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Discovery, Understanding, and Bioapplication of Organic Fluorophore: A Case Study with an Indolizine-Based Novel Fluorophore, Seoul-Fluor

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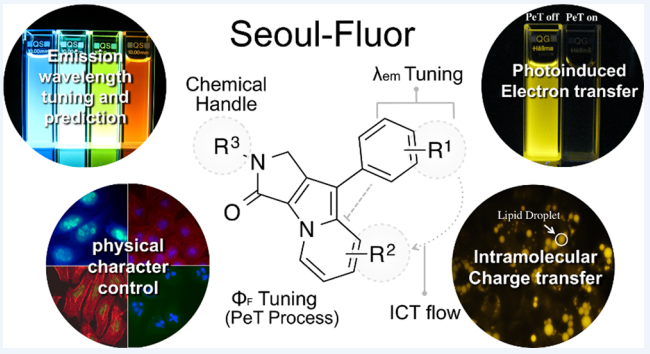
CONSPECTUS: Owing to its high sensitivity and great applicability, the fluorescence phenomenon has been considered as an inevitable research tool in the modern scientific fields of chemistry, biology, materials science, biomedical science, and their interfaces. Many strategies have been pursued to understand and manipulate the photophysical properties of fluorescent materials, but the scientific community has been focused on the repeated application of existing organic fluorophores or the identification of unique fluorescence properties in a trial-and-error basis without systematic studies. Moreover, recent studies are emphasizing the necessity of deeper understanding about the structure−photophysical property relationship of organic fluorophores for the development of better fluorescent probes. Herein, we provide an overview of a novel fluorescent molecular framework, Seoul-Fluor, which can be rationally engineered to furnish a wide variety of fluorophores in terms of the photophysical properties. Seoul-Fluor is built on an indolizine-based fluorescent platform with three different positions to introduce various substituents: R^1 and R^2 substituents for electronic perturbation; R^3 substituent as a functional handle for bioconjugation. Over the past decade, we have demonstrated that the Seoul-Fluor system has (i) tunable and predictable emission wavelength covering a full visible-color range; (ii) controllable quantum yield via photoinduced electron transfer phenomenon; and (iii) environment-sensitive fluorogenic properties that can be modified through intramolecular charge transfer processes. We convincingly demonstrated the prediction of photophysical properties, that is, emission wavelength and quantum yield, through the construction of a systematic set of analogues and the subsequent analysis of their photophysical properties without the highly sophisticated theoretical support. Guided by quantifiable parameters such as the Hammett substituent constants or energy levels of the molecular orbitals, this unique organic fluorophore can serve as a versatile molecular platform for the development of novel fluorescent switchable biosensors and fluorogenic bioprobes. In this Account, we will discuss the discovery and recent progress made on Seoul-Fluor, the rational design of Seoul-Fluor-based bioprobes, and their practical applications to specific biological processes that are facilitated by systematic studies of the structure−photophysical property relationships.



1. INTRODUCTION

The fluorescence phenomenon is an indispensable light-based scientific technique[1] for the study of the nanoscopic,[2] microscopic,[3,4] and macroscopic world[5] in modern biomedical and life sciences. For this technique, fluorescent small molecules are attractive and versatile research tool that has lately received enormous attention from the scientific community.[6,7] To develop novel fluorescent probes for biomedical imaging, it is crucial to have a fundamental understanding of photophysical properties such as absorption (λ_{abs}), emission wavelength (λ_{em}), molar absorptivity (ε), quantum yield (Φ_F), and physicochemical properties[8] on the basis of structural changes in fluorochrome. In addition, contrary to popular belief, the fluorochrome is not an inert color tag but is a molecule that can respond to environmental changes or interact with biomolecules by changing its photophysical properties.[9] Furthermore, chemical properties of the fluorescent compound can significantly influence the fluorescent conjugates.[10−12] Therefore, molecular level understanding of the relationship between its chemical structure and photophysical properties of a given fluorophore will provide a rational molecular design principle to pave a new road for the development of novel fluorescent bioprobes.[13]

Despite significant endeavors, there are only a few fluorochromes such as fluorescein,[8] BODIPY,[14] and cyanine,[15] which have been subjected to systematic studies for their structure−photophysical property relationship (SPPR). This is probably due to the fact that tedious, time-consuming synthetic procedures were required to access the structural analogues of a

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certain fluorochrome. With the introduction of combinatorial chemistry in the 1990s,[16] we now have a better strategy for the systematic synthesis of a series of structural analogues, which alleviates this synthetic obstacle for SPPR studies about fluorochromes.[17,18] In this Account, we provide an overview of our ongoing efforts toward the fundamental understanding of SPPR in a new fluorescent molecular framework, 9-aryl-dihydropyrrolo[3,4-*b*]indolizin-3-one, named Seoul-Fluor (Figure 1), via a combinatorial approach.

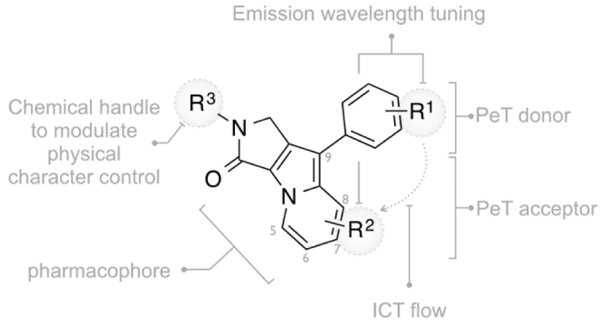

Figure 1. Schematic representation of the structure−photophysical property relationship in Seoul-Fluor system.

2. SYNTHETIC STRATEGIES FOR SEOUL-FLUOR

The discovery of the Seoul-Fluor core skeleton came from our continued efforts to synthesize novel druglike polyheterocycles using a privileged substructure-based diversity-oriented synthesis (pDOS) strategy.[19] During our efforts, we identified 9-aryl-dihydropyrrolo[3,4-*b*]indolizin-3-one as a new fluorescent molecular framework.[20,21] The original synthetic strategy was based on the intramolecular 1,3-dipolar cycloaddition reaction of azomethine ylides with substituted olefins in the presence of a base (Figure 2, top). Using this strategy, various R^1 substituents were introduced to the olefin precursors via reductive amination of cinnamaldehyde derivatives that could be obtained by straightforward sequential reactions. After the acylation of the resulting secondary amine with bromoacetyl bromide, various R^2 substituents were installed via substitution reactions with pyridine derivatives. Following the intramolecular 1,3-dipolar cycloaddition reaction, the resulting nonfluorescent tricyclic adduct was transformed into Seoul-Fluor core skeleton through oxidative aromatization. Although this original synthetic route was designed for the introduction of various R^1 and R^2 substituents, its general applicability was compromised by inherently limited reactivity of intramolecular 1,3-dipolar cycloaddition in the presence of certain R^1 and R^2 substituents.

To improve the efficiency and the versatility of the synthetic strategy, we recently developed the second-generation combinatorial synthetic strategy (Figure 2, bottom).[22] Instead of using substituted olefins, we performed a coinage-metal-catalyzed intramolecular 1,3-dipolar cycloaddition of azomethine ylides with terminal alkynes, which significantly improved the synthetic generality to build the key indolizine core in the presence of various R^2 substituents. In fact, the resulting lactam-embedded indolizine is an excellent substrate for direct C−H activation.[23] Therefore, we were able to robustly introduce a wide range of R^1 substituents with a broader reaction scope at the late stages of the synthetic route through a palladium-mediated cross-coupling reaction via direct C−H activation.

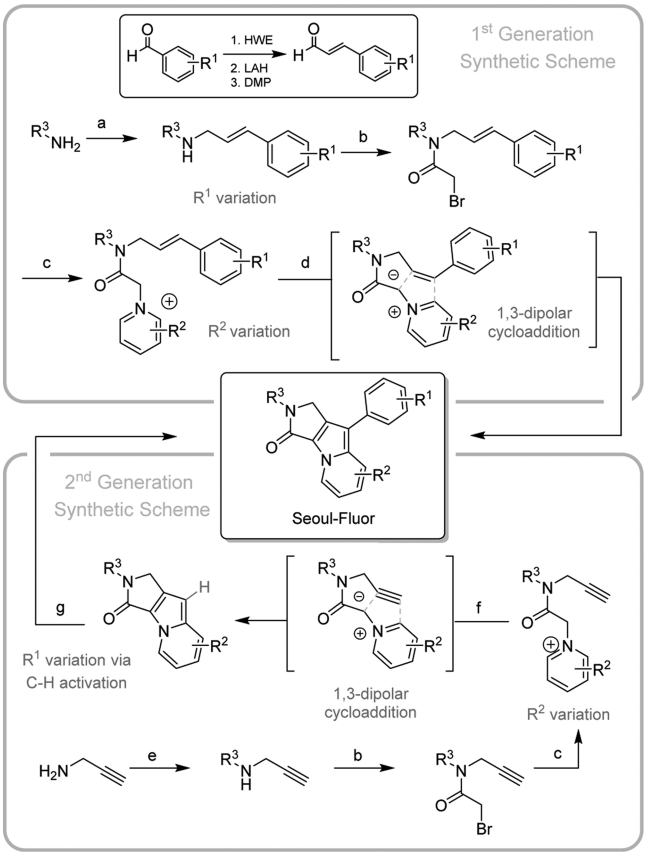

Figure 2. First- (top) and second-generation (bottom) synthetic schemes.

With this second-generation synthetic route in hand, we could efficiently construct a systematic collection of Seoul-Fluor analogues with diverse substituents.

3. STRUCTURE AND PHOTOPHYSICAL PROPERTY RELATIONSHIPS IN SEOUL-FLUOR

3.1. Emission Wavelength (λ_{em})

3.1.1. Tunability. Prior to the systematic synthesis of Seoul-Fluor analogues, we performed in silico analysis of Seoul-Fluor's electronic structures using density functional theory (DFT) calculations. On the basis of this computational study, we predicted the atomic coefficients of the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) of the indolizine-embedded core skeleton (Figure 3b). Since the electron density distribution of the HOMO is dominant at the C-9 position and that of the LUMO is dominant at the C-7 position, we hypothesized that the introduction of an electron-donating group (EDG) at the C-9 position might induce a bathochromic shift of λ_{em} via the preferential elevation of the HOMO energy level relative to that of the LUMO (Figure 3c). Under the same guiding principle, the introduction of an electron-withdrawing group (EWG) at the C-7 position might induce a bathochromic shift via more effective lowering of the LUMO energy level than that of the HOMO (Figure 3c). Guided by these molecular orbital calculations, we designed a library of Seoul-Fluor analogues having various substituents to span a broad range of electronic properties, which was numerically quantified by the Hammett substituent constant (σ_p)[24] at the *para* position of the phenyl group.

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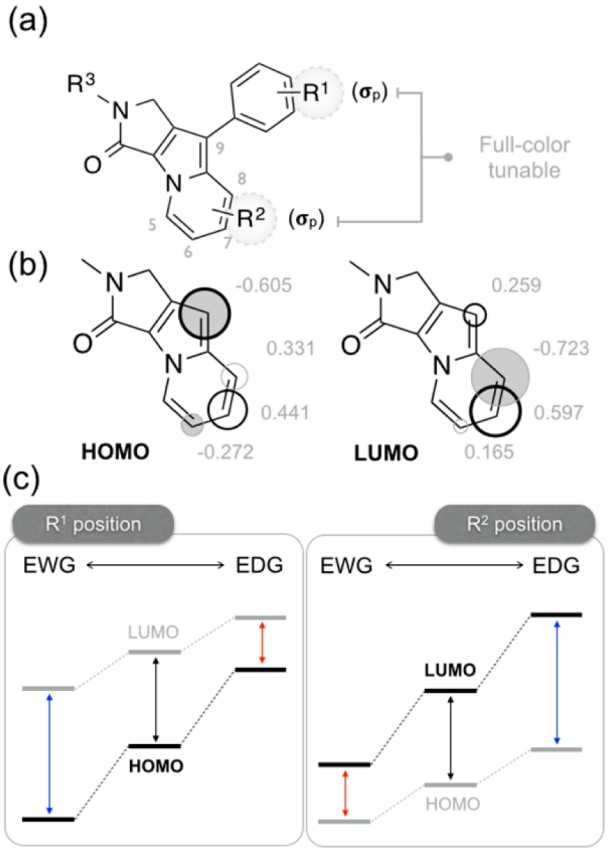
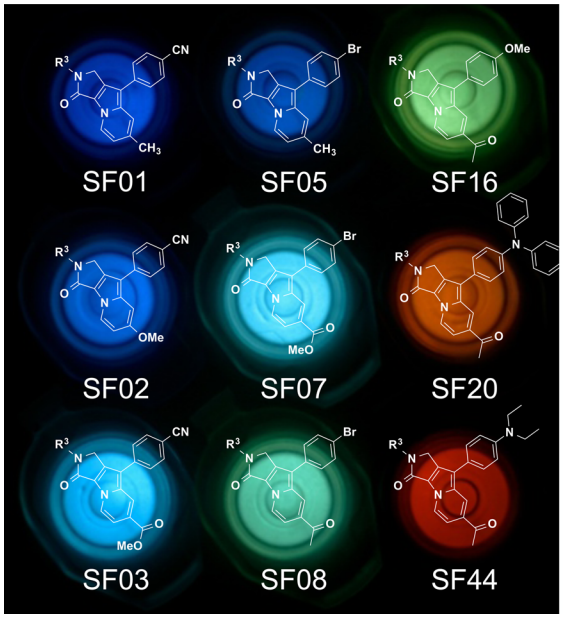


Figure 3. Design principles for full color-tunable λ_{em} in Seoul-Fluor system. (a) Schematic representation of λ_{em} tunability by controlling σ_p of the substituents at the R^1 and R^2 positions. (b) Computational calculation of HOMO and LUMO contour surfaces. (c) According to the dominant molecular orbital diagram, changes of σ_p at the R^1 and R^2 positions induce different shifts of λ_{em}.

A representative collection of Seoul-Fluor analogues, shown in Figure 4, contains diverse substituents ranging from an electron-withdrawing nitrile ($\sigma_p = 0.66$) to an electron-donating dipropylamine ($\sigma_p = -0.93$) group at the R^1 position and from a dimethylamino ($\sigma_p = -0.83$) to an acetyl ($\sigma_p = 0.50$) group at the R^2 position.[21,22] The resulting collection of fluorescent compounds revealed that λ_{em} of Seoul-Fluor can be tuned by simple changes of the substituents at the R^1 and R^2 positions covering full visible colors (445−613 nm). In addition, consistent with our hypothesis, both the decrease of electron-donating ability on R^1 and its increase on R^2 induce a bathochromic shift of the observed λ_{em} values. Although λ_{em} of other donor−π−acceptor (D−A) type fluorochromes are also tunable,[25] they are not full-color tunable, are less systematic, and require relatively big changes in chemical structure.[25] These observations confirmed that our indolizine-based Seoul-Fluor is a unique emission-tunable fluorochrome simply by changing the electronic demand of the peripheral substituents (Figure 3a).

3.1.2. Predictability. After the demonstration of the emission-tunability of Seoul-Fluor, we then questioned whether the λ_{em} of Seoul-Fluor could be predictable. The underlying complexity of photophysical properties makes an accurate theoretical prediction of the λ_{em} for any given fluorochrome difficult. But we envisioned it might be possible to extract the general trend of λ_{em} changes from the analogues of fluorescent molecules built on a single core skeleton, since these fluorescent molecules may undergo nonradiative energy loss in a similar manner. In this context, we hypothesized that calculated energy gaps of Seoul-Fluor analogues might provide a digitized trend of λ_{em} changes and with that trend we might be able to predict λ_{em} of new fluorescent molecules within a single indolizine core skeleton.

To test our hypothesis, we calculated the energy gap between the S_0 and S_1 states using time-dependent (TD) DFT method. To our surprise, the scatter plot of wavenumber ($1/\lambda_{em}$, cm^{-1}) versus the calculated S_1-S_0 energy gap (eV) shows an excellent linear correlation (Figure 5a). Extracted simple linear



	R^1	σ_p	R^2	λ_{Abs}	λ_{Em}	Δ(eV)	Φ	logε
SF01	CN	0.66	Me	344	445	3.29	0.67	4.4
SF02	CN	0.66	OMe	344	460	3.23	0.58	4.1
SF03	CN	0.66	CO_2CH_3	394	487	3.07	0.99	4.2
SF05	Br	0.23	Me	318	462	3.48	0.33	4.3
SF07	Br	0.23	CO_2CH_3	393	492	3.2	0.90	4.1
SF08	Br	0.23	$COCH_3$	405	502	2.98	0.90	4.1
SF16	OMe	-0.27	$COCH_3$	412	526	2.84	0.69	4.1
SF20	$N(Ph)_2$	-0.22	$COCH_3$	428	579	2.49	0.40	4.1
SF44	$N(Et)_2$	-0.72	$COCH_3$	445	611	2.54	0.20	4.0

Figure 4. Photographic image, chemical structures, and photophysical properties of representative Seoul-Fluor analogues. Photographic image was reproduced with permission from ref 34 (Copyright 2012 Royal Society of Chemistry).

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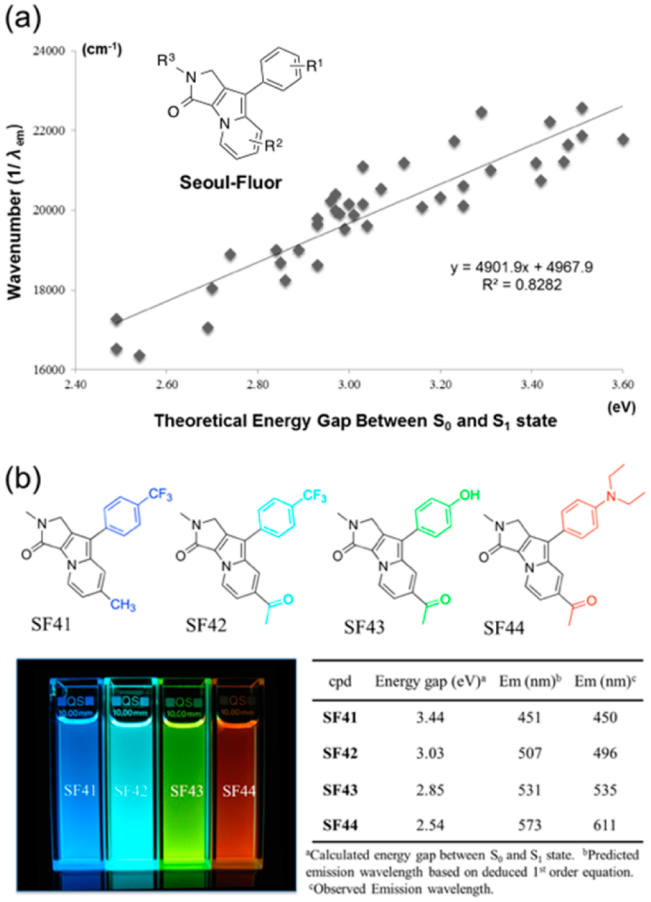

cpd	Energy gap (eV)[a]	Em (nm)[b]	Em (nm)[c]
SF41	3.44	451	450
SF42	3.03	507	496
SF43	2.85	531	535
SF44	2.54	573	611

[a]Calculated energy gap between S_0 and S_1 state. [b]Predicted emission wavelength based on deduced 1st order equation. [c]Observed Emission wavelength.

Figure 5. Predictable emission wavelength (λ_{em}). (a) Scatter plot of theoretical energy gap (S_0 and S_1 states) with observed wavenumbers ($1/\lambda_{em}$) in the Seoul-Fluor analogues. (b) Designed chemical structure of Seoul-Fluor analogues and their predicted and observed λ_{em} values. Photographic image, table of energy gaps, and predicted and observed λ_{em} of each compound were reproduced with permission from ref 21. Copyright 2011 American Chemical Society.

regression confirmed that the observed λ_{em} values of Seoul-Fluor analogues matched with their predicted values within a ±30 nm error range. The predictability of λ_{em} in the Seoul-Fluor system was further supported by the rational design of new Seoul-Fluor analogues with the desired λ_{em} values and the subsequent synthesis. For instance, we designed Seoul-Fluor analogues with substituents having a wide range of σ_p values, that is, trifluoromethyl (0.54), acetyl (0.50), methyl (−0.17), hydroxyl (−0.34), and diethylamino (−0.72) groups at the R^1 and R^2 positions. The resulting collection of "virtual structures" was subjected to in silico calculations of the energy gap between their S_0 and S_1 states. The corresponding λ_{em} values were then predicted from the empirical first-order relationship (Figure 5a). Remarkably, the observed λ_{em} values of the designed Seoul-Fluor analogues matched quite well with desired λ_{em} window, ranging from blue to red color, with only a 14 nm average deviation (Figure 5b). In conclusion, systematic study of single fluorescent core skeleton allowed the extraction of digitized correlation between computational calculation result and experimental values, facilitating reliable predictability for λ_{em} in Seoul-Fluor system.

3.2. Emission Quantum Yield (Φ_F)

Φ_F is one of the most important photophysical properties for determining the brightness of fluorescent molecules. Understanding the fundamental features of Φ_F, therefore, opens a new route for the rational design principle of versatile fluorescent bioprobes via the modulation of their brightness. For instance, understanding SPPR about quantum yields in fluorescein and BODIPY fluorophore paves the way to develop novel fluorescent sensors for monitoring diverse biological events.[8,26−29] After the successful SPPR understanding of λ_{em}, we pursued a systematic study about Φ_F in Seoul-Fluor system. First, we examined the positional effects of the substituents on Φ_F (Figure 6a). In the case of the R^1 group, regioisomers with either bromo or methoxy substituents do not show any significant differences in the Φ_F values (Figure 6b, left). However, regioisomers of the R^2 groups (bromo or methoxy) on indolizine moiety showed drastic differences in Φ_F values (Figure 6b, right). More importantly, we observed further intriguing changes in the Φ_F parameter upon perturbation of

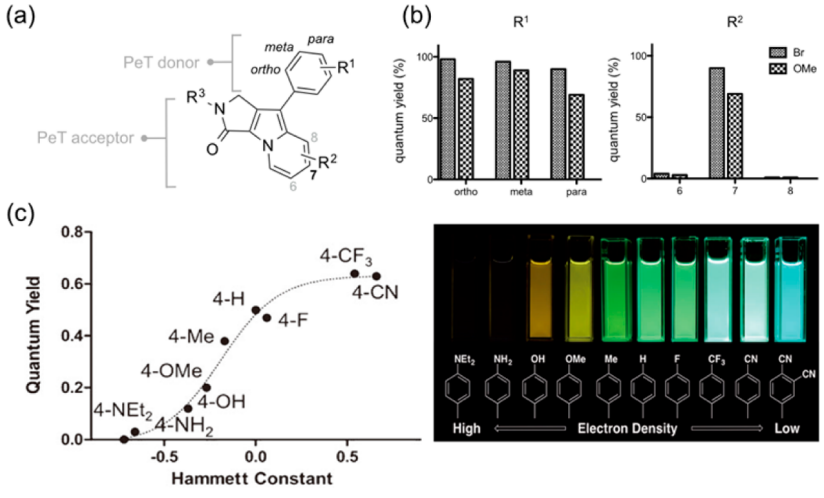

Figure 6. Systematic perturbation of quantum yield (Φ_F) in the Seoul-Fluor system. (a) The R^1-containing aryl moiety is a PeT donor, and the R^2-containing indolizine core skeleton is a PeT acceptor. (b) Positional effects on Φ_F. (c) Scatter plot and photographic illustration of the relationship between electron density in the PeT donor (quantified by Hammett constant, σ_p) and Φ_F. Reproduced with permission from ref 22 (Copyright 2014 Wiley VCH).

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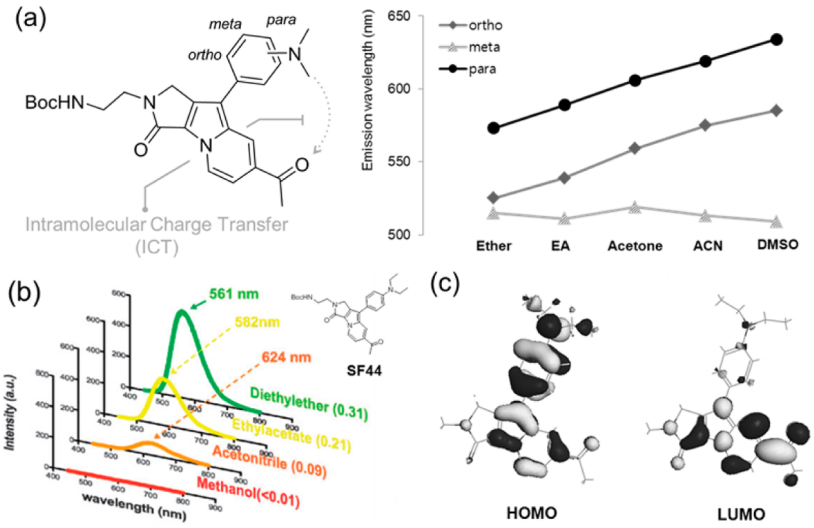

Figure 7. Intramolecular charge transfer (ICT) process in the Seoul-Fluor system. (a) Schematic representation of the ICT process. There is a preferred direction of ICT flow in the Seoul-Fluor system (left). Scatter plot of λ_{em} in various solvents with different polarities (right). (b) Positive solvatochromism of SF44 along with a decrease in Φ_F. Image is reproduced with permission from ref 39 (Copyright 2012 Royal Society of Chemistry). (c) Schematic representation of the contour surface of the HOMO and the LUMO of SF44. Image was reproduced with permission from ref 21. Copyright 2011 American Chemical Society.

the electron richness at the *para* position of the R^1 substituents,[22] which is similar to the PeT phenomenon[30] of other fluorochromes.[26−28] Since photoinduced electron transfer (PeT)-based fluorescence quenching occurs through electron transfer from a PeT donor to its acceptor, we considered the phenyl moiety of Seoul-Fluor as a PeT donor and the indolizine core as a PeT acceptor. The scatter plot of Φ_F against σ_p of the R^1 substituents verifies the PeT process in the Seoul-Fluor system: PeT-based fluorescence quenching was observed when the electron richness was increased at the R^1 substituents, quantified by the calculated HOMO energy level (E_h) of the PeT donor. For instance, changes of R^1 substituents from 4-NEt_2 (σ_p = −0.72, E_h = −0.160) to 4-OH (σ_p = −0.37, E_h = −0.200), 4-F (σ_p = 0.06, E_h = −0.225), and 3,4-$(CN)_2$ (σ_p = N/A, E_h = −0.262) cause the increase in Φ_F from 0.00 to 0.12, 0.47, and 0.72, respectively (Figure 6c). We further validated the PeT process in Seoul-Fluor by direct detection of single-electron species, transiently generated upon electron transfer from the PeT donor to the acceptor, using electron paramagnetic resonance (EPR) spectroscopy.[22] Therefore, along with λ_{em}, we are now able to predict and modulate the brightness of the fluorescence by controlling the Φ_F values of Seoul-Fluor-based fluorescent molecules via simple guidance by σ_p or E_h of the substituents at the R^1 position.

3.3. Intramolecular Charge Transfer (ICT)

One of the key photophysical properties that we are looking for is the environment-dependent changes in λ_{em} or fluorescence brightness. During our endeavors to explore these fluorescent properties, we identified that Seoul-Fluor analogues containing an amine moiety at the R^1 position and an acetyl group at the R^2 position exhibit interesting solvent-dependent fluorescent properties, that is, solvatochromism (Figure 7a, left).[31] For instance, the SF44, consisted of *p*-diethylamino and acetyl moieties at the R^1 and R^2 position, respectively, has positive solvatochromism along with fluorogenic nature in response to the external environmental change (Figure 7b). Considering the D−A molecular architecture of SF44, we postulated that this positive solvatochromism might be caused by the push−pull interactions between the electron-donating amine moiety and the electron-withdrawing acetyl moiety, which results in intramolecular charge transfer (ICT) flow in the excited state.[30] Therefore, dipole−dipole interactions between the indolizine ring and the solvent in the excited state might cause a positive solvatochromism of λ_{em}. Solvatochromism of each regioisomers of the amine derivative at the R^1 position confirmed our hypothesis; *ortho* and *para* isomers, which can effectively donate electrons toward the acetyl group by the resonance effect, show more enhanced solvatochromism than the *meta* isomer (Figure 7a, right). The computational calculation of molecular orbital contour surface further confirm the push−pull interaction and their direction in the indolizine core of Seoul-Fluor;[21] for instance, D−A type Seoul-Fluor analogues, SF44 and SF24 having EDG on R^1 and EWG on R^2, have drastically different electronic distribution between HOMO and LUMO (Figure 7c) than that of SF01 and SF42, which have EDG on R^2 and EWG on R^1 position or have no EDG moiety.[21] Therefore, this ICT process seems to prefer to flow from the R^1 to the R^2 group in Seoul-Fluor system.

SF44 shows not only the positive solvatochromism for λ_{em}, but also decrease in the Φ_F upon increase of solvent polarity. Possible explanation is that fluorescence quenching occurs via intermolecular hydrogen bonding of the carbonyl oxygen at the C-7 position with polar solvents (alcohol or water)[32] and this inverse correlation of Φ_F with solvent polarity allows us to design novel fluorescent bioprobes that can respond to the environmental changes in a cellular system or in a whole organism.

3.4. Orthogonal Chemical Handles

For the covalent labeling of various biomolecules such as proteins of interest, antibodies, or nucleic acids with fluorescent dyes,[33] it is crucial to have an appropriate chemical handle, orthogonal to the photophysical properties. In the Seoul-Fluor system, the R^1 and R^2 substituents attached to the indolizine core skeleton can affect photophysical properties such as λ_{em} and Φ_F. On the other hand, the R^3 group of the β-lactam ring is independent to the fluorescence properties of Seoul-Fluor and,

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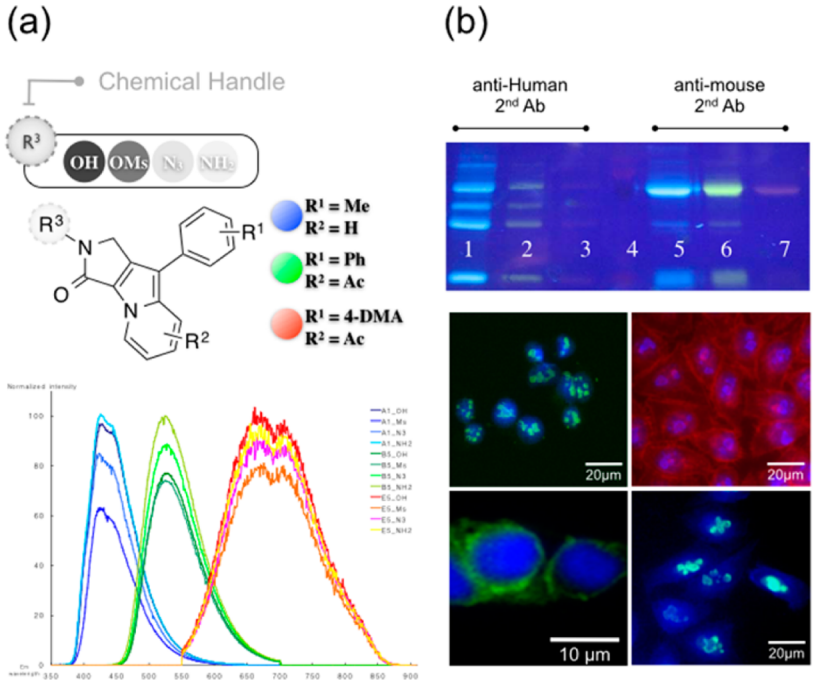

Figure 8. (a) Versatile chemical handle in Seoul-Fluor system, orthogonal to its photophysical properties. (b) Immunofluorescence image with a specific antibody labeled with Seoul-Fluor analogues. Images were reproduced with permission from ref 20. Copyright 2008 American Chemical Society.

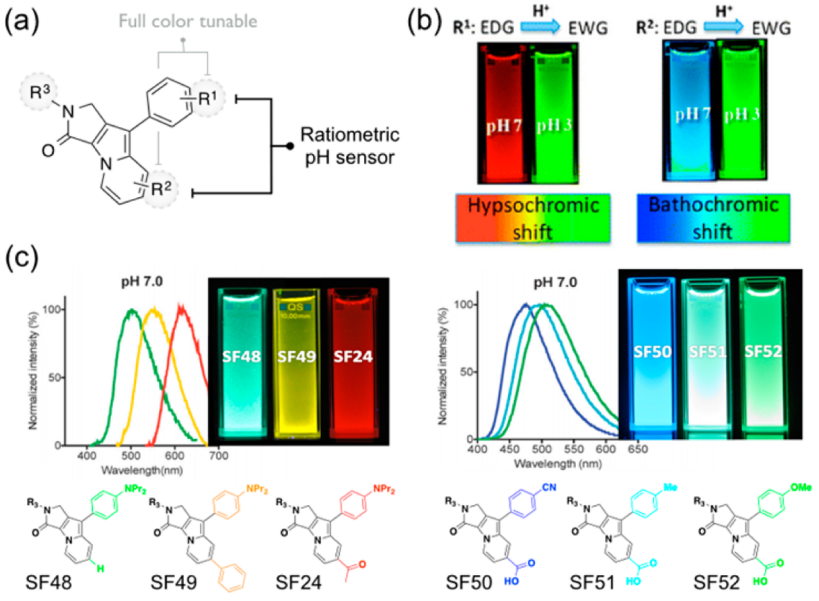

Figure 9. (a) Full color-tunable and predictable λ_{em} in the Seoul-Fluor system allows the rational development of a ratiometric pH sensor. (b) Protonation of pH-responsive elements at the R^1 and R^2 positions results in a hypsochromic and bathochromic shift of λ_{em}, respectively. (c) Normalized emission spectra and photographic images of Seoul-Fluor-based fluorescent pH sensors. Images were reproduced with permission from ref 34 (Copyright 2011 Royal Society of Chemistry).

therefore, the R^3 position can serve as a perfect functional handle to introduce various chemical linkers for conjugation with small molecules or biomacromolecules, without changing the photophysical properties of Seoul-Fluors. As shown in Figure 8a, the λ_{em} of Seoul-Fluor analogues tolerates the introduction of various functional groups at the R^3 position.[20] With this chemical handle, we successfully demonstrated the specific labeling of antihuman and antimouse secondary antibodies with Seoul-Fluor analogues and, subsequently, bioimaging with the resulting fluorescent antibody for immunohistochemical staining of biomarkers (Figure 8b).

4. APPLICATION OF SEOUL-FLUOR AS BIOPROBES

We envisioned that our SPPR understanding of Seoul-Fluor system should contribute to the rational design of novel fluorescent bioprobes. In the following section, we will describe how we applied each of the following photophysical properties of Seoul-Fluor for the development of fluorescent bioprobes/

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sensors: (1) tunable λ_{em} for ratiometric pH sensors, (2) controllable Φ_F for specific bioprobes toward the protein tyrosine phosphatase (PTP) and reactive oxygen species (ROS), and (3) ICT for fluorescent bioprobes toward lipid droplet (LD).

4.1. pH Sensor: Seoul-Fluor as a Ratiometric Sensor

As a proof-of-concept study, we first focused on the tunability of λ_{em}, one of the most unique photophysical properties of Seoul-Fluor. As described above, the λ_{em} of Seoul-Fluor can be tuned by the electronic nature of the substituents at the R^1 and R^2 positions. Using this tunability, we could construct a ratiometric fluorescent pH sensors by introducing pH-responsive components on Seoul-Fluor.[34] Changing the pH can impact completely different photophysical changes depending on what and where the pH-responsive functional groups are on Seoul-Fluor. Accordingly, we designed Seoul-Fluor analogues that can undergo either hypsochromic or bathochromic shifts of λ_{em} according to the pH-responsive groups at the R^1 or R^2 positions (Figure 9a). For instance, the introduction of an amine moiety at the R^1 position causes a hypsochromic shift of λ_{em} upon lowering of the solution pH. Protonation of the tertiary amine group alters the electronic character of the R^1 substituent from electron-rich to an electron-poor moiety (Figure 9b). In contrast, a carboxylic acid moiety at the R^2 position of Seoul-Fluor results in a bathochromic shift of λ_{em} upon protonation of the carboxylate group. This is due to the increase in the electron-withdrawing strength at the R^2 position.

The emission-tunable nature of Seoul-Fluor allows for a pH-dependent emission shift as well as fine control over λ_{em} through the positioning of various substituents at the R^1 or R^2 position (Figure 9c). For instance, various substituents, ranging from hydrogen to phenyl and acetyl at the R^2 position or from cyano to methyl and methoxy at the R^1 position, elicited drastic λ_{em} changes at neutral pH 7.0 with identical pH-response dipropylamino group at the R^1 or carboxylic acid moiety at the R^2 position, respectively. Obviously, these compounds showed the same pattern of pH-dependent λ_{em} changes depending on which pH-responsive group is introduced to the Seoul-Fluor system. In addition, the dynamic range of pH can be tuned via the introduction of pH-responsive functional groups with different pK_a values. Therefore, these observations confirmed that the Seoul-Fluor system can serve as a platform for fluorescent ratiometric pH sensors and can be tailored through the rational design covering full-color emission wavelength with a desired working pH range.

4.2. Seoul-Fluor-Based BioProbe (*Sf*BP): Seoul-Fluor as a Unique Turn-On/Off Fluorescent Sensor

Our in-depth SPPR study of Seoul-Fluor analogues, especially for Φ_F, allowed the rational design of fluorescent on/off-switchable bioprobes for specific molecular events (Figure 10a). First of all, we reported *Sf*BP for phosphotyrosine phosphatases (PTPs) with 93-fold change in brightness ($\varepsilon\Phi_F$).[35] The introduction of the *O*-phosphate moiety at the R^1 position to mimic the phosphotyrosine moiety allowed *Sf*BP to function as a substrate for PTPs. The cleavage of the P−O bond by specific PTPase and the subsequent phenoxide formation under the basic conditions leads to the PeT-type quenching of the fluorescence signal. In fact, the indolizine moiety has been known as a pharmacophore and is frequently observed in various bioactive small molecules.[36] Thus, we envisioned the specific interaction of *Sf*BP with certain PTPs. Through in vitro screening against 65 different class I human

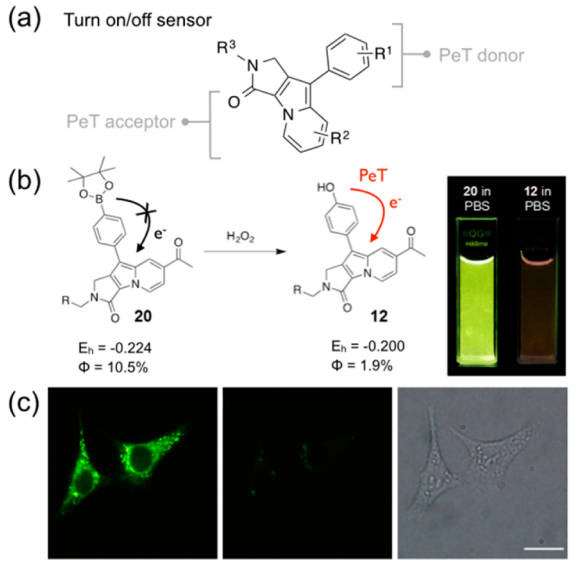

Figure 10. PeT process in the Seoul-Fluor system. (a) PeT process allows the rational development of fluorescent turn-on/off sensors. (b) Chemical structure changes from **20** to **12** upon exposure to H_2O_2 as a reactive oxygen species (ROS), which results in fluorescence quenching due to a PeT process in the Seoul-Fluor system (Left). Photographic images of **20** and **12** (right). (c) Fluorescence image (left, middle) and bright field image (right) of HeLa cells pretreated with **20**, before (left) and after (middle) addition of 1% H_2O_2. Reproduced with permission from ref 22 (Copyright 2014 Wiley VCH).

PTPs (30 classical PTPs and 35 dual-specific PTPs), we found *Sf*BP showed an excellent substrate specificity for vaccinia H1-related (VHR) phosphatase (aka DUSP-3) with up to 93-fold change in the brightness ($\varepsilon\Phi_F$) from 8.3 to 778.4. This confirmed the higher potential of the pharmacophore-embedded Seoul-Fluor system for novel fluorescent biosensors.

Furthermore, we developed a new turn-off H_2O_2 sensor on the basis of our understanding of the PeT process in Seoul-Fluor systems (Figure 10b).[22] Guided by our calculations of the E_h of the PeT donor, phenylboronic acid pinacol ester (**20**) was introduced at the R^1 position in Seoul-Fluor as a recognition motif for reactive oxygen species (ROS). Response to cellular ROS, boronate ester **20** (E_h = −0.224) is spontaneously transformed into the alcohol **12** (E_h = −0.200), which causes a drastic decrease in Φ_F. This result suggests that we can qualitatively monitor the cellular ROS levels by using **20** in live cells (Figure 10c). Therefore, our fundamental understanding of SPPR, especially for Φ_F, can build the foundation for a new, efficient method to develop novel fluorogenic biosensors.

4.3. Lipid Droplet (LD) Bioprobe: An Environment-Sensitive Fluorescent Bioprobe

An LD is a distinctively hydrophobic organelle inside cells, which is surrounded by a phospholipid monolayer and sequesters cellular fat and neutral lipids from the cytosol.[37] Traditionally, LDs were recognized as a simple warehouse for lipid storage, but recent studies support the crucial role of LD as a dynamic subcellular organelle for regulating lipid metabolism, which is closely related to various metabolic diseases. Therefore, continuous and specific monitoring of cellular LDs is receiving increasing attention in biological research. Envisioning a fluorescence turn-on sensor to monitor cellular LDs, we screened Seoul-Fluor analogues against

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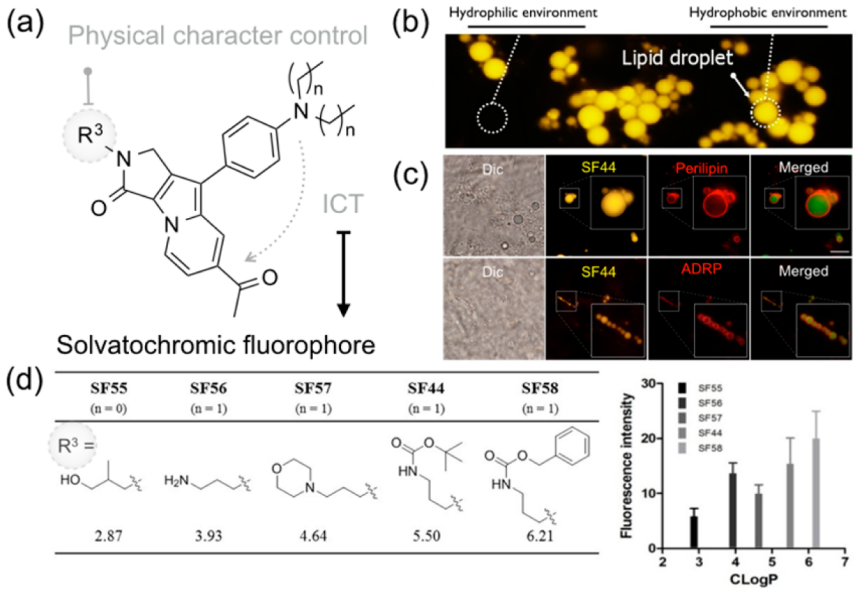

Figure 11. (a) ICT process in Seoul-Fluor system allows the development of an environment-sensitive fluorescent bioprobe. (b) Hydrophobic lipid droplets (LDs) are selectively stained with SF44, fluorogenic LD bioprobe, without additional washing or fixation steps. (c) Immunofluorescent images of perilipin and adipose differentiation-related protein (ADRP) of differentiated 3T3-L1 adipocyte costained with SF44. The scale bar represents 20 μm. (d) Chemical structures and cLogP values of SF44 analogues (left). Controlling the physical properties of bioprobes results in the rational optimization of an LD fluorescent bioprobe (right). Reproduced with permission from refs 38 and 43 (Copyright 2013 Royal Society of Chemistry).

adipocyte cells and found that SF44 selectively stains the LDs in a fluorogenic manner. One possible mechanism here is that hydrophobic nature of LDs makes SF44 selectively turn on its fluorescence via the ICT process (Figure 7b), but not in the other hydrophilic cellular compartments. (Figure 11b).[38] Another possibility is that the viscous LD environment reduces nonradiative energy decay via preventing rotation of phenyl ring, PET process or twisted ICT (TICT) process in SF44, as shown in BODIPY,[39,40] fluorescein,[27] or other D−A type fluorescent molecules.[41,42] Overall, simple addition of SF44 allows excellent signal-to-noise ratio (Figure 11b) and a selective staining pattern for cellular LDs, confirmed with immunofluorescent staining of LD markers (Figure 11c), within a few minutes under physiological conditions, without additional washing, fixation and genetic modification.

To optimize the fluorescent LD bioprobe, we designed four different SF44 analogues with different cLogP values (Figure 11d).[43] All analogues have similar photophysical properties including λ_{em}, Φ_F, and solvatochromism. On the other hand, these SF44 analogues exhibited significantly different staining patterns for cellular LDs in mammalian cells. A linear correlation was observed between their cLogP values and the fluorescent intensity of the cellular LDs (Figure 11d). In our cellular imaging experiments, we observed that the presence of lipophilic substituents at the R^3 position with high cLogP values induces a higher accumulation of SF44 analogues on the hydrophobic LDs inside the cells, which resulted in the enhancement of the fluorescence signal for specific LD monitoring.

In this study, we confirmed that understanding of SPPR about ICT in the Seoul-Fluor system provides an additional principle for the development of fluorescent turn-on/off sensors. Furthermore, the R^3 position can serve not only as a versatile functional handle for specific labeling of biomolecules, but also for the modification of the physical and chemical properties of Seoul-Fluor, without perturbation of its inherent photophysical properties.

5. CONCLUDING REMARKS AND FUTURE PERSPECTIVE

Owing to its unique advantage, fluorescence phenomenon has been extensively studied in various strategies, and the scientific community has been working on the discovery of novel fluorescent materials and their applications in various fields. Since their molecular frameworks govern the fluorescence properties of organic fluorophores, the existing organic fluorophores have been repeatedly used for the development of fluorescence biosensors without a detailed understanding of SPPR. In addition, due to the complicated molecular mechanism of fluorescence, it is not easy to theoretically predict the photophysical properties of fluorochromes using in silico analysis in general.

This Account has focused on the discovery and systematic study of the Seoul-Fluor system, an attractive fluorescent core skeleton with a readily adaptable molecular design. The structural and photophysical characteristics of Seoul-Fluor can be summarized as follows: (1) it is synthetically versatile; (2) it has a tunable and predictable λ_{em}; (3) fluorescence Φ_F can be controlled via the PeT process; (4) it undergoes a photo-induced ICT process along with fluorogenicity; and (5) its versatile chemical handle can be modified without alteration of its photophysical properties. The fundamental understanding of SPPR in the Seoul-Fluor system allowed the development of diverse bioprobes via rational design to afford the desired photophysical properties. Based on our understanding of SPPR of λ_{em}, Φ_F, PeT, and ICT processes, we successfully designed and developed a ratiometric pH sensor, a specific PTP sensor, an ROS sensor, and an LD bioprobe. In addition, we convincingly demonstrated the prediction of photophysical properties of Seoul-Fluor, that is, λ_{em} and Φ_F, through the

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construction of a systematic set of analogues and the subsequent systematic pattern recognition of their photophysical properties. Therefore, we envision that our systematic study on Seoul-Fluor, with the development of novel bioprobes, can pave a new road for the rational design of novel fluorescent sensors and their applications in various biological fields.

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