclodextrin cavity [203].

Thus, the ability of the dyes operating on the basis of ICT mechanism to generate the two-band or wavelength-shifted emission is an extremely valuable property for the design of wavelength-ratiometric reporters.

Electrochromism and electrochromic modulation of ESIPT

When the dye interacts with electric field that is created macroscopically (e.g. between two electrodes) or microscopically (by a closely located ionized group), such interaction changes the energies of electronic transitions and results in spectral shifts. This phenomenon is caused by direct interaction of the ground- and excited-state dipoles with the applied electric field and is known as *electrochromism* (also called as *Stark effect*) [204]. In the description of this phenomenon, very popular is the mesoscopic model. It considers the fluorophore as a point dipole with the dipole moment $\Delta\vec{\mu}$ and the electric field as a vector $\vec{F}$ that averages all the fields influencing the fluorophore. They interact in a dielectric medium characterized by an efficient dielectric constant ε_{ef} that provides partial screening these interactions. Based on this simple model, one can obtain the following expression describing the spectral shifts $h\Delta\nu_{\mathrm{obs}}$ in energy units:

$$h\Delta\nu_{obs} = -\left(1/\varepsilon_{ef}\right)\cdot|\Delta\vec{\mu}|\cdot\left|\vec{F}\right|\cos(\theta) \qquad (10))$$

where θ is the angle between $\Delta\vec{\mu}$ and $\vec{F}$ vectors. Thus, in the simplest dipole approximation the magnitude of the shift is proportional to the electric field vector and to the change of dipole moment associated with the spectroscopic transition with the account of their relative orientation in space. The maximal effect should be observed when the dye exhibits substantial change of its dipole moment $\vec{\mu}$ on electronic excitation, which implies a substantial redistribution of the electronic charge density. Furthermore, it should be located in low-polar environment (low ε_{ef}) and oriented parallel ($\cos\theta=1$) or anti-parallel ($\cos\theta=-1$) to the electric field.

A group of styryl dyes and particularly 4-dialkylaminostyrylpyridinium derivatives with electron-donor and electron-acceptor substituents at the opposite ends of their rod-shaped aromatic conjugated moieties are among the best known electrochromic dyes [205, 206]. They exhibit the following excited state reaction producing the strong change of the dipole moment (Fig. 9):

These dyes were applied for recording the electrostatic potential in biomembranes. The shifts are commonly recorded in excitation spectra, where the strong variations of fluorescence intensities are observed at two slopes of these spectra (as depicted in Fig. 6a). The magnitude of this two-wavelength intensity ratio is rather small, about 7–10% per 100 mV. Still in biomembranes they detect the electrostatic fields produced by charged groups, adsorbed ions and oriented dipoles of lipids. The more convenient for detection are the shifts in fluorescence spectra, but for these

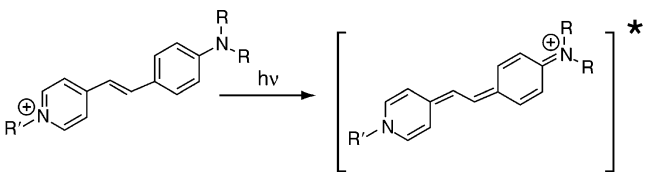

Fig. 9 Excited-state transformations of a typical electrochromic styryl dye. Dramatic redistribution of electronic charge allows observing strong spectroscopic shifts under the influence of electric field acting in the direction parallel or antiparallel to the long axis of the molecule

dyes they are much smaller [35] due to the involvement of dielectric relaxations during fluorescence lifetime.

In order to observe electrochromic effect as the ratio of two intensities in emission spectra and to increase dramatically the sensitivity of this ratiometric response, we suggested to couple electrochromic effects with the ESIPT reaction. The idea was to transform small electrochromic shifts of initially excited spectral form (N*) into a strong variation of relative intensities of two well-resolved emission bands (as depicted in Fig. 6b). These two bands can be generated in designed 3-hydroxychromone derivatives [207, 208]. Similarly to styryl voltage sensors, they produce strong dipoles in the N* excited state with the shifts in excitation spectra of similar magnitude. Influencing the ESIPT reaction, the electric field provides switching from strongly electrochromic N* form to low electrochromic T* form, as it is shown below (Fig. 10). As we observe, the variations of electrostatic potential on a molecular level result in significant changes of relative intensities of two fluorescence bands. The properties of these dyes will be discussed in more detail below.

Since the ESIPT reaction in these dyes is so sensitive to electric field perturbations, they can be used as basic elements in molecular sensors and probes that detect the binding of charged compounds and also on a more general scale, where the sensing effect is coupled with relocation of proximal charges. Being tested on biomembranes and their phospholipid models [209], they are expected to find application in sensing devices that involve charged interfaces or sensing surfaces of nanoparticles.

Excited-state intramolecular proton transfer (ESIPT)

ESIPT is the reaction that can produce emissive species with dramatic (often by 100 nm and more) long-wavelength shifts of fluorescence spectra. This fact is very attractive for the design of λ-ratiometric reporters, but realization of such opportunity is not easy. ESIPT is commonly a very fast reaction proceeding along the intramolecular H-bond preexisting in the ground state that requires keeping proton donor and acceptor in close proximity [210]. Therefore in many dyes demonstrating it, only the ESIPT reaction product band is observed in fluorescence spectra. Since these dyes are not applicable for designing the λ-ratiometric fluorescence reporters, our choice has to be concentrated on the systems exhibiting interplay between normal (N*) and produced by ESIPT tautomer (T*) emissions. Two emission bands belonging to normal and ESIPT emissions can be observed in a limited number of cases.

(a). When the ESIPT reaction is *gated by transformations between two or more ground-state forms*. Then, when excited, one of the forms, with intramolecular H-bonding, will exhibit ESIPT, and other forms, in

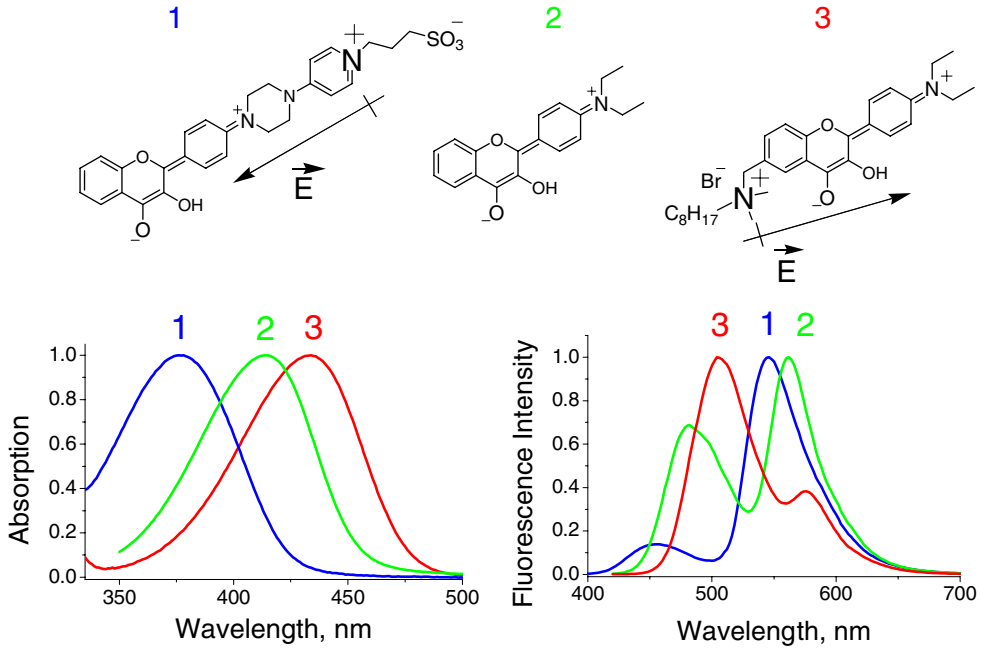

Fig. 10 The effect of covalently attached positively but electronically uncoupled charged groups on absorption and fluorescence spectra of strongly dipolar 3HC dye. The two bands in fluorescence emission appear as a result of ESIPT reaction. The dye (*1*), in which the charged group is attached from the side of electron-donor dialkylamino group of dye (*2*) possesses the absorption spectrum strongly shifted to the blue. Its fluorescence spectrum possesses greatly decreased intensity of the N* band that is also shifted to the blue. The opposite effect is observed for dye (*3*), in which the positively charged group is attached through a spacer to the opposite side

which this bond is disrupted, display normal emission. This is the case of benzimidaazole, benzoxazole and benzothiazole derivatives that got the common name benzazoles [211–213]. In these compounds the ratiometric sensing signal can be obtained on switching between these ground-state forms.

(b). When the ESIPT reaction *exhibits slow kinetics* on the time scale of emission. This can be due to intermolecular H-bonding perturbations as observed for parent 3-hydroxyflavone [214] and 3-hydroxyquinolones [215]. Then the factors that influence the barrier for ESIPT reaction (such as temperature and viscosity) can modulate the intermolecular interactions with H-bond donors (including water), generating fluorescence reporting signal. The compounds in which the ESIPT reaction is retarded to allow observing LE emission can be also used for sensing. Meantime, they are less attractive because their ratiometric response to the influence of various dynamic quenchers cannot be easily controlled.

(c). When the initially excited normal (N*) state is *stabilized by intermolecular interactions* to become of the same energy as the product tautomer (T*) state. Then the ESIPT reaction becomes reversible and equilibrium between two forms can be established on a time scale faster than the emission. This is the case of designed 3-hydroxychromone derivatives, in which the stabilization of the N* state occurs due to its transformation into ICT state by introduction of electron-donating substituents [216–218]. The rapidly established equilibrium between two excited-state populations [164] allows variations of relative intensities of correspondent fluorescence bands as a function of polarity [219] and local electric field [208] with the absence of perturbations that can be produced by variations of temperature [220] and the presence of dynamic quenchers [221].

The dyes of benzazole type, such as 2-(2′-hydroxyphenyl)benzimidazole (HBI), 2-(2′-hydroxyphenyl)benzoxazole (HBO), and 2-(2′-hydroxyphenyl)benzothiazole (HBT) exhibit ESIPT that depends on relative configuration of molecular fragments. These dyes can be denoted as HBX compound series, where –X– in the benzazole ring stands for –NH–, –O– and –S– (Fig. 11). In their ground states in nonpolar and aprotic environment their *enol-cis* form is the most stable conformer that on excitation undergoes ESIPT to form the *keto* tautomer T*. This transition gives rise to a strongly (by more than 100 nm) Stokes-shifted emission [222]. Other conformers that are more stable in more polar and protic environments (for example, an *enol-anti* open rotamer) do not undergo ESIPT because of significant distance between donor and acceptor sites and lack of intramolecular H-bond connecting them. These conformers are responsible for the fluorescence band of normal emission, N*, observed at shorter wavelengths. Tuning of ratiometric response to particular intermolecular interactions can be provided by probe design, particularly, by variations in location of amino substituents [223] or fusion with naphthalene group [224]. Strong variation of spectroscopic properties can be achieved by introduction of electron-donating or withdrawing substituents in *para* position of N atom in benzothiazolyl ring [225].

The ground-state transformations of these molecules governing the ESIPT reaction allow many possibilities in providing the λ-ratiometric response in sensing. Covalent labeling of proteins with these dyes with the retention of their two-band response was described [226]. This opens many possibilities for using labeled peptides and proteins as efficient sensors.

Presently the most efficient applications of benzazole dyes are observed in ion sensing. It was shown that blocking ESIPT in HBI derivative by attaching *tert*-butyldimethylsilyl chloride to hydroxyl group can be selectively removed by fluoride ions that can be used for determination of fluoride in aqueous media [227].

The fluorescence of HBO aggregates demonstrates a fluorescent ratiometric change in a range of temperature from 15 to 60 °C and this property can be used for the development of molecular thermometers [228].

An interesting aspect of application of benzazole dyes is their incorporation into polymeric molecules and nanostructures. It was shown that when benzothiasole dye is

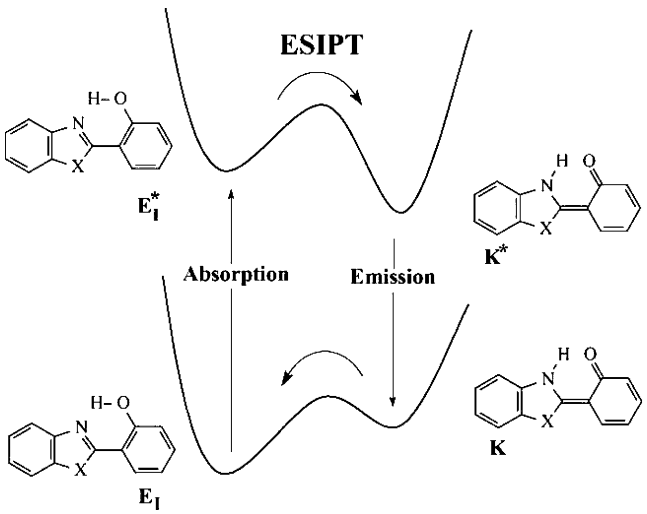

Fig. 11 The ESIPT mechanism of benzazole dyes [226]. Only E_I *enol-cys* conformer with intact intramolecular H-bond can exhibit this reaction resulting in strongly Stokes-shifted fluorescence emission. A number of non-productive conformers can be observed that can be stabilized by intermolecular H-bonding. They produce normal emission at shorter wavelengths

incorporated into silica-based hybrid materials, it retains two-band emission with ratiometric sensitivity to polarity [229]. A ratiometric fluorescent sensor for zinc ions based on derivative of benzoxazole, 4-benzoxazol-2′-yl-3-hydroxyphenyl allyl ether, co-polymerized with synthetic polymer and immobilized on glass slides was described [230]. Upon binding with Zn^{++}, the ESIPT process is disrupted resulting in a 46 nm blue-shift of fluorescence emission. A photoluminescent amorphous conjugated polymer with an HBO unit in the molecular main chain was synthesized [231]. It emits green light, both in solution and as a solid, with a fluorescence emission maximum at 518 nm from an excited *keto* tautomer generated in ESIPT reaction. The polymer shows different fluorescence emission spectra in solvents with different polarities because of intramolecular hydrogen-bond interruption and provides sensitive detection of metal cations.

Since ionization of proton-donor groups in benzazole compounds makes ESIPT not possible, the switching of intensity between anionic and ESIPT forms can be used for pH probing and for the development of sensors with ratiometric response that is coupled with this ionization [152].

The 3-hydroxychromones (3HCs) are the classical dyes demonstrating ESIPT mechanism [232], see Fig. 12. In contrast to benzazoles, their rigid skeleton eliminates the possibility of forming the ground-state conformers. Because of this great advantage their major mechanism of two-band response is different. Meantime, they possess some features that make them similar to benzazoles. Intermolecular H-

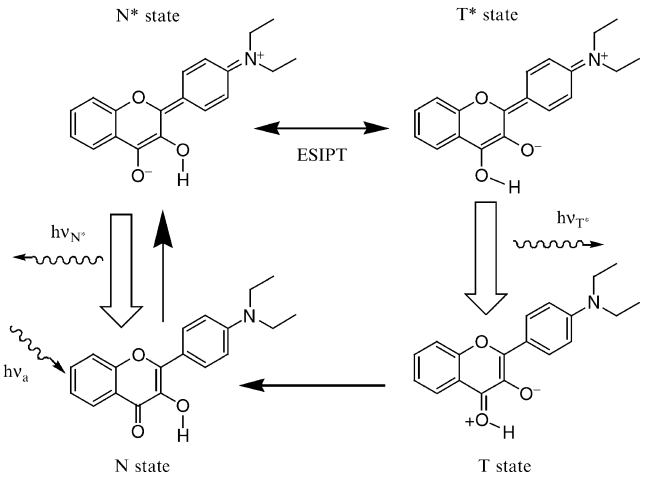

Fig. 12 The ESIPT reaction in 3HC derivative 4′-dialkylamino-3-hydroxyflavone. The high excited-state dipole moment of the N* form is provided by diakylamino group (electron donor) and carbonyl group (electron acceptor). In the tautomer T* form due to the reaction of proton transfer the charge distribution is more symmetric and therefore it is less sensitive to variations in intermolecular interactions. Therefore the sensitivity of two N* and T* forms to all types of intermolecular interactions is different producing different variations in positions and intensities of correspondent fluorescence bands

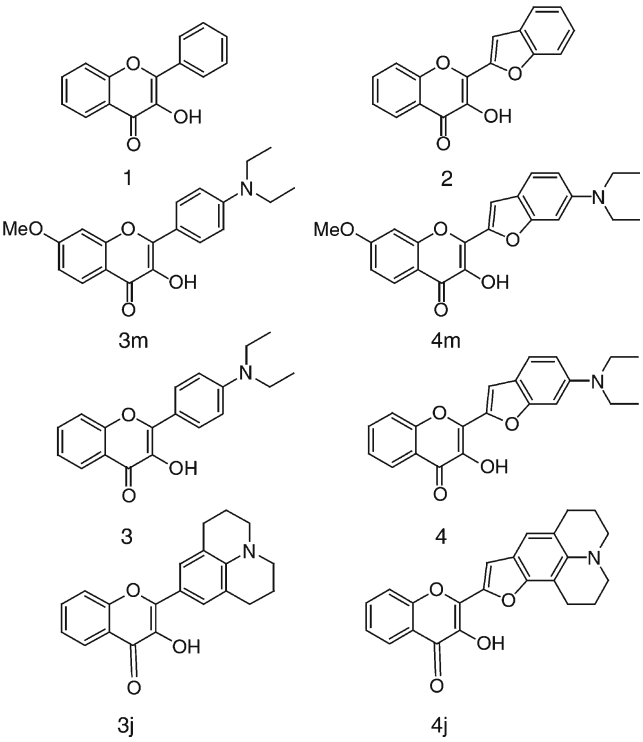

Fig. 13 Two series of 3-hydroxychromone dyes exhibiting dramatic variation of wavelength-ratiometric response to polarity of molecular environment [218, 258]

bonding to their 4-carbonils provides perturbation that suppresses the ESIPT reaction [164], as well as deprotonation of 3-hydroxyl at high pH [153]. In both these cases there appears intensive fluorescent band located at intermediate wavelengths between N* and T* emissions. In the absence of their contributions or when they are properly accounted, 3HCs with substitutions in position 2 for π-conjugated electron-donor groups (such as dialkylaminophenyl) or the groups possessing high electronic density (such as furyl or thiophenyl) (Fig. 13) demonstrate extreme two-band ratiometric sensitivity to solvent polarity. This sensitivity appears as interplay of ICT emission from N* state and the ESIPT emission from the tautomer T* state [216–218]. Both emissions originate from the same ground state and the two bands belong to the species that exhibit and do not exhibit the ESIPT reaction. These species are in dynamic equilibrium that can be shifted if one of the forms is stronger stabilized by intermolecular interactions. In 3HCs it is the N* form, so that variations in distribution of its π-electronic density by introduction of substituents produce dramatic influence on its ratiometric response adjusting the full-scale switching between two emissions within the narrow ranges of polarity [218], Fig. 14.

Due to these unique features, the 3HC dyes have found numerous applications. As the polarity-sensitive probes [167, 219] they demonstrate the variations of two-band relative intensities (F_{N^*}/F_{T^*}) by several orders of magnitude

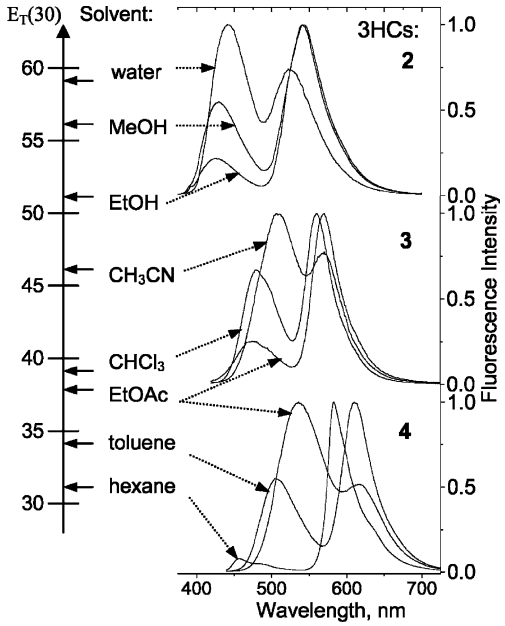

Fig. 14 Fluorescence spectra of 3-hydroxychromones **2**, **3** and **4** (Fig. 13) showing that the two-band ratiometric response of these dyes is adapted to different ranges of solvent polarities—polar, medium-polar and low-polar, respectively. The arrows show the position of solvent on a polarity scale ET(30) presented as an ordinate to the left and the correspondent fluorescence spectrum [218]

even within the narrow polarity ranges. Because of that they became useful tools in the studies of such diverse systems as supercritical liquids [233], polymers [234], direct [235] and reverse [236] micelles, and also of models of biological membranes [209, 237, 238]. They probe cyclodextrin microcavities [239] and ligand-binding sites of proteins [240]. They are used to detect ions [241].

In this broad range of applications the most important is the study of interactions with and between biological macromolecules [242] since they open many possibilities in sensing and imaging technologies. The strong ratiometric signal was detected on interaction of labeled antigenic peptide with specific antibody [243], on enzyme binding with its inhibitor [244] and on temperature and pressure dependent rearrangement of subunit structure of α-crystallin [245, 246]. It was also shown that using labeled spermin as a probe it is easy to distinguish the binding to a single-stranded and to double-stranded DNA [247], and that the labeled peptide corresponding to the zinc finger domain of the HIV-1 nucleocapsid protein on interaction with target oligonucleotides displays strong sequence-dependent fluorescence response [248]. Oligonucleotides with the natural bases replaced by 3HCs [249] have good prospect in DNA and RNA sensing technologies.

The other well developed field of application of 3HC dyes is the study of membranes of intact living cells. These membranes are characterized by steep (on nanometer scale) gradients of polarity and hydration. In addition, electric fields change steeply and non-monotonously across the membrane [250]. Presently only the designed 3HC dyes with substituents that allow orienting the fluorophore and its locating at different depth allow consistent characterization of biomembrane with these parameters [209]. Examples of this application will be presented in the sub-section that follows.

Multiparametric λ-ratiometric reporting

Operating with spectroscopic data only it is hard to characterize separately different types of intermolecular interactions. Meantime, such possibility exists. Addressing Fig. 6, we may consider the possibility of combining the cases depicted in its parts (a) and (b). The scheme (a) illustrates the principle of ratiometry based on variation of ground-state transformations of reporter dye and of its interactions, and (b), suggests ratiometry on the basis of transformations in the excited-state. In principle, the ground-state and excited-state transformations can be governed by different types of molecular forces. Therefore the proper reporter design may allow not only combining these effects but also obtaining separate information on these interactions making the fluorescence response at least two-dimensional.

This idea was realized with 3-hydroxychromone dyes exhibiting ground-state equilibrium between the species with and without intermolecular H-bonds characterizing *proticity* (H-bond donor potential) of the environment [164] and also an excited-state equilibrium between ICT and ESIPT states indicating *polarity* of the environment [219], as shown in Fig. 15. When these dyes are applied to test the unknown properties of their environment, one can observe three partially overlapped emission bands, and their excited-wavelength-dependent deconvolution allows obtaining independently the polarity and hydrogen bonding potential of this environment [251]. The validity of this deconvolution is increased when an important principle based on the mechanism of this reaction is applied. The N* and T* bands generate from the same ground state and therefore the ratio of intensities F_{N*}/F_{T*} should not depend on excitation wavelength, whereas the H-bonded complex formed in the ground state can be recognized by a different excitation spectrum. The global deconvolution of spectra that involves excitation and emission wavelength dependencies allows obtaining all three spectral forms and independent analysis of two types of intermolecular interactions. The switching between two ground-state forms of the same dye may be considered as the switching between the distinct species that possess their characteristic excitation spectra. These forms should be different in a sense that one should participate and the other not participate in an excited-state reaction. Following this

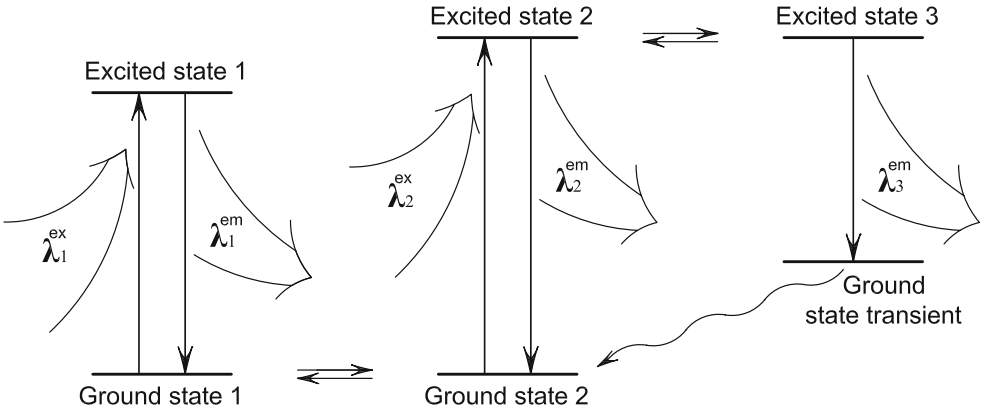

Fig. 15 General scheme of ground-state and excited-state transformations and emissions in the case of a reversible excited-state reaction that involves only one of the two ground-state species (from ref. [251], modified)

principle and with proper selection of reporter dye one can strongly increase the possibilities of designing the fluorescence reporters with advanced properties.

An example of simultaneous measurement of proticity and polarity was demonstrated recently in the studies of α-synuclein aggregates that are known to appear in certain nerve cells on Parkinson's disease and are thought to be in the origin of this pathology [252]. The studies of aggregates formed by wild-type protein and its three mutated forms demonstrated strong differences in polarity and hydration between them based on analysis of response of 3HC dye.

The 3HC dyes and the multiparametric analysis of their response have found important applications in the studies of living cells, their membranes and their phospholipid models, where very steep gradients of both hydration and polarity are observed across the membrane bilayers [209, 250]. The first observation suggesting deconvolution of fluorescence spectra into three bands was made in the studies of small 3HC dye in lipid bilayers of different composition [253]. These studies suggested different (more shallow) location of small dye molecule in the bilayer when it forms the H-bond in water. In recent studies the dyes containing substituent groups that allow locating the fluorophore at different depths and orientations in the outer leaflet of cell membrane were applied. This allowed comparison of structural transformations occurring in model phospholipid membranes with that in living cells occurring on depletion and then addition of cholesterol [254].

It is interesting that the same family of 3HC dyes allows observing the pH-dependent [153] and dependent on the presence of strong proton acceptors in the medium [255] effects of dye deprotonation that, displaying a different excitation spectrum, generate additional fluorescence band. Observations in the titration range show that the response to deprotonation by the appearance of this additional band and the two-wavelength response to the change of polarity/electrostatics can be independent. This opens new dimension in the studies of molecular interactions and also suggests the design of new probes for fluorescence sensing and imaging.

Concluding the section on λ-ratiometric measurements with a single dye we indicate that the problem here is not only in finding the dyes with ground-state and excited-state transformations providing band-shifting and generation of new bands. These new or shifted bands should be of comparable intensities to original bands, otherwise the stronger signal could hamper the measurement of intensity of the weaker one and the ratiometric reading cannot be reliably assessed. The presented examples demonstrate clearly that this problem can be overcome.

General conclusions

The primary goal of λ-ratiometry is to use the simplest possible instrumentation for providing the self-referenced ratiometric measurements that are not possible with single-channel recording of fluorescence intensity. In contrast to anisotropy and lifetime sensing (that may not need additional referencing), the reference in intensity sensing should come from additional measurement of intensity at different wavelength. If the ratio of recorded intensities changes on intermolecular interactions occurring on target binding or on target spatial distribution that forms the image, then it is easy to use this responsive parameter as a self-referenced signal. Here we critically discussed different possibilities for that. We can introduce an inert molecular or nano-sized reference that can emit at different wavelength than the reporting dye. Alternatively, there can be a couple of active reporters forming an excimer or exciplex or interacting via FRET mechanism with the formation of new bands. We demonstrated also that 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/or generation of new bands. In the case of wavelength shifting, the intensity readings can be taken at two slopes of the bands

where there is the strongest change of intensity. If the new bands are generated, the greater efficiency can be achieved with intensity readings at band maxima. In this type of two-band ratiometric sensing because the signal comes from a single type of the dye (in contrast to FRET) and the forms emitting at two wavelengths may have the same lifetimes (in contrast to wavelength-shifting), the internally calibrated signal has the advantage to be resistant to any uncontrolled quenching effect.

In this Review we have given a general perspective on λ-ratiometry as a tool for research and development in sensing and imaging. We can observe how rich are the possibilities if we consider it not only as a tool but as a general strategy that involves the design of fluorescence reporters, the choice of mechanism of their response and of the mechanism coupling this response with the target binding event. Numerous examples from the literature allow stating that the benefits of this general strategy offering many possibilities for this choice are still not realized in full. We believe that their knowledge should stimulate the progress. And finally we emphasize that being convinced that the most attractive prospectus is offered by λ-ratiometric response of a single dye, there are many possibilities to enhance and modulate it in a required direction by synthesis of diversity-oriented dye libraries [256], using new emitting and supporting materials [88], plasmonic enhancement [257] and FRET from properly designed donors.

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