

Progress in Tuning Emission of the Excited-State Intramolecular Proton Transfer (ESIPT)-Based Fluorescent Probes

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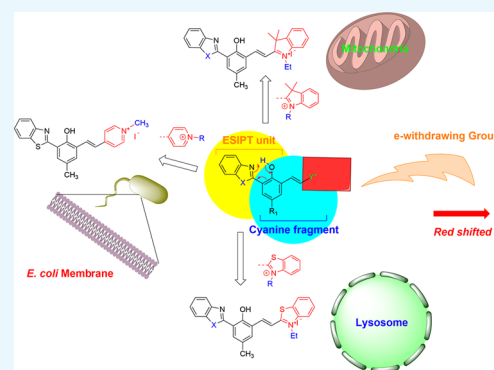
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ABSTRACT: In this review, we will summarize our recent progress in the design and application of novel organic sensors with emission in the near-infrared region (600–900 nm). By coupling different functional groups with excited-state intramolecular proton transfer (ESIPT) segments, new probes are developed to achieve a large Stokes shift, high sensitivity, and selectivity and to tune the emission toward the near-infrared region. The developed probes exhibit attractive optical properties for bioimaging and environmental science applications. In addition, we further discuss the photophysical properties of ESIPT dyes and how their fluorescence could be affected by structural/environmental factors, which should be considered during the development of robust ESIPT-based fluorescence probes. Their potential applications as imaging reagents are illustrated for intracellular membranes, mitochondria, lysosomes, and some biomolecules.



1. INTRODUCTION

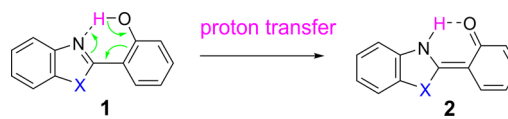
Fluorescence organic dyes have been widely used in biology, pharmacology, and environmental science as selective sensors for specific analytes for more than a century.¹ The novel fluorescent dyes with excellent photostability, high fluorescence quantum yield, high sensitivity, and selectivity are desirable for medical diagnostics, cell biology applications, and drug delivery in recent years, especially after the global pandemic.² The species to be detected can react with sensors^{3,4} or interact through host–guest interaction⁵ and then give a fluorescence signal. Some molecular probes that emit in the near-infrared (NIR) region are ideal to be developed for analytical and biological applications such as in cell imaging. In comparison with commercial dyes that emit in the UV/vis range, NIR dyes have advantages of deep tissue penetration, less tissue damage, and minimal interference caused by tissue absorption and autofluorescence.^{6–10}

Donor–acceptor-involved fluorescence mechanisms for certain dyes have been well-investigated over the past decade. Typically, there are three well-known mechanisms which include PET (photoinduced electron transfer), FRET (Förster resonance energy transfer), and ICT (internal charge transfer).¹ Traditional dyes based on these three mechanisms always have a noticeable restriction. Their characteristic small Stokes shift ($\Delta\lambda < 25$ nm) causes self-quenching and impedes their application in species detection, as the detected signals may not accurately reflect the analyte concentrations.^{11,12}

Excited-state intramolecular proton transfer (ESIPT), discovered by Weller in the 1950s,¹³ involves a four-level photochemical process, which leads to low energy fluorescence

with a large Stokes shift and enhanced quantum yield.^{1,14} The molecules typically contain a hydrogen bond donor (–OH) and exist in an *enol* form in the ground state. When the enol tautomer **1** is excited upon photon absorption, the hydrogen atom is transferred to the –C=N– group (a proton acceptor), resulting in the excited *keto* tautomer **2** that gives long-wavelength emission (Scheme 1).¹⁵ 2-(2'-Hydroxyphenyl)-

Scheme 1. Keto and Enol Tautomerization in ESIPT



benzoxazole (HBO, X = O in **1**) and 2-(2'-hydroxyphenyl)-benzothiazole (HBT, X = S in **1**) and their derivatives are the most common ESIPT chromophores.¹ However, the emission of such designs is often limited to blue, green, and orange-red colors.

The proton transfer from the phenol to the –C=N– group can be viewed simply as an acid–base reaction, as the phenolic proton is known to become more acidic in the excited state

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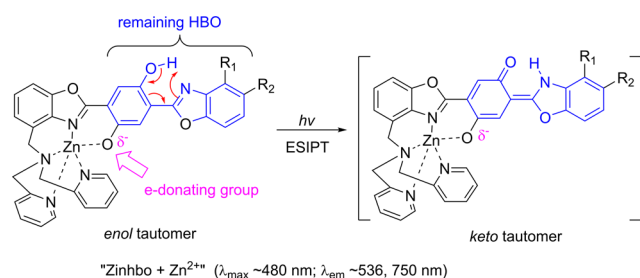


($pK_a \sim 5.7$).¹⁶ The acidity of the phenolic proton could be influenced by a suitable electron-withdrawing or electron-donating substituent, which could actively perturb the proton transfer process.^{7–10}

2. ESIPT-BASED FLUORESCENT PROBES AND THEIR APPLICATIONS

In the effort of seeking ESIPT fluorophores with NIR emission and large Stokes shifts, our group has recently synthesized a series of Zinhbo compounds,⁵ in which a strong e-donating group (i.e., $-\text{OH}$) is placed at the *para*-position of the phenol group that is involved for ESIPT (Scheme 2). Interestingly, its

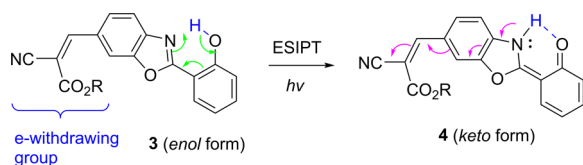
Scheme 2. Keto and Enol Tautomerization in Zinhbo-Zn²⁺



zinc complex (i.e., “Zinhbo-Zn²⁺”) gives NIR emission while retaining the dual emission feature of ESIPT fluorophores (with λ_{em} at ~ 536 and 750 nm from its enol and keto tautomers, respectively). These compounds have been demonstrated to be useful for intracellular sensing of Zn²⁺ cations via live cell fluorescence imaging.

An alternative strategy to tune the optical absorption/emission is attaching an electron-withdrawing group to ESIPT chromophores. Among very limited examples is HBO derivative **3**, in which a strong electron-withdrawing group is attached to the benzoxazole segment.¹⁷ Compound **3** is reported to exhibit $\lambda_{abs} \approx 370$ nm, which is only slightly affected by solvent polarity. Whereas its enol form gives emission at $\lambda_{em} \approx 430$ nm, its keto form gives solvent-dependent emission ($\lambda_{em} \approx 525$ nm in cyclohexane and ~ 600 nm in ether). Interestingly, the ESIPT enables a strong donor–acceptor interaction, as shown by the arrows in **4** (Scheme 3).

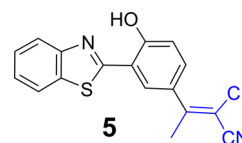
Scheme 3. ESIPT-Induced Tautomerization of 3



However, the compound exhibits low fluorescence quantum yield ($\phi_f = 2.6 \times 10^{-3}$ in cyclohexane) and a radiative lifetime of ~ 0.725 ns in nonpolar solvent with the absence of keto emission.

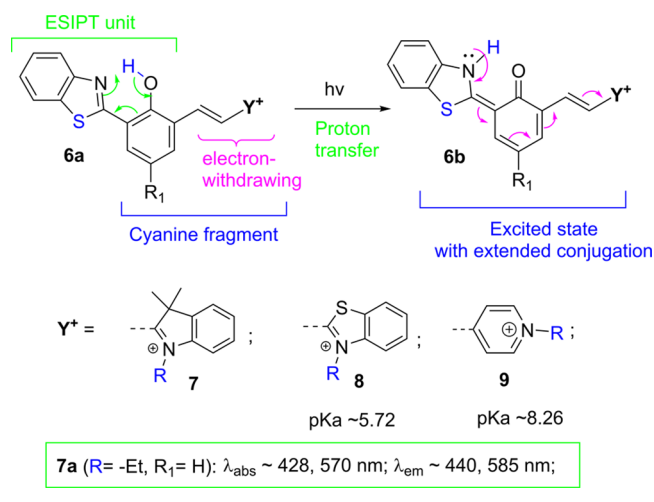
It is assumed that the electronic impact can be achieved by introducing a substituent on the phenol ring of the HBO unit. One example is **5** (Scheme 4), which is reported to exhibit two absorption bands ($\lambda_{abs} = 382$ and 458 nm) and very weak fluorescence ($\lambda_{em} \approx 510$ nm) in DMSO/H₂O solution (8:2, v/v) at 25 °C.⁴ It appears that the classical e-withdrawing groups may play a very limited role in tuning the emission toward longer wavelength.

Scheme 4. Structure of 5



Another strategy is to couple the traditional cyanine dyes with ESIPT chromophores. As a typical class of dyes, cyanines possess significant advantages including high fluorescence quantum yields and tunable emission,¹⁸ which have made them popular in various applications for decades. However, conventional cyanine dyes display small Stokes shifts, e.g., often less than 30 nm.¹⁹ It can be assumed that the combination of ESIPT chromophore with cyanine structures could lead to attractive new dyes that exhibit long-wavelength emission (of cyanines), while still giving ESIPT emission with a large Stokes shifts. As illustrated in Scheme 5, a cyanine

Scheme 5. ESIPT Probes with Different Acceptors



terminal group (Y^+) is attached to the *ortho*-position of the central phenol ring. In the ground state of **6a**, the ESIPT and cyanine fragments share the central phenol ring. However, the benzoxazole (part of the ESIPT unit) has a poor electronic connection with the cyanine terminal group ($-\text{CH}=\text{CH}-Y^+$), as they are placed at 2,6-position of the central phenol. The electronic connection between the two groups is greatly enhanced in the excited keto state **6b**, thereby generating ESIPT emission with a large Stokes shift. The presence of an e-withdrawing group could also enhance the acidity of the phenolic proton, thereby increasing the ESIPT emission.

Recently, Paul et al. reported that **7a** was used for hydrogen sulfide detection.²⁰ A later study from our group¹⁰ suggests that the absorption peaks at ~ 428 and 570 nm could be attributed to the neutral form $\text{Ar}-\text{OH}$ and its anionic form $\text{Ar}-\text{O}^-$, respectively. By comparison with its model compound (**10**), the study revealed that the absorption, λ_{abs} , of **8** is basically determined by the cyanine fragment (Figure 1). However, the presence of the ESIPT unit in **8** causes a significant bathochromic shift in emission, by about ~ 100 nm in comparison to that with **10**.⁸

Strong emission from only the keto tautomer of **8** is observed (Figure 1), in contrast to traditional ESIPT dyes that give emission from both enol and keto tautomers.²¹ It appears

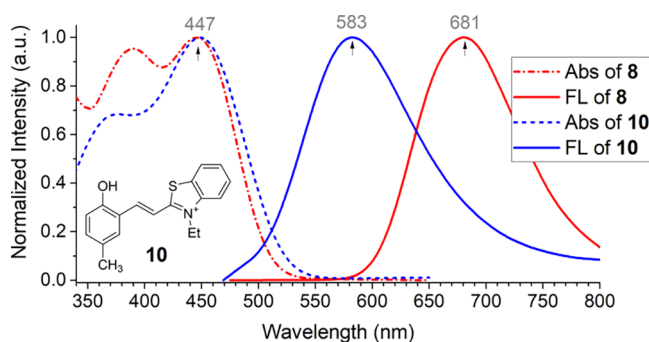


Figure 1. UV–vis absorption and fluorescence spectra of compounds **8** ($X = S$, red curves) and **10** (blue curve) in CH_2Cl_2 . Replotted with permission from ref **8**. Copyright 2017 Royal Society of Chemistry.

that the e-withdrawing group increases the acidity of phenolic proton and facilitates the proton transfer. In a collaborated effort, study from Guo et al. further revealed the details of the photophysical process, showing the fluorescence lifetime of 1.75 ns for **8** in CH_3CN .¹⁴ These findings consistently indicate that the ESIPT is a rapid process. Although this class of new fluorescent dyes have strong donor–acceptor interaction in the excited state, as illustrated in **6b**, they exhibit reasonably high fluorescence quantum yields ($\phi_{\text{fl}} \approx 0.20$ – 0.35), which opens the possibility for various applications.

The synthesis of additional examples allows us to further evaluate the properties of ESIPT–cyanine dyes. As shown in **Table 1**, the absorption λ_{abs} values change with the type of the

Table 1. Photophysical Properties of ESIPT–Cyanine Dyes in DCM and Their Intracellular Selectivity

Compound	$\lambda_{\text{abs}} / \lambda_{\text{em}}$	Φ	Selectivity
	430/675 ($X = O$) 447/681 ($X = S$)	0.28 0.32	Lysosome
	458/689	0.27	Mitochondria
	422/684	0.34	Membrane/ Neuromast

e-withdrawing groups (compare **11**, **12**, and **9**), consistent with the assumption that the dye's conjugation length is basically defined by the cyanine segment.¹⁰

Temperature Effect on Emission. As seen from **6**, the ESIPT–cyanine dyes include an ESIPT unit and a cyanine segment. Once the ESIPT occurs in the excited state (upon photon absorption), it enables a strong donor–acceptor interaction (shown in **6b**). Low-temperature fluorescence has been used to evaluate the impact of the cyanine segment.^{7,8} As illustrated in **Figure 2a**, fluorescence at 712 nm can be observed from the ethanol solution of **8** at room temperature. As the temperature is decreased by cooling the solution with liquid nitrogen, the fluorescence signal is increased significantly, and the emission peak is blue-shifted to 635 nm. The

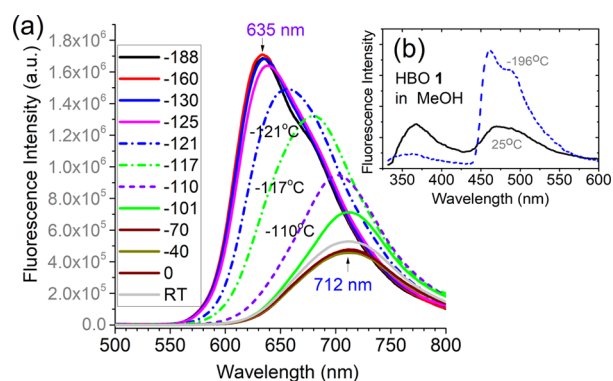


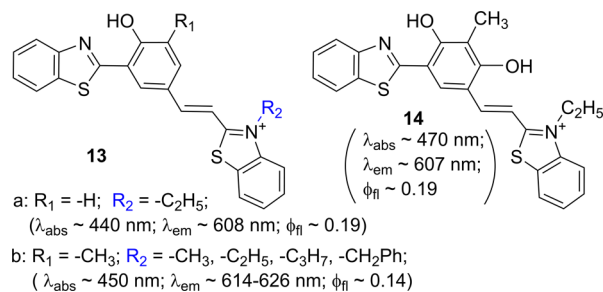
Figure 2. (a) Fluorescence spectra of **8** in EtOH ($3 \mu\text{M}$) at different temperatures in degrees Celsius ($^{\circ}\text{C}$). Replotted with permission from ref **8**. Copyright 2017 Royal Society of Chemistry. (b) Fluorescence spectra of HBO **1** in MeOH excited at 320 nm at room temperature and at -196°C . Reproduced from ref **15**. Copyright 1994 American Chemical Society.

spectral blue shift occurs in a narrow range of temperature from -100 to -125°C , as the solution is frozen to prevent intramolecular charge transfer.⁸ It should be noted that the keto emission predominates at low temperature (**Figure 2b**), as reported from HBO **1** in MeOH solution in a previous study.¹⁵ Since ESIPT will not be restricted by low temperature (as observed from spectra of **1**), the emission peak at 635 nm, observed from low-temperature fluorescence spectra of **8**, is likely from the keto tautomer, and the observed spectral blue shift can be attributed mainly to ICT.

Effect of Regiochemistry. The e-withdrawing group in **6** is at the *ortho*-position of the phenol ring. Recently, our group examined the possibility of moving the e-withdrawing group to the *para*-position of the phenol ring by synthesis of **13**.²² In CH_2Cl_2 , **13a** exhibited the fluorescence $\lambda_{\text{em}} \approx 608 \text{ nm}$ ($\phi_{\text{fl}} \approx 0.17$), which was notably blue-shifted in comparison to that with **8**. Introduction of the second hydroxy group, as seen in **14**, could generate a notable bathochromic shift in λ_{abs} (in CH_2Cl_2). However, the emission of **14** was slightly blue-shifted, thus decreasing the Stokes shift. The results clearly illustrate that the optical properties of this system are tunable. At a similar time, Rodembusch et al.²³ reported the synthesis of **13b** (with $\text{R}_2 = -\text{CH}_3$ and $-\text{C}_8\text{H}_{17}$), which was shown to be a useful pH sensor, although their conclusion is different (in terms of ESIPT event and Stokes shift) (**Scheme 6**).

Biological Cell Studies. Despite interests in using ESIPT for molecular probe design in recent year,¹ little is known about using ESIPT probes for imaging subcellular organelles until recently (in 2017).⁸ It should be noted that the phenolic

Scheme 6. Structure and Photophysical Properties of **13** and **14**



proton becomes acidic, with $pK_a \approx 5.7$ estimated from **8**, as a consequence of attaching the cyanine fragment. The ability of **8** to give a large fluorescence turn on in an acidic environment ($pH < 6$) leads to its application for fluorescence imaging of intracellular organelles, i.e., lysosomes, which are acidic ($pH \approx 4-5$) for optimal function.²⁴

The most attractive features of the reported ESIPT–cyanine dyes are their potential in molecular imaging, due to their unique photophysical properties (large Stokes shift), excellent biocompatibility, and high cell penetration ability.^{8–10,22} The fluorescence of **13b** ($R_2 = -CH_3$) was sensitive to its environmental pH, with $pK_a \approx 6.01$.²² Benefiting from its large fluorescence turn on upon protonation ($Ar-O^- + H^+ \rightarrow ArOH$), probe **13b** exhibits selectivity toward intracellular lysosomes similar to that of its regioisomer **8**. However, the low pK_a is not the sole reason for the observed lysosome selectivity, as both **8** ($pK_a \sim 5.72$, $X = S$) and **11** ($pK_a \sim 8.72$, $X = O$) are targeting lysosomes.^{8,10} The intriguing lysosome selectivity of **11** is clearly evidenced by a co-staining experiment with commercial MitoTracker Green and LysoTracker Red (Figure 3), and the probe exhibits excellent colocalization with the known LysoTracker (Figure 3D,H).

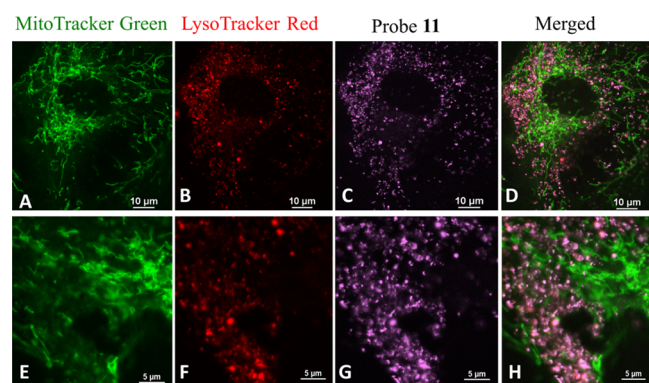


Figure 3. Confocal fluorescence images of normal human lung fibroblast (NHLF) cells co-stained with MitoTracker Green (A), LysoTracker Red (B), probe **11** (C), and their overlap (D). Images E–H are 4 times digitally enhanced for A–D, respectively. Reproduced from ref 10. Copyright 2019 American Chemical Society.

Since ESIPT–cyanine dyes contain two segments, one fundamental question is whether the ESIPT or cyanine segment has a larger impact on the intracellular selectivity. In one experiment, the $-OH$ group in **13b** was converted to a $-OCH_3$ group, and the resulting derivative maintained the lysosome selectivity.²² The same lysosome selectivity is also observed from **8** (with HBT) and **11** (with HBO).^{8,10} The result suggests that the ESIPT unit might have less influence on the cellular selectivity.

When the benzothiazolium terminal group in **11** was replaced by the indolizium group, however, the resulting **7** became the mitochondria-targeting probe.¹⁰ The drastic switch in intracellular selectivity can be seen clearly by the remarkable colocalization of **12** with MitoTracker Green (Figure 4), in sharp contrast to **11** (Figure 3D). The mitochondrial selectivity of **12** can be attributed to its Coulombic attraction toward a negative potential gradient across the mitochondrial matrix. Interestingly, the ESIPT–cyanine dye **9** with a pyridinium group stained the membrane of *E. coli* cells and does not show any selectivity toward eukaryotic cells in vivo;

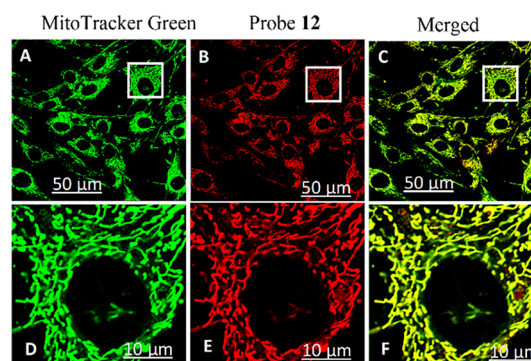
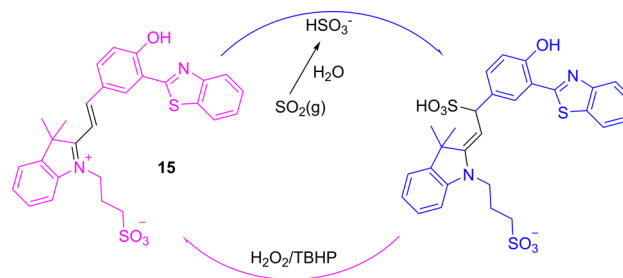


Figure 4. Images of a normal human lung fibroblast (NHLF) stained with (A) LysoTracker Green, (B) probe **12**, and (C) their overlap. Images D–F are 4 times digitally enhanced images of the region covered by white rectangular box in A–C, respectively. Reproduced from ref 10. Copyright 2019 American Chemical Society.

however, the dye selectively stained neuromast hair cells and supporting cells in zebrafish.⁷ Therefore, the cyanine terminal groups play a vital role in tuning the cellular selectivity.

Sensor Application Based on Selective Reactions. The structure of ESIPT–cyanine dyes is well-suitable for conjugated addition (Michael addition reaction), as a vinyl bond is connected to a strong e -withdrawing group (called a Michael acceptor). Such a structural feature can be used for the detection of various Michael donors, such as biologically important bisulfite and thiols. Recently, **7a** has been used for hydrogen sulfide detection,²⁰ and **15** has also been demonstrated as a reversible colorimetric and ratiometric probe for SO_2/HSO_3^- (Scheme 7) in cancer cells (MCF-7).³

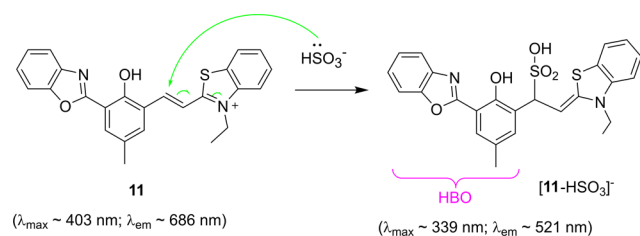
Scheme 7. Structure of **15** and the Proposed Mechanism for the H_2O_2/H_2O_2 (TBHP)-Induced Redox Cycle



Similarly, Chen et al. reported the ratiometric fluorescence detection of ROS (reactive oxygen species) such as HOCl/OCl⁻ in HeLa cells using probe **9**.²⁵ During the process, addition of the ROS to the conjugated π -bond breaks the extended π -conjugation, and the emission is given by only the ESIPT chromophore, which is hypsochromically shifted from the original probe.

While the previous studies have been focusing on using the probes with HBT as the ESIPT unit, our group examined the possible use of **11** for bisulfite detection in NHLF cells as it became available.¹⁰ In 1:1 EtOH/PBS buffer, the absorption ($\lambda_{max} = 410$ nm) and fluorescence ($\lambda_{em} = 668$ nm) of **11** gradually blue-shifted along with the formation of its adduct [**11**- $H_2SO_3^-$] (Scheme 8), showing a good linear response.

Scheme 8. Reaction of 11 with Bisulfite



3. CONCLUSION

Excited-state intramolecular proton transfer (ESIPT) provides a unique photochemical process to generate fluorescence with a large Stokes shift. Continuous efforts in developing the NIR-emitting ESIPT-based probes could lead to valuable molecular probes for specific recognition of targets, including in vivo cellular imaging. In this review, we summarize the recent progress in using various strategies to tune the ESIPT emission toward longer wavelengths. The study using the substituent effect incorporates an ESIPT mechanism with strong electronic donor–donor or donor–acceptor interaction in order to shift the emission into the desirable NIR region ($\sim 700 \text{ nm}$). Coupling the ESIPT and cyanine fragments has led to various new probes. The role of each part in the structure has been discussed. They have shown attractive applications in cellular imaging for recognizing intracellular organelles. As more and more ESIPT–cyanine dyes are synthesized, we anticipate continuous discovery of novel fluorophores that exhibit attractive optical properties to meet the need for molecular imaging applications.

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Notes

The authors declare no competing financial interest.

Biographies



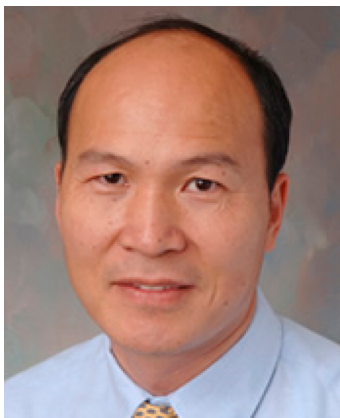
Yonghao Li is a Ph.D. candidate in Chemistry at the University of Akron, USA. He received his B.Sc. in Chemistry in 2017 from Shandong University, China, and is currently pursuing his Ph.D. degree in Organic Chemistry. His research interest is mainly in the synthesis of ESIPT-based conjugated heterocyclic compounds, especially novel cyanine dyes and their application in bioimaging.



Dr. Dipendra Dahal did his master's degree in Organic Chemistry from the Tribhuvan University, Nepal, and his M.S. and Ph. D. in Chemistry from The University of Akron, USA. He is currently a postdoctoral research fellow at the Center for Blood Oxygen Transport and Hemostasis (CBOH) at the University of Maryland Baltimore, with experience in the Organic Chemistry laboratory, synthesis, characterization, and application of molecular probes, fluorescence sensors, medicinal chemistry, and drug discovery and drug delivery. His research interests involve fluorescence probes, synthesis of drugs, and prodrugs for biomedical applications, therapeutics, and drug delivery.



Chathura Abeywickrama received his Ph.D. in 2018 from The University of Akron under the supervision of Professor Yi Pang, focusing on developing small molecule fluorescent probes for bioimaging applications. He is currently working as an Organic Chemist at St. Jude Children's Research Hospital. His research interests are following developing photostable fluorescent probes for small-molecule FRET imaging, development of hybrid fluorophore systems and organelle-targeted small molecule fluorescent probes, and understanding structure–selectivity relationships of molecular probes.



Dr. Yi Pang received his Ph.D. degree in Organic Chemistry in 1990 from Iowa State University. He worked as postdoctoral fellow in Ames Laboratory from 1991 to 1993. He's currently a professor in the Department of Chemistry, University of Akron, with a research position in the Maurice Morton Institute of Polymer Science. His current research interests include synthesis of luminescent polymers and fluorescent molecular probes and their potential applications for imaging biologically important species.

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ABBREVIATIONS

ESIPT, excited states intramolecular proton transfer; NIR, near-infrared; PET, photoinduced electron transfer; FRET, Förster resonance energy transfer; ICT, internal charge transfer; HBO, 2-(2'-hydroxyphenyl)benzoxazole; HBT, 2-(2'-hydroxyphenyl)benzothiazole; NMR, nuclear magnetic resonance; DCM, dichloromethane; NHLF, normal human lung fibroblast; ROS, reactive oxygen species

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