基于分子工程能量转移构建的比率型双光子荧光探针在生物成像中的应用

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摘 要：设计和合成可被应用于生物系统中各种分析物的比例检测与成像的基于能量转移二元体系的比率型双光子荧光探针是一项至关重要的任务。因此，对近 10 a 基于荧光共振能量转移（FRET）或跨键能量转移（TBET）构建的比率型双光子荧光探针在生物成像中的应用进行综述。未来的研究方向是基于 FRET/TBET 构建新型双光子比率型荧光探针，并将其应用于生物分析和疾病诊断领域。

关键词：分子工程；能量转移；比率型双光子荧光探针；荧光共振能量转移；跨键能量转移

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0 Introduction

Organic small-molecule fluorescent probe-based imaging is a widely used technology to study biological events with high spatiotemporal resolution, because of the good cell permeability, and fast biological target response speed of small molecule fluorescent probes, which are important for in vivo detection and cell-imaging reagents[1-5]. So far, thousands of one-photons (OP) probes have been developed for one-photon microscopy fluorescence (OPMF) imaging. However, most of these fluorescent probes have been developed by employing common fluorophores, such as fluorescein, coumarin, BODIPY, and rhodamine with OP-excited at UV-visible light (350~550 nm), which resulted in photobleaching, interference from autofluorescence in cells and tissues (such as flavin adenine dinucleotide and nicotinamide adenine dinucleotide), and shallow penetration depth (<100 μm)[6].

During the past two decades, two-photon microscopy fluorescence(TPMF), which utilizes two near-infrared(NIR) photons as the excitation source, has emerged as a novel, attractive imaging tool for biomedical research[7-8]. Compared with OPMF, TPMF offers several advantages, such as increased penetration depth (>500 μm), minimized fluorescence background, and less light scattering and tissue injury[9-10]. However, most previously reported TP fluorescent probes were designed on the basis of single-

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emission intensity changes, which could be affected by instrumental efficiency, environmental conditions, and the concentration of probe molecules[11-14]. Fortunately, the ratiometric fluorescent probes can overcome these drawbacks, such as interferences by a built-in correction of the dual-emission peaks. Several strategies, including internal charge transfer (ICT), fluorescence resonance energy transfer (FRET), and through-bond energy transfer (TBET) have been adopted to design ratiometric probes. For ratiometric probes based on FRET/TBET, the donor is linked with the acceptor by special linking group[15-17]. As a result, such probes showed high energy transfer efficiency, two well-resolved emission peaks with high imaging resolution, less cross talk between channels because the large stokes shift between donor and acceptor[18-20].

Although in the current literature, some reviews have been published on use of TP probe for the specific response and targeting analysis, they mainly focus on metal ions, organelles, enzymes and other analytic objects[6, 10, 21-24]. In this review, we will summarize recently used energy transfer strategies to develop ratiometric TP probes for biological imaging applications. Thus this review mainly focuses on energy transfer-based ratiometric TP probes. Single TP fluorophore-based ratiometric TP fluorescent probes by ICT strategy, and could also be used for a similar purpose, but they do not belong to the energy transfer strategy-based ratiometric TP fluorescent probes, and will not be included in this review. Firstly, because the TP fluorophore was adopted as a donor for energy transfer systems, we will begin with a description of the classical TP fluorophores. Secondly, some of the fundamental concepts and photophysical properties of TP fluorophores are described. Thirdly, we will further provide an overview of energy transfer strategies used to design ratiometric TP probes and highlight some reported probes that were used to detect biological targets in vivo. Finally, current challenges and opportunities of energy transfer-based ratiometric TP fluorescent probes will be outlined.

1 Common two-photon fluorophores

There are several types of TP fluorophore skeletons used for designing TP fluorescent probes, such as derivatives of coumarin, naphthalene, quinoline, 4-substituted-1,8-naphthalimide, fluorene, carbazole, styrene and so on. Through these nucleoluses of compound for designing some two-photon fluorophore with a structure of electron donor-π- electron acceptor (D-π-A), D-π-D, A-π-A, D-π-A-π-D, et al[25]. Fig. 1 shows the nucleoluses of compound applied for the design of TP fluorophore, which show good photophysical properties, including large TP absorption cross-section value, excellent photostability. Moreover, through modification of the functional groups, they are easy to design of some two-photon probes. However, the variety of TP fluorophores is still limited, most of the existing TP fluorophores have short-emission wavelengths ⩽ 650 nm, and the quantitation of these probes is primarily based on single-emission intensity changes which tends to be affected by a variety of environmental factors, so much effort is necessary for the development of novel TP fluorophores with improved photophysical properties. And biological imaging applications could be affected by instrumental efficiency, environmental conditions, and the concentration of probe molecules, and limited penetration depth in tissues imaging.

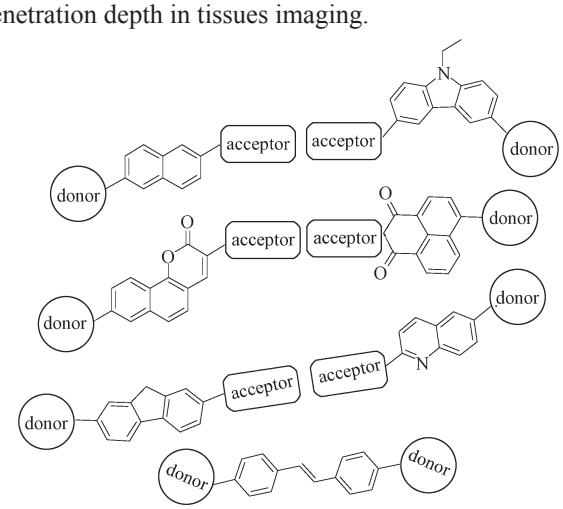


Fig. 1 Representative TP fluorophore skeletons applied for the design of TP probes

2 Photophysical properties of TP fluorophores

2.1 TP absorption action cross-section (TPA$\Phi\delta$)

The two-photon absorption cross-section (TPAδ)

is an important property for evaluating a TP fluorescent probe. So, TPA δ of the probe needs to be well studied. In general, a large conjugated system and a rigid coplanar structure are believed to be essential for large TPAδ. The TP excited fluorescence was measured with a Ti: sapphire femtosecond oscillator (Spectra Physics Mai Tai) as the excitation source. The output laser pulses have a tunable central wavelength of 690 to 1 020 nm with pulse duration of less than 100%fs and repetition rate of 80.5 MHz. The laser beam was focused onto the samples using a lens with a focal length of 3.0 cm. The emission was collected at an angle of 90 ° to the direction of the excitation beam to minimize scattering. The emission signal was directed into a CCD (Princeton Instruments, Pixis 400 B) coupled monochromator(IsoPlane160) with an optical fiber. The TPAδ of the sample(s) at each wavelength was calculated according to Equation(1), and rhodamine B in CH_3OH was used as the reference(r)[26-27].

$$\delta_s=\delta_r(S_s\Phi_r\phi_r c_r)/(S_r\Phi_s\phi_s c_s), \quad (1)$$

Where S is the integrated fluorescence intensity, Φ is the fluorescence quantum yield, c is the concentration of sample (s) and reference (r), and ϕ is the collection efficiency of the experimental set up. The uncertainty in the measurement of cross sections is ≤ 15%.

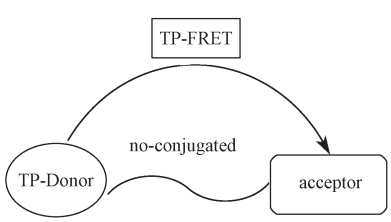
2.2 Photostability

Two-photon microscopy (TPM) adopts two near-infrared photons as the excitation source, and the TP probe-based fluorescent imaging can provide improved three-dimensional spatial localization and increased imaging depth. However, when some probes are used for long real-time imaging in vivo, the photostability of the probes need to be well studied. Generally speaking, the photostability of TP fluorescent probes under irradiation of a 100 W mercury lamp in vitro to investigate the degree of attenuation the fluorescence intensity.

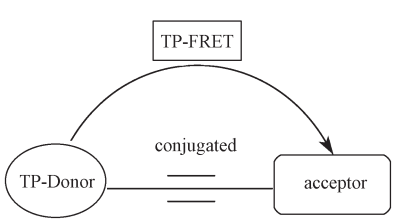
3 Molecular engineering of energy transfer-based ratiometric TP dyads

Fluorescence resonance energy transfer (FRET) and through bond energy transfer (TBET) mechanisms involve energy transfer between a pair of fluorophores (energy donor and acceptor)[28-31]. When applied in sensing applications, the ratio of these two emissions modulated by the target analytes between the emission of the relative short wavelengths donor and the longer wavelengths acceptor. A high FRET efficiency system is generally required substantial spectral overlap between the donor emission and the acceptor absorption bands. As a consequence of this photophysical requirement, FRET-based dyads are typically linked by a nonconjugated spacer with energy transfer occurring through space (Fig. 2a). Since Stryer and Haugland exploited FRET as a ‘‘spectroscopic ruler’’[32], FRET has emerged as a critical tool to construct large number of ratiometric fluorescent probes for the analysis of many of the analytes.

Early on, a systematic study of TBET was undertaken by Verhoeven and co-workers[33]. For TBET-based dyads, the donor is linked directly by an electronically conjugated bond with the acceptor, and energy transfer occurs through a conjugated bond without the need for spectral overlap, thus preventing donor and acceptor fragments from becoming planar. As a result, such probes showed high energy transfer efficiency, two well-resolved emission peaks with high imaging resolution, less cross talk between channels. Owing to no spectral overlap between donor and acceptor is needed in TBET, dramatic amenability to molecular design (Fig. 2b).



a) FRET-based ratiometric TP fluorescent probe



b) TBET-based ratiometric TP fluorescent probe

Fig. 2 Using TBET or FRET strategy to construct ratiometric two-photon fluorescent probes

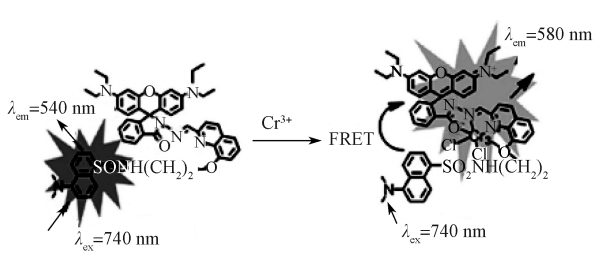
3.1 TP-Fluorescence resonance energy transfer dyads(TP-FRET)

However, although the traditional FRET-ratiometric fluorescent probe has two emission peaks, with good resolution, and can overcome the environmental and other factors of interference, they belong to the OP-excited at short wavelengths in the UV-visible region (400~600 nm), which resulted in photobleaching, interference from autofluorescence in cells and tissues, and shallow penetration depth (<100 μm)[6]. A much more feasible alternative would be utilizing TP probe-based fluorescent imaging, which is an emerging technique that can realize deep-tissue imaging with prolonged observation time. Despite the advantages of TPM, such as increased penetration depth (>500 μm), minimized fluorescence background, and less light scattering and tissue damage, most TP probes are designed on the basis of single emission intensity changes. Furthermore, choosing a suitable TP-excited fluorophore for design of energy transfer-based ratiometric TP fluorescent probes remains challenging. In recent years, some energy transfer-based ratiometric TP fluorescent probes have been developed.

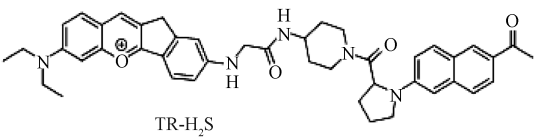
3.1.1 Via regulation of the xanthene dyes as the acceptor′ switch effect to achieve ratiometric TP detection and imaging applications

The FRET-based ratiometric TP fluorescent probe DANS-RB for selectively detecting Cr^{3+} in semi-aqueous solution (Fig. 3, probe 1)[34]. The energy transfer from the dansylamide (a TP fluorophore as energy donor) to the rhodamine (energy acceptor) after the addition of Cr^{3+} ions. In the absence of Cr^{3+}, the acceptor existed in a closed-ring, colorless and nonfluorescent lactam form, and only the green fluorescence of the donor was observed, because no energy transfer was observed from the donor to acceptor. Once, in the presence of Cr^{3+}, the nonfluorescent lactam form of the acceptor changed to the conjugated fluorescent xanthene form. The cell imaging experiment demonstrated DANS-RB was a good candidate for monitoring the intracellular Cr^{3+} level with the ratiometric TP fluorescent images. The FRET-based ratiometric TP fluorescent probe TR-H_2S for deep-tissue imaging of H_2S, was designed by conjugating TP fluorophore acedan as the donor and deep red fluorophore anthocyanidin-analog as the acceptor for H_2S-responsive moiety (Fig. 3, probe 2)[35]. This probe shows large emission shift 125 nm with two well-resolved emission bands. Moreover, TR-H_2S was successfully applied for ratiometric TP imaging of H_2S in living cells and tissues with imaging depths from 90~180 μm. Based on TR-H_2S detection mechanism, almost at the same time, a ratiometric TP fluorescent probe (Fig. 3, probe 3) for SO_2 was developed and reported by two research groups[36-37]. Probe 3 was applied for ratiometric TPM imaging, and the results revealed the endogenous SO_2 derivates within mitochondria of living systems with respect to the enzymatic reaction of $Na_2S_2O_3$ and glutathione(GSH), demonstrating that probe 3 would be an effective molecule tool to study the metabolism of sulfur-containing species in biomedical research[37]. In order to better study the lysosomal pH changes, FRET-based ratiometric TP fluorescent probe NRLys (Fig. 3, probe 4) for lysosomal pH was reported[38]. The FRET scaffold based on a TP fluorophore (D-π-A-structured naphthalimide derivative) and a rhodamine B fluorophore are directly connected by a flexible piperidinelinker. The experiments demonstrated that the probe 4 was a reliable and specific probe for labeling lysosomes in living cells and tissues with two well-resolved emission peaks separated by 60 nm. Probe 4 showed high ratiometric imaging resolution and deep-tissueimaging depth of over 180 μm. P-Np-Rhod (Fig. 3, probe 5) was the first reported ratiometric TP fluorescent probe for nitroxyl (HNO)[39]. In this FRET system, a naphthalene derivative served as the TP-excitation donor and a rhodol fluorophore was chosen as the energy acceptor and (diphenylphosphino)-benzoate moiety, which acted as the recognition moiety for HNO and fluorescence intensity control, which was directly tethered to the hydroxyl position of the acceptor. In the absence of HNO, the rhodol existed in a nonfluorescent lactone form and the FRET signal was cut off. In the presence of HNO,

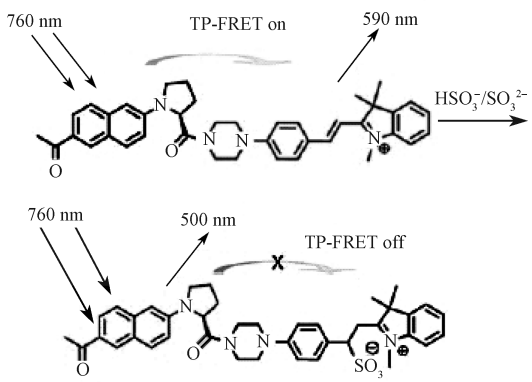
the hydroxyl was released by 2-(diphenylphosphino)-benzoate moiety reaction with HNO. And then the closed spirolactone form was converted to a conjugated fluorescent xanthene form to achieve FRET. P-Np-Rhod could respond quickly to HNO with large emission shift (93 nm), high selectivity, and high sensitivity. More importantly, P-Np-Rhod was successfully applied for TP fluorescence imaging of HNO in living tissues with less cross-talk between the blue and yellow emission channels. Followed by the P-Np-Rhod, a ratiometric TP fluorescent probe for HNO, termed TP-Rho-HNO (Fig. 3, probe 6), which consists of benzo[h]chromene-rhodol scaffold as TP energy transfer cassette with phosphine moiety as specific HNO recognition unit[40]. The newly proposed probe has been successfully applied in ratiometric TP bioimaging of endogenous HNO in the human umbilical vein cells (HUVECs) and as well as in rat brain tissues. A ratiometric TP probe, TPR-S (Fig. 3, probe 7) for hydrogen polysulfides had been developed above FRET-based platform[41]. TPR-S showed a FRET, which emitted a blue fluorescence of the donor and yellow fluorescence of the acceptor. In the presence of hydrogen polysulfides, the closed spirolactone form of acceptor was changed into a conjugated xanthene form, and then the FRET was on, and then a remarkable fluorescence intensity ratiometric (I_{541}/I_{448}) enhancement (about 62 folds) was observed and a detection limit of 1.0 μmol/L was achieved for H_2S_n in buffered aqueous solution. In living systems, H_2S_n could be formed from CSE mediated cysteine metabolism, and CSE could be overexpressed when induced by inflammatory mediators and LPS. TPR-S was successfully applied for monitoring H_2S_n formation in this process in LPS-induced organ injury samples (Fig. 4).



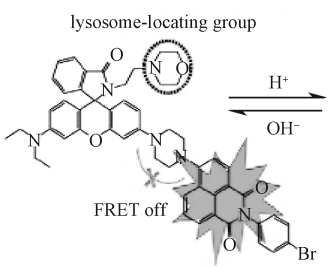
a) Probe 1

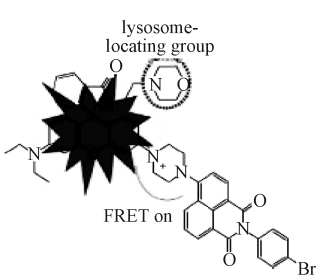


b) Probe 2

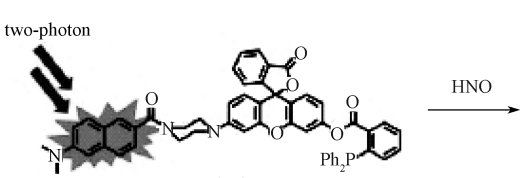


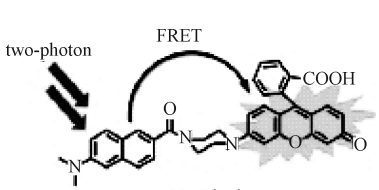
c) Probe 3



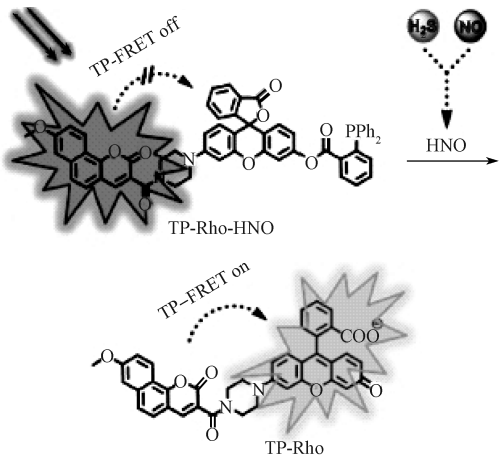


d) Probe 4

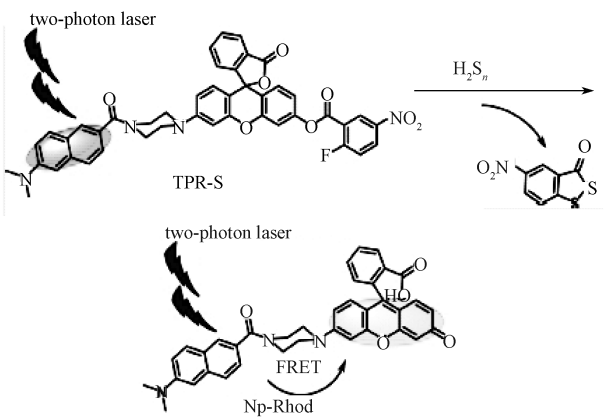




e) Probe 5

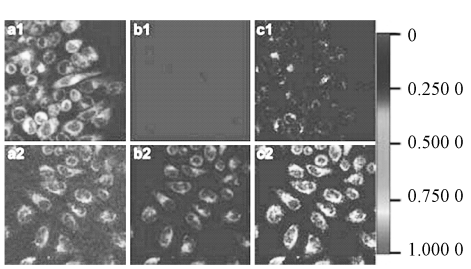


f) Probe 6

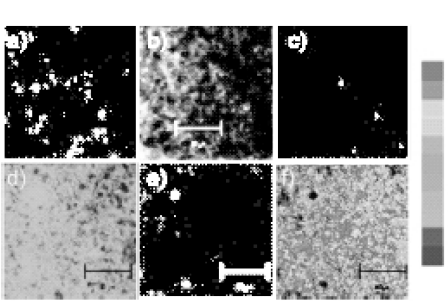


g) Probe 7

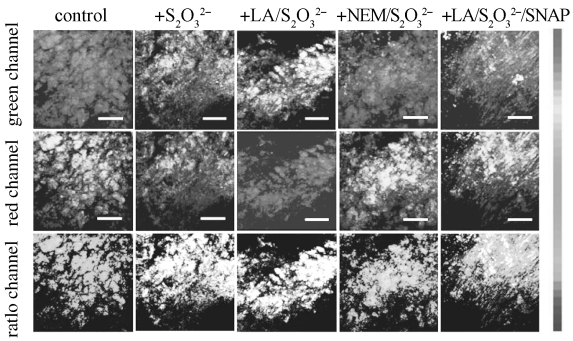
Fig. 3 D-π-A-structured of TP fluorophore (as the FRET donor)-based ratiometric TP fluorescent probe



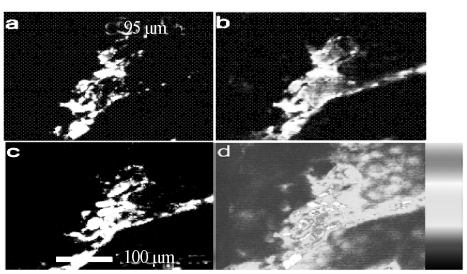
a) Probe DANS-RB for monitoring the intracellular Cr^{3+} level with the ratiometric TP fluorescent images



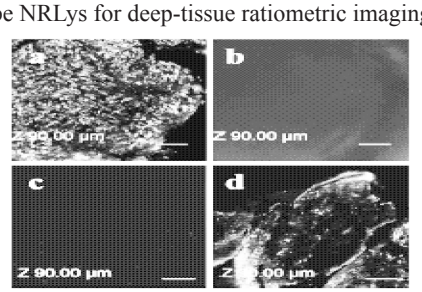
b) Probe TR-H_2S for deep-tissue ratiometric imaging of H_2S



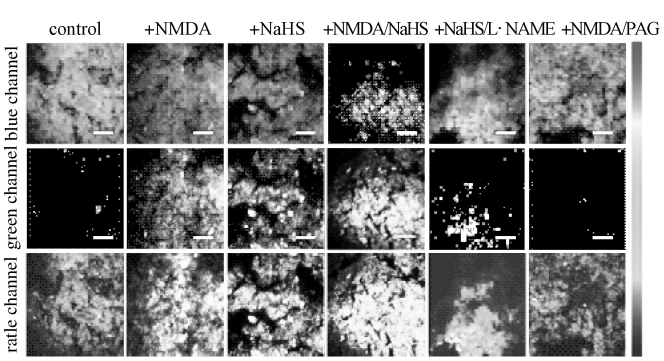
c) Probe TP-FRET was applied for tissues ratiometric TPM imaging of SO_2



d) Probe NRLys for deep-tissue ratiometric imaging of H^+



e) Probe P-Np-Rhod was applied for ratiometric TPM imaging of HNO in living tissues



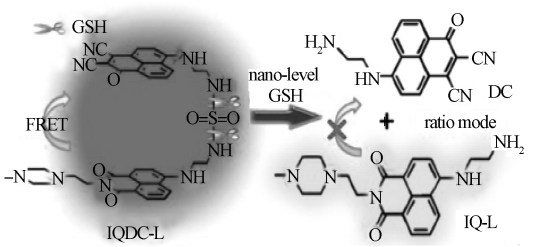
f) Probe TP-Rho-HNO was applied for ratiometric TPM imaging of HNO in living tissues

Fig. 4 The FRET-based dyads for cells and tissues ratiometric TPMF imaging applications

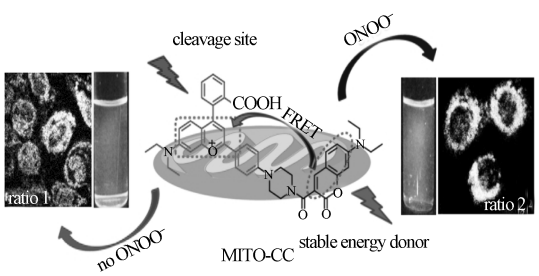
3.1.2 Via damaging the molecular structure to release the TP fluorophore to achieve ratiometric TP detection and imaging applications

Because ultratrace change of reduced glutathione

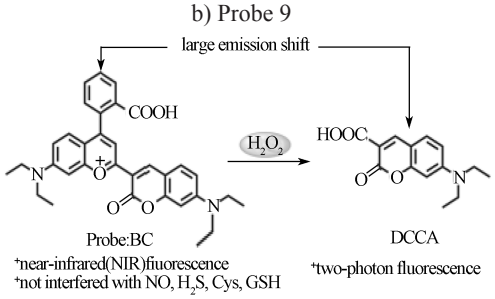
(GSH) can weaken coagulation function of platelet (PLT), ratiometric TP fluorescent probe for screening PLT from peripheral blood and quantitative imaging of GSH are more beneficial and effective for monitoring coagulation function of PLT. A ratiometric TP GSH-specific fluorescent probe IQDC-L was reported[42], which using sulfuric diamide as the linker, and resulted in not only specific selectivity for GSH, but also FRET occurring in probe. In presence of GSH, "S—N" in sulfonamide group was cut off, and FRET was inhibited. Furthermore, fluorescence intensities at 520 and 595 nm presented linear change on ratio mode in the range of GSH (2.0~65.0 nmol/L) with the lowest detection 0.083 nmol/L. Thus, IQDC-L could be used to screen PLT from peripheral blood and in situ image ultratrace GSH. A new ratiometric fluorescent probe BC with a novel H_2O_2 response unit (Fig. 5, probe 10) was developed[43], which using the simultaneous NIR and TP imaging of H_2O_2 in the living systems. Via the Baeyer-Villiger oxidative rearrangement reaction in which the ketone can react with H_2O_2 at mild conditions, and the oxonium group was served as a novel H_2O_2 response site and exhibited NIR emission. The dyans contained a CS fluorophore and a coumarin fluorophore via the π -conjugated system to be connected (emission at the NIR region) (Fig. 5, probe 10). When responded to H_2O_2, BC released a TP dye and exhibited an extremely large emission shift of 221 nm, and displayed high sensitivity and selectivity for detecting H_2O_2 in the aqueous condition. BC could be applied for sensing exogenous and endogenous H_2O_2 in the living cells and tissues. Importantly, BC was employed to image H_2O_2 in zebrafish at both the NIR and TP modes. Peroxynitrite ($ONOO^-$) is a kind of reactive oxygen species (ROS) with super activity of oxidization and nitration, and overproduction of $ONOO^-$ is associated with pathogenesis of many diseases. A new ratiometric TP fluorescent probe MITO-CC (Fig. 5, probe 9) for $ONOO^-$ based on FRET mechanism by combining rational design strategy and dye-screening approach[44]. The ratiometric probe exhibits not only outstanding sensitivity (11.30 nmol/L), fast response (within 20 s) toward $ONOO^-$ but also high selectivity upon other various biological ROS and RNS in a physiological pH aqueous solution. The perfect performance in both cells and tissues imaging illustrated that probe MITO-CC can be applied to monitor endogenous $ONOO^-$ with minimal cytotoxicity by TP fluorescence confocal microscopy (TPFM). Moreover, probe MITO-CC is capable of monitoring $ONOO^-$ produced by LPS stimulation in the inflamed mouse model (Fig. 6, probe 8~10) .



a) Probe 8

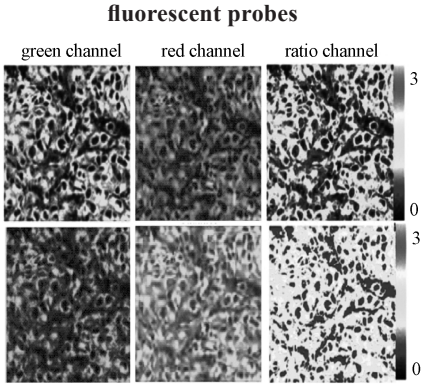


b) Probe 9

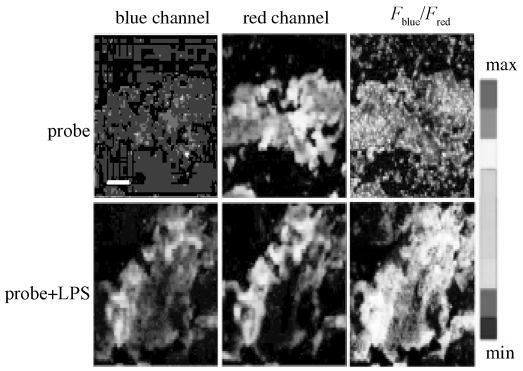


c) Probe 10

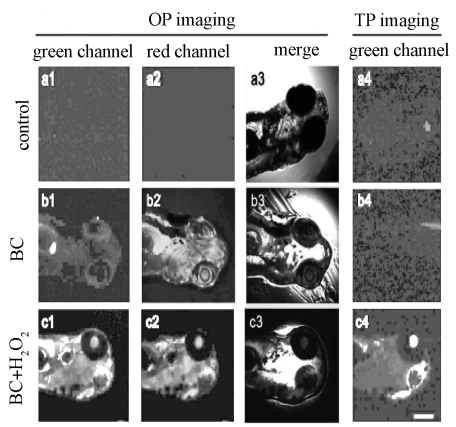
Fig. 5 Via damaging the molecular structure to release the TP fluorophore-based ratiometric TP fluorescent probes



a) Probe IQDC-L for monitoring the intracellular GSH level with the ratiometric TP fluorescent images



b) Probe MITO-CC was applied for tissues ratiometric TPMF imaging of $ONOO^-$

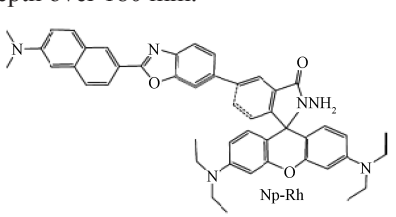


c) Probe BC was used NIR and TP modes in zebrafish for ratiometric imaging of H_2O_2

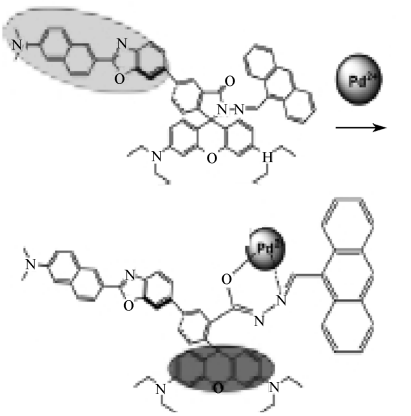
Fig. 6 Via damaging the molecular structure to release the TP fluorophore for cells and tissues ratiometric TPMF imaging applications

3.2 TP-Through-Bond energy transfer dyads (TP-TBET)

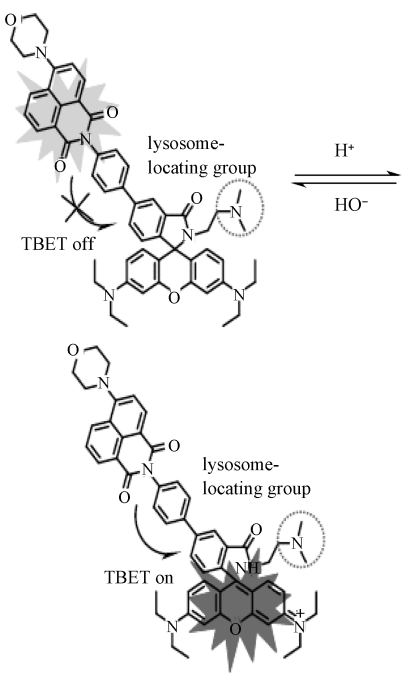
Through-bond energy transfer (TBET) is very similar to FRET, but not exactly the same. For TBET-based dyads, the donor is linked directly by an electronically conjugated bond with the acceptor, and energy transfer occurs through a conjugated bond without the need for spectral overlap, thus preventing donor and acceptor fragments from becoming planar. As a result, such probes showed high energy transfer efficiency, two well-resolved emission peaks with high imaging resolution, less cross talk between channels. Owing to no spectral overlap between donor and acceptor is needed in TBET, dramatic amenability to molecular design. Based on these strategies, the first TBET-based ratiometric TP fluorescent probe has been developed (Fig. 7, probe 11)[45]. The proposed probe, which well combined the advantages of TP fluorescent imaging with TBET, was designed by directly conjugating naphthalene derivative (a TP fluorophoe) as the TBET donor to rhodamine spirolactam as the TBET acceptor. And this platform was later applied for designing a probe Np-Rh for Cu^{2+}. In the absence of Cu^{2+} ions, the acceptor existed in a closed-ring, colorless and nonfluorescent lactam form, and only the cyan fluorescence of the donor was observed, because no energy transfer was observed from the donor to acceptor. In the presence of Cu^{2+} ions, the nonfluorescent lactam form of the acceptor was converted to the conjugated fluorescent xanthene form. With the increase of the concentration of Cu^{2+} ions, the donor's characteristic emission peak (475 nm) gradually disappeared, while the characteristic emission peak (575 nm) of rhodamine B grew. This new probe was then applied for TP imaging of living cells and tissues, which showed high ratiometric imaging resolution and deep tissue-imaging depth of 180 μm (Fig. 8a). A ratiometric TP fluorescent probe, Np-Rh-Pd, for Pd^{2+} ion detection and bioimaging applications was developed based on this TBET platform[46]. Followed by these two jobs, a ratiometric TP fluorescent probe for imaging of lysosomal pH in live cells and tissues, termed Np-Rh-Lys was developed[47]. Specifically, a two-photon fluorophore (D-π-A-structured naphthalimide derivative) as the donor and a rhodamine B fluorophore as the acceptor were directly connected, and dimethylamino moiety served as a lysosomal targeting-group, which demonstrate the new probe Np-Rh-Lys was a reliable and specific probe for labeling lysosomes in living cells with two well-resolved emission peaks separated by 80 nm, and which showed high ratiometric imaging resolution and deep-tissue imaging depth over 180 mm.



a) Probe 11

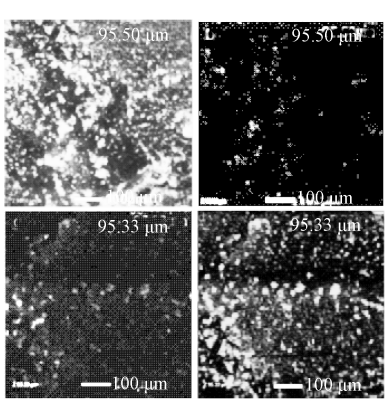


b) Probe 12

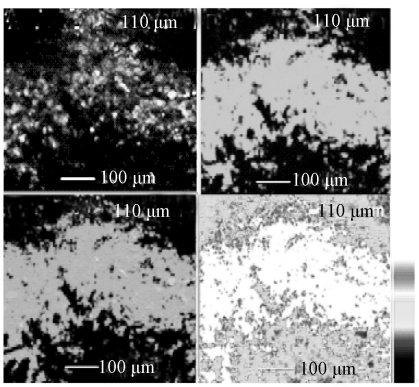


c) Probe 13

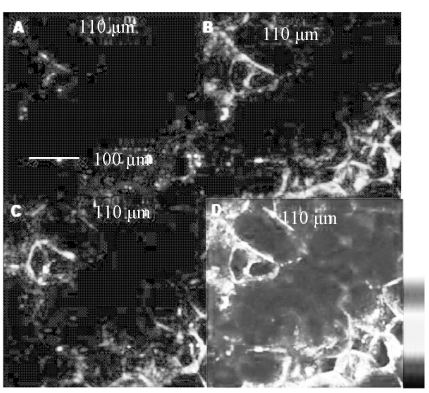
Fig. 7 D-π-A-structured of TP fluorophore (as the TBET donor)-based ratiometric TP fluorescent probe



a) Probe Np-Rh was applied for the liver tissues of rat ratiometric TPMF imaging of Cu^{2+} ions



b) Probe Np-Rh-Pd was applied for the liver tissues of rat ratiometric TPMF imaging of Pd^{2+} ions



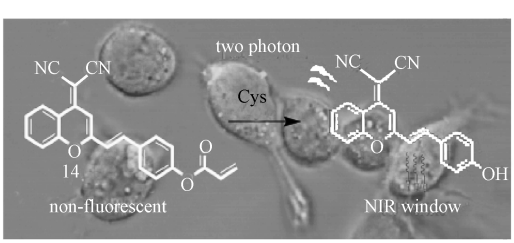
c) Np-Rh-Lys was applied for the tissues of onion ratiometric TPMF imaging of H^+ ions

Fig. 8 The TBET-based dyads for tissues ratiometric TPMF imaging and applications

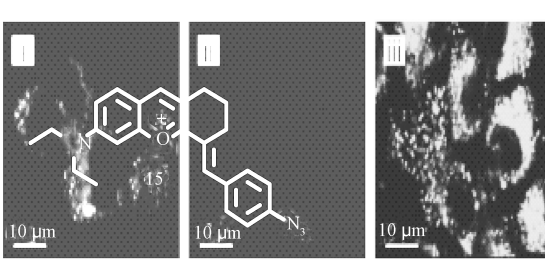
3.3 Other types of TP fluorophores with near-infrared emission

However, despite employing near-infrared (NIR) excitation, most previously reported two-photon exhibited visible emissions (400~600 nm), which might strictly limit their tissue penetration depth. Therefore, the development of a two-photon probe emitting in the NIR region and showing deep penetration depth is highly desired and challenging. Recently, several TP fluorescent probes based on 4-(dicyanomethylene)-styryl-4H pyran dyes and benzylidene-6-(diethylamino)-1,2,3,4 tetrahydroxanthylium hypochlorite delivative with near-infrared emission (NIRE) have also been developed[48-51]. Sensing of biological cysteine (Cys) has aroused dynamic interest due to its essential roles in human physiology. The two-photon excitable (850 nm) NIR

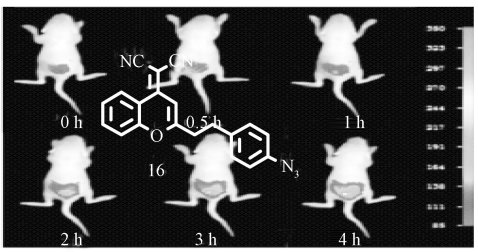
emission (702 nm) TP fluorescent probe TP-NIR (Fig. 9, probe 14) was designed and synthesized for selective detection of Cys. The probe itself shows is turned on by reaction with Cys in aqueous buffer, and displays greater sensitivity and selectivity for Cys over glutathione (GSH) and homocysteine (Hcy). Moreover, the large Stokes shift, NIR excitation, and NIR emission make this probe suitable for biological imaging[48]. Hydrogen sulfide (H_2S), a newly recognized transmitter, is synthesized in mammalian tissues from cysteine and homocysteine through the action of enzymes such as cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). It reduces mucosal inflammation, protects gastrointestinal mucosa from injury, and augments tissue repair. Malfunction of H_2S homeostasis has been implicated in colitis hypertension, cardiomyopathy, atherosclerosis, diabetic endothelial dysfunction, and diabetic nephropathy. In order to get deeper understanding to the chemical and biological properties of it, highly sensitive and highly selective detection techniques for tracking endogenous it are urgently desirable, since the complex manifestations of it in both physiological and pathological states. In Fig. 9, probe 15, probe 16 and probe 17 were the three two-photon excitable NIR emission TP fluorescent probe for selective detection of endogenous H_2S[49-51].



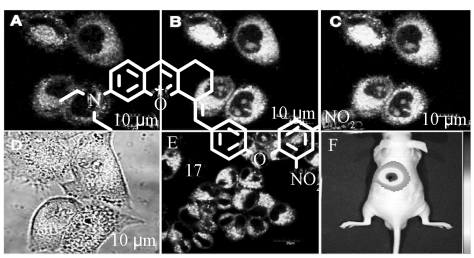
a) TP fluorescent probe TP-NIR (probe 14) was applied for the Hep 3B cells TPMF imaging of Cys



b) TP fluorescent probe 4 (probe 15) was applied for the HeLa cells TPMF imaging of H_2S



c) TP fluorescent probe 1 (probe 16) was applied for imaging of H_2S in the nude mice



d) TP fluorescent probe TP-NIR-HS (probe 17) was applied for imaging of H_2S in HeLa cells and nude mice

Fig. 9 The two-photon excitable NIR emission TP fluorescent probe for TPMF imaging and applications

4 Conclusions and outlook

In this review, we focused on the recent major contributions from the molecular engineering of energy transfer strategy-based ratiometric TP fluorescent probe. They included the systems of FRET and TBET-based ratiometric TP fluorescent. The ingenious design of the probes well combined the advantages of TP fluorescent imaging with ratiometric imaging, which made them have high detection sensitivity, and practicability. On one hand, the energy transfer-based ratiometric TP probes are ideal, although they are difficult to design and synthesize, they are still worth constantly exploring. On the other hand, ratiometric TP probes with both excitation and emission wavelength in the far-red to NIR region are more desired for deep-tissue imaging. Although ratiometric TP probes used NIR laser pulses as the excitation source, some probes with emission wavelength in the farred to NIR region are still highly desired. It is our hope that this review can provide an overview of current research effort and provide a mechanistic framework for the creation of novel ratiometric TP

fluorescent probes. We believe that with the development of novel ratiometric TP probes with excellent properties and design strategies will appear, and they will find wide application in various areas.

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Molecular Engineering of Energy Transfer-Based Ratiometric Two-Photon Fluorescent Probes for Bioimaging Applications

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Abstract：Design and synthesis of energy transfer dyads-based ratiometric TP fluorescent probe for the ratiometric detection and imaging of various analytes in biological systems is a vital task. A general introduction to molecular engineering of the fluorescence resonance energy transfer (FRET), and through-bond energy transfer (TBET)-based ratiometric TP fluorescent probe for bioimaging applications during the past decade were reviewed. It was suggested that more attention would be committed to development of new FRET/TBET-based ratiometric TP dyads and applications of two-photon fluorescence microscope (TPFM) in areas of bioanalysis and disease diagnosis.

Keywords：molecular engineering；energy transfer；ratiometric two-photon fluorescent probe；fluorescence resonance energy transfer；through-bond energy transfer