

**MJ** MULTISCIA  
JOURNALS PUBLISHERS

# FRONTIERS IN CHEMICAL AND LIFE SCIENCES



**ISSN: ( 3065- 4238 )**

<https://multisciajournals.com/journals/index.php/fcls>  
editor.fcls@gmail.com

# Chloride ion, anion, and biomolecule ratiometric fluorescent probes based on small molecules

Pappulu Nayak, Chandraian  
Department of Chemical and Life Sciences

## Article Info

Received: 16-02-2026    Revised: 03-03-2026    Accepted: 14-03-2026    Published: 10-04-2026

## Abstract

Many fields, from environmental monitoring to the biological sciences, rely on the accurate quantitative detection of individual analytes. By using optical sensing, we may take less intrusive pictures, monitor several samples in simultaneously, and conduct non-invasive studies within biological environments. Ratiometric fluorescence sensing is one of the optical sensing approaches that is being studied since it might provide quantitative and exact evaluations. The self-calibration that comes from monitoring two or more emissions is reflected in its inherent dependability and high sensitivity, two of its benefits. Sensing, imaging, and biological applications have led to the development of several ratiometric sensing probes using tiny fluorescent molecules. Analytes such as cations, anions, and biomolecules in both aqueous and biological samples may be detected ratiometrically using tiny fluorescent probes, which are detailed in this study highlight. The purpose of this spotlight is to provide examples, not all of them.

## Introduction

An important area of research in supramolecular and biological chemistry is the development of sensor systems for the efficient and fast detection of analytes. Due to their technological simplicity, rapid reaction time, and great sensitivity, fluorescent probes have garnered interest for analytical sensing and optical imaging. No matter the nature of the analyte—chemical, environmental, or biological—the ideal fluorescence sensing system would be able to reliably provide a fluorescent response under the analytical circumstances. In order to conduct a highly selective quantitative analysis of an analyte, fluorescent sensing usually necessitates the production of tiny molecules. A number of factors that are not related to the analyte, such as instrumental parameters, the surrounding microenvironment, the local concentration of the probe molecule, photobleaching, etc., can impede the analysis, making it difficult to quantify a target analyte using single-emission fluorescent probes. Take use of the so-called ratiometric technique to overcome these issues and ensure dependability. Changes in the intensity of two or more emission bands, caused by analytes, are the basis of ratiometric fluorescence sensors. An efficient internal referencing system that boosts sensitivity and quantification is the end product. Internal charge transfer (ICT), excited-state intramolecular proton transfer (ESIPT), fluorescence resonance energy transfer (FRET), through-bond energy

transfer (TBET), and monomer-excimer formation are some of the strategies that have been studied extensively within the context of ratiometric fluorescence sensing. In fact, to date, ratiometric fluorescent probes have permitted the quantitative determination of target analytes, as well as the fluorescence imaging of toxic substances associated with various human diseases. In this highlight, we summarize some recent advances made in the area of ratiometric fluorescent sensor design. Particular emphasis is placed on the chemical features of successful probes and their use in biological applications where appropriate.

### ICT-based ratiometric fluorescent probes

In the excited state, probes based on internal charge transfer (ICT) have a "push-pull"  $\pi$ -electron system that is formed by an electron-donating unit conjugated to an electron-accepting unit inside a single molecule. Cation sensing has made heavy use of them. When the probe's electron-donating component comes into contact with a cation, its electron-donating nature diminishes. The absorption spectra then become blue as a result of this. In contrast, a red shift in the absorption spectra is seen as a result of the ICT being more developed when an electron-accepting portion interacts with a cation. Figure 1a also shows that lifetimes and quantum yields of fluorescence undergo variations. It was Valeur who first proposed the idea of using ICT for cation sensing.<sup>15</sup> Imaging target analytes has been made This

probe could be used effectively for the ratiometric sensing of Cd<sup>2+</sup> in living cells.<sup>20</sup> In seminal work, the Shinkai and James groups have prepared saccharide sensing ICT probes that show promise for use in real world applications.<sup>21,22</sup>

Disulfide-carbamate based naphthalimide derivatives have been widely exploited as ratiometric fluorescent probes for biological thiol detection.<sup>23-28</sup> In the presence of thiols, the disulfide bond is readily cleaved to release 4-aminonaphthalimide. This bond scission results in a red shifted emission and an obvious colour change from colourless to yellow. Using this basic approach, biological thiols, e.g., glutathione, cysteine, and thioredoxin could be quantified both in aqueous solution and in living cells. Detection proved possible at the

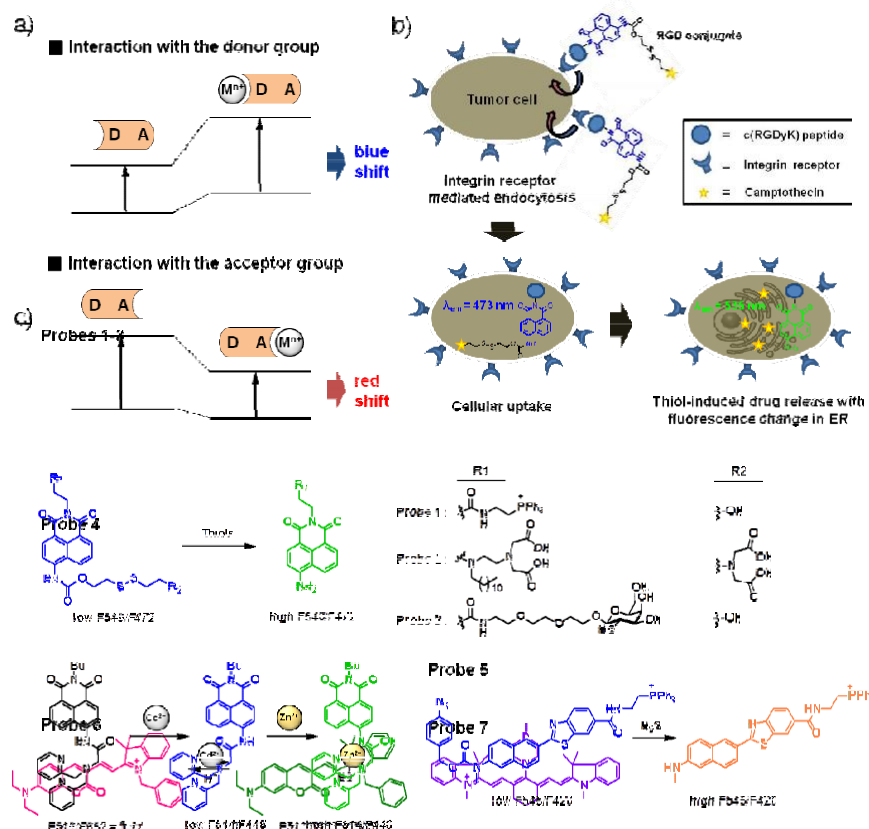
Two-photon microscopy (TPM) has emerged as a useful technique for the ratiometric imaging of species in live cells and tissues. TPM, which employs two near-IR photons as the excitation source, provides several **localized in mitochondria and allows a real-time imaging of intracellular H<sub>2</sub>S.**

Cyanine dyes have been also widely used in bioimaging studies. These dyes have emission and absorption maxima that fall in the near-IR region (i.e., 650-900 nm). Such photophysical features are

advantages over other optical-based sensing methods, including a relatively deep penetration depth (> 500 μm), a localized excitation, and longer observation times. Cho et al. designed several two-photon probes (e.g., Probe 5) based on aminonaphthalene derivatives for the ratiometric detection of H<sup>+</sup> and thiols.<sup>32,33</sup> These probes could be used to estimate the pH value and thiol levels in cells and tissues, including mouse brain.

A hybrid fluorophore (Probe 6) constructed by combining coumarin and merocyanine was developed for the ratiometric sensing of H<sub>2</sub>S.<sup>34</sup> This probe shows a strong absorption band due to its ICT character. It displays two well-resolved emission bands arising from the coumarin and merocyanine subunits, respectively. In the presence of H<sub>2</sub>S, the intensity of the merocyanine emission decreases while that of the coumarin emission increases. When dissolved in phosphate-buffered saline (PBS, pH 7.4), the solution color was found to change from dark blue to very pale blue upon exposure to H<sub>2</sub>S.

advantageous *in vitro* and *in vivo* imaging applications since biological samples display weak autofluorescence in the NIR region. Kircher *et al*

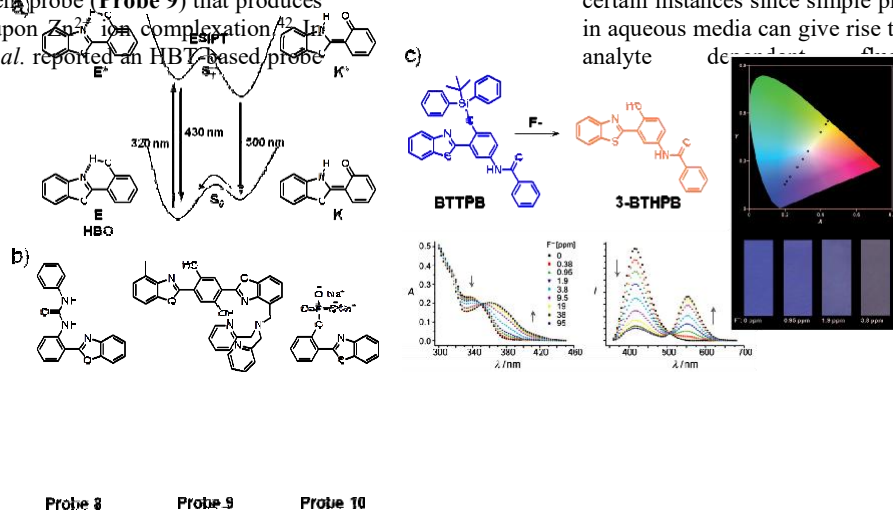


**Fig. 1** (a) Schematic representation of the spectral shifts expected for ICT-based sensors as the result of cation binding to the constituent electron donor and electron acceptor groups. (b) A naphthalimide-based ICT molecule that functions as a theragnostic agent. (c) Structures of the ICT-based molecules and corresponding ratiometric fluorescence responses, **Probes 1-7**. The illustration in Figure (b) is reproduced with permission of the American Chemical Society from ref. 28. Copyright © 2012.

### ESIPT-based ratiometric fluorescent probes

Excited-state intramolecular proton transfer (ESIPT) typically involves a fast proton transfer from a hydroxyl (or amino) unit to a carbonyl oxygen (or imine nitrogen) atom in the excited state of a fluorophore. For example, 2-(2'-hydroxyphenyl)benzoxazole (HBO) exists preferentially in the *enol* form (E) and is stabilized by an intramolecular six-membered ring H-bonding motif in the ground state. Upon excitation at 320 nm, the excited *enol* form (E\*) is converted into the excited *keto* tautomer (K\*) as a result of ESIPT. This process gives rise to an emission band (~ 500 nm) with a large Stokes shift. After decaying to the ground state, the *keto* form (K) reverts back to the *enol* form through a reverse proton transfer. Other excited *enol* molecules that do not undergo ESIPT typically display emission

Several other ESIPT dyes, including 2-(20-hydroxyphenyl)-benzoxazole (HBO), 2-(20-hydroxyphenyl)benzothiazole (HBT), *N*-(3-(benzo[d]thiazol-2-yl)-4-(hydroxyphenyl)benzamide (3-BTHPB) and 3-hydroxyflavone (3HF) have been developed as ratiometric fluorescent probes. For example, Demchenko and Mely reported 3HF-based ESIPT probes that serve as fluorescent biomembrane probes that permit the ratiometric detection of apoptosis.<sup>40</sup> Peng *et al.* reported HBO-based ESIPT probes (**Probe 8**) for the detection of fluoride and acetate ions.<sup>41</sup> Metal ion binding can also trigger an ESIPT effect. For instance, Pang *et al.* reported a HBO-based fluorescence probe (**Probe 9**) that produces an ESIPT emission upon Zn<sup>2+</sup> ion complexation.<sup>42</sup> In other studies, Kim *et al.* reported an HBT-based probe



**Fig. 2** (a) An illustration of ESIPT showing the photo- and thermal driven changes that occur upon photoexcitation of 2-(20-hydroxyphenyl)-benzoxazole (HBO). (b) Chemical structures of ESIPT-based molecules, **Probes 8-10**. (c) Structure of **BTHPB**, a probe fluoride ions and spectral traces show the analyte-induced absorption and emission changes observed upon exposure to F<sup>-</sup> in micellar suspensions in water. CIE 1931 (x,y) chromaticity diagram of test papers for the detection of NaF that are based on 3-BTHPB as derived from fluorescence spectra recorded at different analyte concentrations. The absorption and emission spectra and the CIE chromaticity diagram are reproduced with permission of the Wiley from ref. 44. Copyright © 2010.

### FRET/TBET-based ratiometric fluorescent probes

bands at higher energies (e.g., ~ 430 nm). Under many conditions, ESIPT fluorophores show two emission bands originating from the *enol* and *keto* forms. Variations in the relative intensities of these bands can provide the basis for ratiometric sensing systems (Figure 2a).<sup>13,14</sup> Perturbations to the intramolecular arrangement through intermolecular H-bonding interactions or changes in solvent polarity have been extensively studied.<sup>38</sup> Moreover, environment-dependent modulations in the optical features provide the background for several applications of ESIPT dyes, including as polarity-sensitive probes in analytical chemistry, molecular logic gates, luminescent materials, as readout elements in polymer science, markers in colloidal chemistry, and biochemical markers.<sup>39</sup>

**(Probe 10)** that produces an *enol*-derived emission feature as the result of inhibiting ESIPT via phosphorylation of the HBT hydroxyl group.<sup>43</sup> Upon selective enzymatic (MKP-6; a protein tyrosine phosphatase) hydrolysis, however, ESIPT occurs to give a *keto* tautomer-based emission. Similarly, the probe (**BTHPB**), where the hydroxyl group was protected by *tert*-butyldiphenylchlorosilane, was found effective for fluoride anion detection in micellar mixtures (Figure 2c). Sensitivities at the ppb level were observed and extensions to easy-to-prepare test papers proved easy to effect.<sup>44</sup> Unfortunately, ESIPT-based ratiometric detection has proved limited in certain instances since simple protonation of the probe in aqueous media can give rise to unexpected and non-analyte dependent fluorescence changes

Fluorescence resonance energy transfer (FRET) and through-bond energy transfer (TBET) mechanisms involve energy transfer between a pair of fluorophores that act as energy donors and acceptors, respectively.<sup>1,2,11,12</sup> As applied in sensing applications, the emission of the donor at relative short wavelength serves to activate emission of the acceptor at longer wavelength with the ratio of these two emissions modulated by the target analytes. Substantial spectral overlap between the donor emission and the acceptor absorption bands is generally required to show a high FRET efficiency.<sup>11,12</sup> As a consequence of this photophysical requirement, FRET-based dyads are typically linked by a nonconjugated spacer with energy transfer occurring through space (Figure 3a). Since Stryer and Haugland exploited FRET as a “spectroscopic ruler”,<sup>45</sup> FRET has emerged as a critical tool for the analysis of DNA structures, nucleic acid regulation, protein structure, function analysis, and immunoassays.

In the case of TBET-based dyads, the donor and acceptor are linked by an electronically conjugated bond. This prevents the donor and acceptor fragments from becoming planar. Energy transfer can thus occur through a linking bond or bonds without the need for spectral overlap (Figure 3b).<sup>11</sup> Early on, a systematic study of TBET was undertaken by Verhoeven and co-workers.<sup>46</sup>

Subsequent to these pioneering efforts, many FRET/TBET-based fluorescent probes have been put forward for the ratiometric detection of metal cations. Metal ions, such as  $\text{Hg}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Ag}^+$ , and  $\text{Cu}^{2+}$ , generally act as excited state quenchers *via* electron transfer or heavy metal ion effects. As a result, the fluorescence of the probe is quenched. In early work, Akkaya *et al.* designed several FRET-based “cassettes” having  $\pi$ -extended BODIPY derivatives and demonstrated their use in  $\text{Hg}^{2+}$  ion sensing (**Probes 11** and **12**).<sup>47</sup> The energy transfer efficiency is enhanced in the presence of  $\text{Hg}^{2+}$  ions. This increase produces a correlated ratiometric change in the fluorescent emission features. Burgess *et al.* reported a design concept for TBET cassettes that could be used in many molecular biology and biotechnology applications.<sup>48</sup> In one proof-of-concept study, involving a series of TBET molecules bearing a BOPIPYPY-based donor and a cyanine acceptor (**Probes 13-15**),<sup>49</sup> the Burgess group demonstrated how upon excitation of the BODIPY moiety the excitation energy is transferred to the cyanine dye acceptor through an acetylene bridge. The emission wavelength was found to depend on the structure of the cyanine acceptor and could be varied from approximately from 600 to 800 nm. Hence, this provides a large spectral window for imaging and was considered to be a useful feature. This set of probes could be encapsulated in calcium phosphate/silicate nanoparticles, which could be dispersed freely in water and applied to intracellular imaging.

Rhodamine has a time-honoured role in sensing. Its use dates to the early days when Czarnik *et al.* reported the application of a rhodamine derivative for  $\text{Cu}(\text{II})$  sensing.<sup>50</sup> Rhodamine is generally colourless and non-fluorescent in its spiro-ring closed form. In contrast, the ring-opened form of rhodamine is red and characterized by a strong fluorescence. The rhodamine ring-opening process can be activated by metal complexation, analyte binding, substrate-induced reactions, or exposure to acidic pH.<sup>51</sup> In a typical

FRET system, ring-opening of rhodamine is used to control an Off-On signal by inducing a spectral overlap between the emission of the donor dye and the absorption of the rhodamine acceptor. To date, a number of the rhodamine-based FRET sensors have been reported. These include rhodamine-dansyl dyads for  $\text{Cu}^{2+}$  sensing, rhodamine-coumarin dyads for nitric oxide and HOCl detection (**Probe 16**), rhodamine-BODIPY dyads for  $\text{Hg}^{2+}$  sensing or for the analysis of thiol-containing analytes, such as cysteine, etc. Some of these systems have been used to effect the ratiometric imaging of certain analytes in living cells.<sup>11,52</sup> When used in a TBET system, the rhodamine ring-opening process activates a through bond energy transfer event.<sup>11</sup> This has been exploited by Tan *et al.* who developed a rhodamine-based two-photon TBET probe (**Probe 17**) that allowed for the two-photon imaging of living cells and tissues. This system permitted high resolution ratiometric imaging with good tissue penetration (up to 180  $\mu\text{m}$ ).<sup>53</sup>

In another study, FRET-based probes having two  $\text{Zn}^{2+}$  binding sites and containing coumarin as an energy donor and xanthene as an

## Summary and outlook

Because of their versatility, ease of use, and seeming simplicity, fluorescent probes have been the center of a lot of research interest lately in the fields of chemistry, biology, and environmental science. Since they may allow for better accuracy under circumstances of quantitative analysis, ratiometric fluorescent probes are of special interest among the probes now being investigated. Using well-designed small compounds, we have shown many methods in this Highlight for producing a ratiometric fluorescence signal. Additionally, we have shown how ratiometric fluorescent probes based on traditional molecular motifs may be developed to tackle various imaging and sensing problems. When it comes to developing ratiometric probes based on small molecules, some of the most important factors currently include monomer-excimer formation, excited-state intramolecular proton transfer (ESIPT), fluorescence resonance energy transfer (FRET), through-bond energy transfer (TBET), and internal charge transfer (ICT). Fluorescent probes that enable ratiometric sensing and imaging of harmful substances and important cellular components, including small chemical species associated with different human diseases, have been developed by utilizing these mechanistic modalities in combination with suitable fluorophores, analyte-specific receptors, or reactive sites. With any luck, this Highlight will serve as a synopsis of important past and present studies and a structural blueprint for the development of novel ratiometric fluorescence probes. More efficient and cost-effective sensor systems may be the result of research spurred by this spotlight, which might lead to the creation of novel sensing techniques such combined organic-inorganic constructions.

## References

1. Chem. Rev. 1997, 97, 1515, by A. P. de Silva, H. Q. N. Gunaratne, T. Gunnlaugsson, A. J. M. Huxley, C. P. McCoy, J. T. Rademacher, and T. E. Rice.
2. In 2007, J. S. Kim and D. T. Quang published Chem. Rev., 107, 3780. Chemical Review, 2008, 108, 3443, by E. M. Nolan and S. J. Lippard.

4. Chem. Soc. Rev. 2010, 39, 2120, by X. Chen, Y. Zhou, X. Peng, and J. Yoon.  
Fifthly, in 2011, Y. Zhou, Z. Xu, and J. Yoon published in Chem. Soc. Rev. 40, 2222.  
Chem. Soc. Rev. 2011, 40, 79, by H. N. Kim, Z. Guo, W. Zhu, J. Yoon, and H. Tian.  
6. Chemical Society Reviews, 2012, 41, 3210, by H. N. Kim, W. X. Ren, J. S. Kim, and J. Yoon.  
Chemical Review, 2003, 103, 4419 (R. Martinez-Manez and F. Sancenon, 2003).  
"Chem. Rev. 2010, 110, 6280" by D. T. Quang and J. S. Kim, number 9.  
10. In the 2012 issue of Chem. Rev., X. Chen, T. Pradhan, F. Wang, J. S. Kim, and J. Yoon discuss the topic at 1910.  
Chemical Society Reviews 2013, 42, 29 (J. Fan, M. Hu, P. Zhan, and X. Peng).  
Analyst 2012, 137, 4885, by Y. Feng, J. Cheng, L. Zhou, X. Zhou, and H. Xiang.  
Chemical Society Reviews, 2011, 40, 3483, by J. Wu, W. Liu, J. Ge, H. Zhang, and P. Wang.  
Chem. Soc. Rev. 2013, 42, 1379, by A. P. Demchenko, K.-C. Tang, and P.-T. Chou.  
Coordinated Chemical Review 2000, 205, 3 (B. Valeur and I. Leray).  
16. Handbook of Fluorescent Probes and Research Products, 10th edition, by R. P. Haugland; Molecular Probes, Inc., Eugene, OR, 2005.  
17. In a 2002 article published in the Journal of the American Chemical Society, S. Maruyama, K. Kikuchi, T. Hirano, Y. Urano, and T. Nagano discuss several topics.  
J. Am. Chem. Soc. 2007, 129, 13447; K., Kensuke, Y. Urano, H. Kojima, and T. Nagano.  
19, K. Kiyose, H. Kojima, Y. Urano, and T. Nagano, Journal of the American Chemical Society, 2006, 128, 6548.  
Article published in the Journal of the American Chemical Society in 2008 by Taki, Desaki, Ojida, Iyoshi, Hirayama, Hamachi, and Yamamoto (130, 12564).  
21. In a 1995 article published in Chemistry Letters, K. R. A. S. Sandanayake, S. Imazu, T. D. James, M. Mikami, and S. Shinkai discuss these findings.  
23. In Tetrahedron Letters 2001, 42, 4553, S. Arimori, L. I. Bosch, C. J. Ward, and T. D. James were authors.  
23. C. E. Kruger, T. Gunlaugsson, F. M. Pfeffer, E. B. Veale, R. M. Duke, Chem. Soc. Rev. 2010, 39, 3936.  
24.1 In a 2010 article published in Chemistry, B. Zhu, X. Zhang, Y. Li, P. Wang, H. Zhang, and X. Zhuang were the authors.  
The authors of the article "J. Am. Chem. Soc. 2012, 134, 1316" are M. H. Lee, J. H. Han, P.-S. Kwon, S. Bhuniya, J. Y. Kim, J. L. Sessler, C. Kang, and J. S. Kim.  
In their 2012 article published in the Journal of the American Chemical Society, M. H. Lee, J. H. Han, J.-H. Lee, H. G. Choi, C. Kang, and J. S. Kim discuss the topic at 134, 17314.  
Article 27 from the Journal of the American Chemical Society, 2014, 136, 8430, authored by M. H. Lee, H. M. Jeon, J. H. Han, N. Park, C. Kang, J. L. Sessler, and J. S. Kim.  
Journal of the American Chemical Society, 2012, 134, 12668, by M. H. Lee, J. Y. Kim, J. H. Han, S. Bhuniya, J. L. Sessler, C. Kang, and J. S. Kim.  
29. In Scientific Reports 2014, 4, 5870, L. Zhang, W.-Q. Meng, L. Lu, Y.-S. Xue, C. Li, F. Zou, Y. Liu and J. Zhao published their work.  
Journal of the American Chemical Society, 2010, 132, 601, by Z. Xu, K.-H. Baek, H. N. Kim, J. Cui, X. Qian, D. R. Spring, I. Shin, and J. Yoon.  
Chemical Communications 2013, 49, 877 (written by M. Kumar, N. Kumar, and V. Bhalla).