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# MODULATION OF FRET THROUGH THE SPECTRAL-OVERLAP STRATEGY

HAIBO YU and YI XIAO*
*State Key Laboratory of Fine Chemicals*
*Dalian University of Technology*
*Dalian 116024, P. R. China*
**xiaoyi@dlut.edu.cn*

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Fluorescence resonance energy transfer (FRET) is an important photophysical mechanism which finds many applications in biophotonics, particularly in biological sensing and imaging. It is well known, there are two major factors that determine the efficiency of FRET. One is the distance between the donor and the acceptor, and the other is the overlap of the donor's emission and the acceptor's absorption spectra. However, while the distance-modulation of FRET is very popular, the spectral-overlap-modulation draws much less attention.

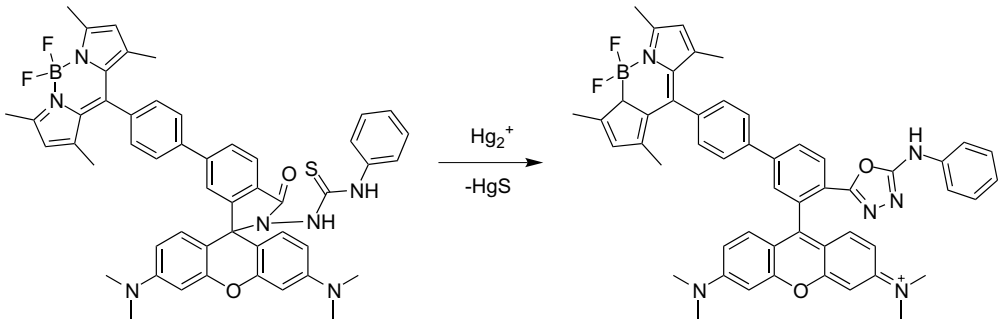
In this talk, I would like to illustrate the importance of the strategy of spectral-overlap-modulation. The presentation will include our works on the construction of highly efficient FRET systems featuring the short and rigid linker between the donor and acceptor moieties, and several typical examples of spectral-overlap-modulation strategy applied in the development of ratiometric sensors.

*Keywords*: FRET; spectral overlap; ratiometric sensors.

## 1. Introduction

Forster Resonance Energy Transfer (FRET) is one of the major mechanisms[1] widely used in biological labeling,[2] fluorescence probes,[3] and molecular beacon in biology,[4] due to its excellent characters such as a single excitation wavelength, a large pseudo-stoke shift and proportional changes of two emission bands. The proportional changes of two emission bands make FRET more widely used in ratiometric approaches. It is well known, there are two major factors that determine the efficiency of FRET. One is the distance between the donor and the acceptor, and the other is the overlap of the donor's emission and the acceptor's absorption spectra. To our knowledge, the distance-modulation of FRET is very popular in biology to study the interactions between biomarcomolecules or their changes in structure and conformation, but we also notice that spectral-overlap-modulation draws much less attention. However, the works of ourselves' and some others have proved that the approach called "spectral overlap-modulated FRET strategy" to be an efficient method to develop ratiometric sensors for small molecular analytes.[5–7] For the sake to popularize this strategy, in this talk, we would like to illustrate two of our efforts toward FRET-based ratiometric sensors.

*Corresponding author.



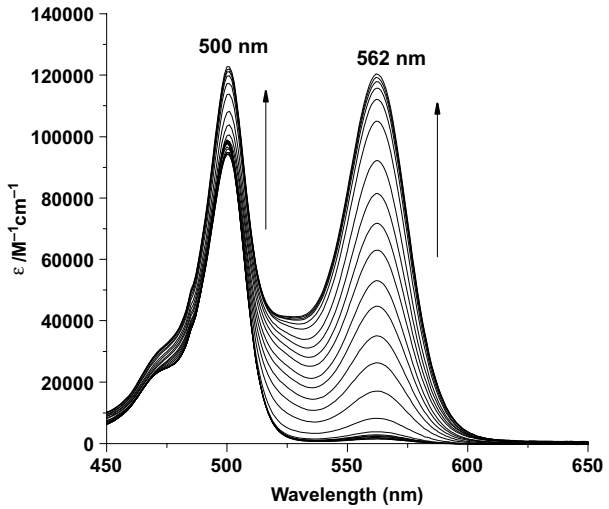

Scheme 1. $Hg^{2+}$-induced ring-opening and cyclization.[6]

## 2. Switch ON FRET: A Probe for Imaging $Hg^{2+}$ in Living Cells[6]

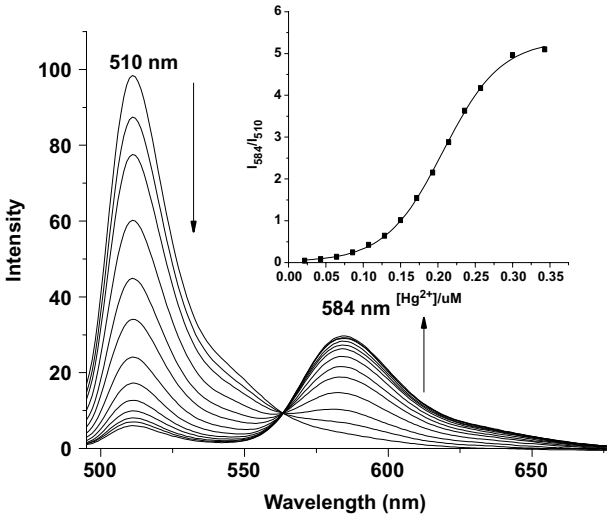
In our design, a short and rigid biphenyl spacer was adopted purposely to satisfy the requirements for constantly high efficiency energy transfer. We prefer **BODIPY** rather than many other fluorophores in the construction of a platform for ratiometric sensors. In our understanding of spectral overlap-modulated FRET strategy, while the rhodamine unit is sensitive to cations, the donor fluorophore should have strong and stable fluorescence insensitive to environmental factors such as polarity and pH, since the donor would simultaneously act as the internal standard for the ratiometric detection. **BODIPY** fulfills this requirement, and should be superior to other donors.

The mechanism of the reaction between **BRP-1** and $Hg^{2+}$ was shown in Scheme 1. The thiosemicarbazides section of **BRP-1** was transformed into a 1,3,4-oxadiazide promoted by $Hg^{2+}$, resulting in the irreversible ring-opening reaction of rhodamine section. The absorption and fluorescence titration of $Hg^{2+}$ was conducted using 0.3 $\mu$M **BRP-1** in ethanol-water (80/20 v/v) at pH 7.2. In the absence of $Hg^{2+}$ the absorption and emission spectra of **BRP-1** only displayed the features of **BODIPY**. Apparently, rhodamine spirolactam did not absorb in visible range and could not accept the energy from **BODIPY**. Upon addition of $Hg^{2+}$, the new absorption band peaked at 562 nm appeared and increased, which indicated rhodamine chromophore was generated. Consequently, the large spectra overlap between **BODIPY**'s emission and rhodamine's absorption switched FRET ON.

As shown in Fig. 1, with increasing $Hg^{2+}$ concentration, **BODIPY**'s emission peaked at 510 nm decreased, and rhodamine emission peaked at 584 nm appeared and gradually increased in




(a)




(b)

Fig. 1. Changes of absorption spectra (a) and emission spectra (b) of **BRP-1** (0.3 $\mu$M) in $C_2H_5OH/H_2O$ (80:20) at pH 7.2 (0.01 M HEPES) upon gradual addition of $Hg^{2+}$ from 0.03−0.35 $\mu$M. Inset: the ratio intensity at 584 and 510 nm upon gradual addition of $Hg^{2+}$.[6]

intensity. Upon addition of $Hg^{2+}$ from 0.03−0.15 $\mu$M, the emission intensity at 510 and 584 nm was linearly proportional to the amount of $Hg^{2+}$, respectively. However, the ratio of emission intensity at 584 and 510 nm was curvilinear function with the $Hg^{2+}$ concentration over the range of 0.03 to 0.3 $\mu$M (6−60 ppb). The response to $Hg^{2+}$ was very fast and all spectra were recorded within 5 min after addition of $Hg^{2+}$.

During the experiment of confocal imaging, Ar laser provided the single excitation wavelength (488 nm) which was suitable to the absorption of **BODIPY** fluorophore. Then, in this experiment fluorescence images were obtained at $515 \pm 15$ nm (green channel) and $590 \pm 25$ nm (red channel), respectively. MCF-7 cells incubated with **BRP-1** (2.5 $\mu$M) for 15 min at room temperature showed a clear green intracellular fluorescence, shown in Fig. 2(a). In green channel a strong green fluorescence could be seen. Meanwhile, there was little red fluorescence in the red channel, which indicated that **BRP-1** mainly exists in the form of spirolactam. From Fig. 2(a) ratio imaging, there were not significant ratio changes of $I_{590 \pm 25}/I_{515 \pm 15}$. When cells stained with **BRP-1** were incubated with $HgCl_2$ (2.5 $\mu$M) for 5 min, the color of MCF-7 cell showed a significant change from green to yellow−orange. In the double-channel imaging, a partial quenching of the green fluorescence intensity and a partial increase in the red fluorescence intensity was observed (Fig. 2(b)). Because only a partial $Hg^{2+}$ reacted to **BRP-1** within 5 min, the pseudo-color of Fig. 2(b) ratio imaging could show real-time responses between $Hg^{2+}$ and **BRP-1** in different cell. When MCF-7 cells stained with **BRP-1** were incubated with $HgCl_2$ for 10 min, the green fluorescence intensity decreased significantly and the red fluorescence intensity remarkably

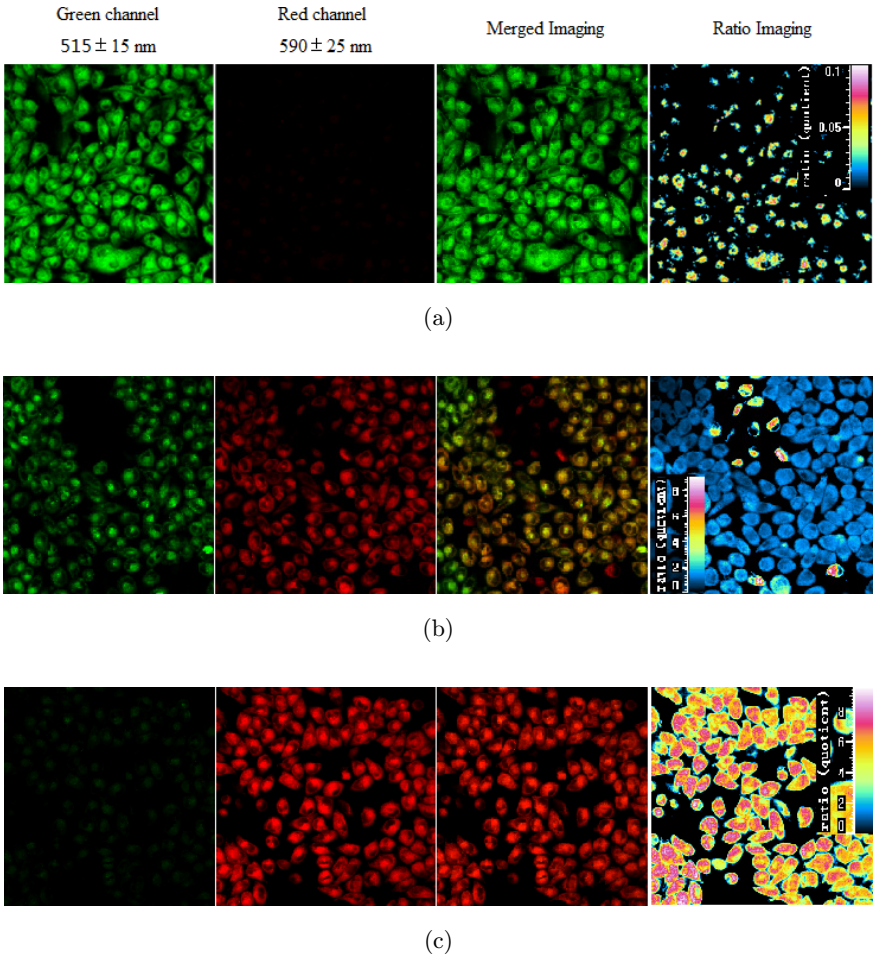



Fig. 2. Confocal fluorescence imaging of $Hg^{2+}$ in MCF-7 cell with **BRP-1** (2.5 $\mu$M) for 15 min. (a) Cells without $Hg^{2+}$; (b) Cells incubated with $HgCl_2$ (2.5 $\mu$M) for 5 min; (c) Cells incubated with $HgCl_2$ (2.5 $\mu$M) for 10 min.[6]

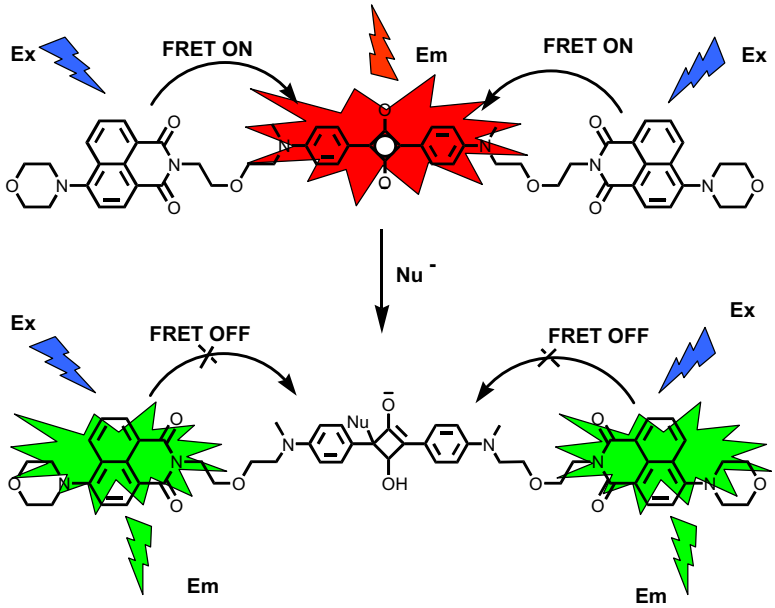
increased (Fig. 2(c)). The pseudo-color of Fig. 2(c) ratio imaging showed different distribution of $Hg^{2+}$ in MCF-7 cell. By ratioing the intensities in the images, it is possible to construct a map showing the local ion concentrations throughout the field of view.

## 3. Switch OFF FRET: A Probe for Nucleophiles[7]

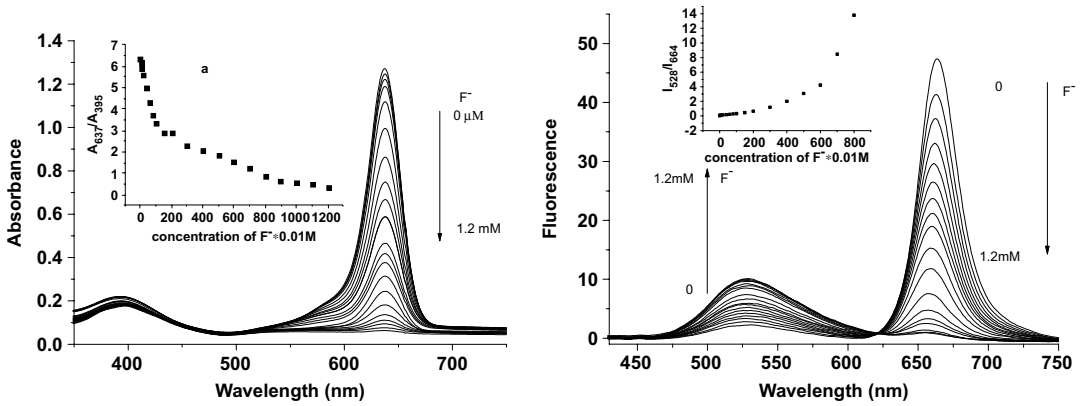
Squaraine is an important fluorescence "turn-off" type chemodoismeter for the recognition of nucleophiles. Due to significant electron-deficiency, squaraine can be readily attacked by nucleophiles such as $F^-$, $CN^-$ and thiol compounds, to generate colorless and non-fluorescent products. Unfortunately, "turn-off" sensors are not very desirable for biological sensing or imaging, for the lower accuracy in quantative detection, compared with ratiometric sensors. Thus it is interesting to transform the "turn-off" squaraine chemodoismeter into a ratiometric one. As illustrated in Scheme 2, we suggested a new design concept to transform the "turn-off" chemodoismeters into ratiometric ones. The nucleophilic analytes attacked on electron-deficient squaraine center and thus FRET process was switched off due to complete cancellation of the spectra overlap. This resulted in the recovering of donor's emission and the quench of the acceptor's emission, simultaneously, which provided the basis of ratiometric detection. For the sake to reveal the feasibility of the above concept, we constructed FRET systems SN-2 composed of a squaraine and two naphthalimide units. Noticeably, the chemically reactive recognition of a nucleophilic analyte is a FRET "switching-off" process, which is a new mode different from the known FRET "switching-on" sensors based on the previous platform of rhodamine spirolactam.

The fluoride concentration titration was conducted using 10 $\mu$M solution of SN-2 in acetonitrile. The responses of SN-2 toward fluoride in its absorption and emission spectra were shown in Fig. 2. With fluoride addition, the absorption band of naphthalimide at 402 nm was not changed, but the absorption band of squaraine section at 636 nm decreased. When the concentration of fluoride ion was up to 5 mM, the squaraine absorption band disappeared. The emission band of squaraine section at 636 nm decreased, and the emission band of naphthalimide at 525 nm increased. So a ratiometric sensor based on FRET "switching-off" was realized. So the proportion of two emission bands could be obtained. The ratio plot of absorption and emission with the concentration of $F^-$ was shown in Fig. 3.

The sensor SN-2 was also chosen to detect cyanide. The changes of spectra were shown in Fig. 4. In the mixture solvent of water and acetonitrile (1/10, V/V), SN-2 showed three obvious absorption peaks at 400, 575, and 641 nm. The short



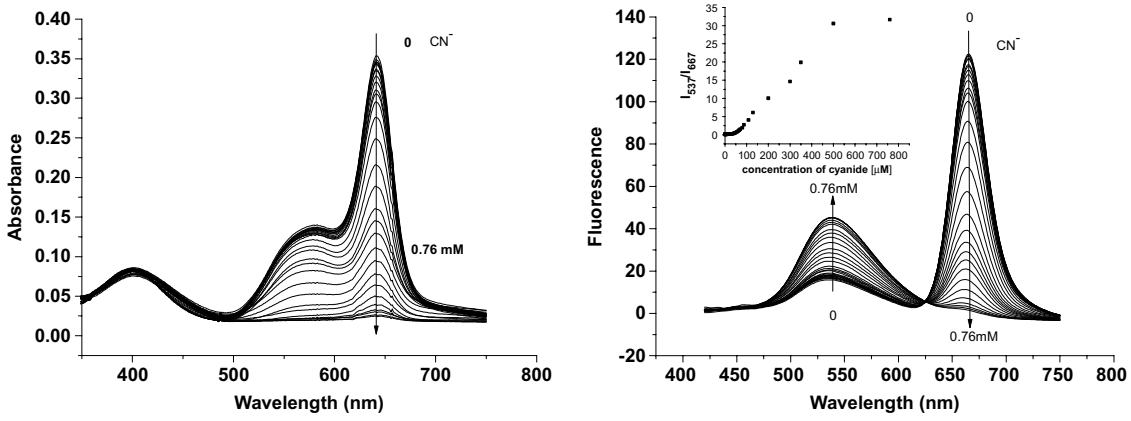

Scheme 2. "Switching-off" FRET: Design concept of squaraine-based ratiometric sensors for nucleophilic analytes.[7]




(a) (b)

Fig. 3. The absorption (a) and fluorescence (b) changes of 10 $\mu$M compound SN-2 in acetonitrile with the increasing concentration (2 $\mu$m−1.2 mM) of fluoride anion. Inset was the ratio plot of absorption (a) and emission (b) with the addition of $F^-$.[7]

wavelength band belonged to naphthalimide and the longer two bands ascribed to squaraine units, respectively. With the addition of cyanide anion, the absorption bands at 575 and 641 nm decreased obviously, when the concentration of cyanide was up to 0.76 mM, the two peaks totally disappeared, but the absorption peaks at 400 nm had no changes. This result indicated that cyanide anion attacked the electron deficient center of squaraine to break the conjugation system, without affecting naphthalimide. The fluorescence properties were shown in Fig. 4(b), excited with the absorption of naphthalimide (400 nm), the SN-2 had two emission bands peaked at 537 and 667 nm, which could be ascribed to naphthalimide and squaraine respectively. Obviously, the emission at 667 nm resulted from the FRET process from naphthalimide to squaraine. However, there remained a weak emission of naphthalimide, which indicated that energy transfer was incomplete. It might be explained by that in highly polar solvent of water−acetonitrile mixtures, the efficiency of energy transfer was lower to some extent. With the addition of the cyanide, the emission at 667 nm decreased sharply followed by the increases of the emission of naphthalimide at 537 nm, this phenomenon indicated that the chemical reaction between the cyanide and the squaraine blocked the FRET process between the two dyes, the fluorescence of naphthalimide recovered. The ratiometric changes of the two fluorescence peaks ($I_{537}/I_{667}$) offered well linear function to the concentration of cyanide between 0.5 and 500 mM as shown in Fig. 4(b) (inset), from which it was easy to get the quantitative information.




(a) (b)

Fig. 4. Absorption spectra (a) and emission spectra (b) of (5 $\mu$M) SN-2 with addition of $CN^-$ (0.4 $\mu$M∼0.76 mM) in the mixture solvent of water and acetonitrile (1/10, V/V). Inset was the ratio plot of emission at 537 and 667 nm.[7]

## 4. Conclusion and Outlook

With the above two examples, we demonstrate that spectral overlap-modulated FRET strategy is very useful in the development of ratiometric sensors which are highly desired in the field of biological sensing and imaging. Using efficient dyes acting as donor and acceptor, and connecting them with suitable linker, highly efficient FRET systems can be constructed. And combining the knowledge of chemodosimeters, it is feasible to modulate the FRET efficiency through the spectral-overlap strategy and thus produce ratiometric sensors with ideally well-separated dual emissions. One of the challenges is the difficulty in chemical syntheses, because those FRET-based molecules have relatively complicated structures. However, we can construct some versatile platforms, from which, only very few steps are further needed to generate the target sensors, and thus, the spectral overlap-modulated FRET strategy can be popularized.

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