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REVIEW

Quantitative analysis of FRET assay in biology —New developments in protein interaction affinity and protease kinetics determinations in the SUMOylation cascade

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Abstract **Förster resonance energy transfer (FRET) techniques have been widely used in biological studies *in vitro* and *in vivo* and are powerful tools for elucidating protein interactions in many regulatory cascades. FRET occurs between oscillating dipoles of two fluorophores with overlapping emission and excitation wavelengths and is dependent on the spectroscopic and geometric properties of the donor-acceptor pair. Various efforts have been made to develop quantitative FRET methods to accurately determine the interaction affinity and kinetics parameters. SUMOylation is an important post-translational protein modification with key roles in multiple biological processes. Conjugating SUMO to substrates requires an enzymatic cascade. Sentrin/SUMO-specific proteases (SENP) act as endopeptidases to process the pre-SUMO or an isopeptidase to deconjugate SUMO from its substrate. Here we also summarize recent developments of theoretical and experimental procedures for determining the protein interaction dissociation constant, K_d, and protease kinetics parameters, k_{cat} and K_m, in the SUMOylation pathway. The general principles of these quantitative FRET-based measurements can be applied to other protein interactions and proteases.**

Keywords quantitative FRET analysis, protein affinity determination, kinetics analysis

Introduction

Förster resonance energy transfer (FRET) is widely used in biological and biomedical research, including cell biology, medical diagnostics, optical imaging and drug discovery. FRET occurs when the donor fluorophore (D) and acceptor fluorophore (A) are close to each other (1–10 nm in general) and in a favorable orientation. This distance is comparable to the size of biomolecules or the distances of molecular interactions. Because of its sensitivity and accuracy, FRET has become a powerful tool for studying the dynamics and interactions of biological molecules, quantitating *in vitro* assays, and monitoring cellular events *in vivo* (Szöllosi et al., 2002; Saucerman et al., 2006; Dams et al., 2007; Gambin and Deniz, 2010; Lu and Wang, 2010; Prasuhn et al., 2010).

Various quantitative methods of FRET signal analysis have been developed in biological research. Energy donor fluorescence is quenched, and acceptor fluorescence is increased. This makes it is easy to characterize the FRET signal by traditional ratiometric methods (Verveer et al., 2000; Eis et al., 2001; Saucerman et al., 2006; Hires et al., 2008). However, the spectral overlap between donor and acceptor that allows FRET to occur is also the cause of FRET signal contamination, including two artifacts. 1) Bleed-through excitation occurs when an acceptor is excited by the donor excitation wavelength and vice versa. 2) Cross-talk in emission detection occurs when the emission of a donor contributes to the signal measured in a setup for acceptor detection, and vice versa. FRET signal characterization, like FRET efficiency and FRET indices, requires corrections for the bleed-through and the cross-talk, which we call fluorescence contamination.

We developed highly sensitive FRET-based assays to study

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the SUMOylation and deSUMOylation processes. Reversible posttranslational modifications are used widely in nature to dynamically regulate protein activity, and small ubiquitin-like modifier (SUMO) covalently modifies a large number of proteins in many important cellular processes, including signal transduction, cell-cycle regulation, cell survival and death, DNA damage, and stress responses (Yeh, 2009). Several diseases, such as pathogenic infection, cancer and neurodegenerative disorders, are associated with altered SUMOylation (Steffan et al., 2004; Yeh, 2009). SUMO conjugation occurs through a cascade of reactions involving E1, E2, and E3 enzymes, SUMO target protein and sentrin-specific protease (SENP).

A full understanding of SUMOylation requires an assay that can easily and quantitatively measure the kinetics and other dynamic aspects of its interactions. FRET technology is an ideal method for this purpose. In this review, we summarize current quantitative FRET analysis methods, and our recent new developments of mathematic algorithms and experimental procedures of quantitative FRET analysis and its application for a protein binding affinity (K_d) and protease kinetics (k_{cat}/K_m) determinations in SUMOylation cascade. These are achieved by FRET assay with the correction of the FRET contamination. In addition, we have developed FRET-based high-throughput screen (HTS) assays for small chemical inhibitor of SUMOylation pathway, which allows reproducible and large-scale application, such as genome-wide and industrial applications. These new developments can be generally expanded to other biological processes.

FRET: concepts and principles

Originally predicted by German scientist Förster (1948), FRET is a physical phenomenon in which dipole-dipole resonance interaction results in energy transfer between excited donor and acceptor chromophores. The interaction occurs between oscillating dipoles with similar resonance frequencies and depends on the spectroscopic and geometric properties of the donor-acceptor pair. In the theory proposed by Förster, the rate constant (k_T) of energy transfer is given by:

$$k_T = r^{-6}\kappa^2 J n^{-4} k_F \times 8.71 \times 10^{23} \sec^{-1},$$

Where r is the distance between the centers of the donor and acceptor chromophores, κ^2 is the orientation factor of the dipole-dipole interaction, J is the overlap integral of donor emission spectrum and acceptor excitation spectrum, n is the refractive index of the medium between the donor and acceptor chromophores, and k_F is the rate constant of fluorescent emission from the donor chromophore.

The efficiency (E) of energy transfer can be represented by:

$$E = \frac{R_0^6}{R_0^6 + r^6},$$

Where R_0 is the Förster radius, the distance between donor and acceptor chromophores when the efficiency of energy transfer is 50%, and $R_0 = (Q_0\kappa^2 J n^{-4})^{1/6} \times 9.7 \times 10^3$Å, where Q_0 is the quantum yield of the donor chromophore in the absence of acceptor; and

$$J = \frac{\int F(\lambda) \in (\lambda)\lambda^4 d\lambda}{\int F(\lambda) d\lambda},$$

where $F(\lambda)$ is the fluorescent intensity of the donor chromophore at wavelength λ and $\in(\lambda)$ is the molar extinction coefficient of the acceptor chromophore.

The orientation factor (κ^2) depends on the relative orientation of the donor and acceptor dipoles and is given by:

$$\kappa^2 = (\cos\alpha - 3\cos\beta\cos\gamma)^2,$$

where α is the angle between the donor and acceptor transition moments, β is the angle between the donor moment and the line joining the centers of the donor and acceptor, and γ is the angle between the acceptor moment and the line joining the centers of the donor and acceptor.

Unlike the molecular distance (r) and spectrum overlap integral (J), the value of κ^2 is often hard to obtain experimentally due to the uncertainty of molecular motions. Stryer (1978) analyzed the probability distribution of donor-acceptor relative angles and showed that, while the theoretical value of κ^2 can vary from 0 to 4, its range is narrower in practice and will introduce no more than 20% variation to the determination of donor-acceptor distance. In most FRET studies, the value of κ^2 is assumed to be 2/3, which corresponds to the ideal case in which both the donor and acceptor chromophores undergo unrestrictive motion (Stryer, 1978; dos Remedios and Moens, 1995).

The theory of FRET has been validated by many subsequent studies, and the effects of different parameters on FRET efficiency have been carefully examined. Stryer and Haugland (1967) synthesized a poly-L-proline with up to 12 residues as the linker to separate a pair of fluorophores, and correlated the observed efficiencies of energy transfer with the reverse sixth power of the donor-acceptor distances. An r^{-6} dependence was also confirmed by Bücher et al. (1967) with fatty acid layers of known dimensions to separate donor and acceptor chromophores. The dependence of FRET efficiency on the spectrum overlap integral (J) was later verified by Haugland et al. (1969): J was varied over 40-fold by changing the solvent and found to have a linear relationship to the energy transfer rate constant.

The inverse sixth power relationship between energy transfer rate and donor-acceptor distance makes FRET efficiency quite sensitive to changes in distance. When the distance of the donor and acceptor narrows from $2R_0$ to R_0, the efficiency of FRET increases from 1.5% to 50%. This high sensitivity serves as the basis of two types of FRET-based applications. First, FRET can be used as a

spectroscopic ruler to precisely measure molecular distance when the donor and acceptor distance is not far from their R_0. Second, the “on” and “off” status of FRET can be monitored to determine the status of molecular interaction.

Quantitative analysis of FRET signal

FRET signal contamination, either through bleed-through or cross talk, affects the FRET signal characterization. Traditional ratiometric methods cannot correct the FRET contamination because they do not distinguish the contributions of donor, acceptor and FRET signals. Various approaches then have been developed to correct the contaminations.

One FRET measurement employs “three-cube FRET” fluorescence microscopy/spectroscopy. To account for the fluorescence contamination, except the detection through an optical FRET filter set selecting acceptor emission during donor excitation (I_{DA} image), two additional images are acquired: acceptor fluorescence during acceptor excitation (I_{AA}) and donor fluorescence during donor excitation (I_{DD}). The crosstalk coefficients of acceptor and donor in the FRET filter set are given as a and d, respectively, which are constant and assume no other crosstalk components (Tron et al., 1984; Gordon et al., 1998). In this way, the sensitized emission, F_c, can be calculated by linear unmixing of the I_{DA} intensity as:

$$F_C = I_{DA} - aI_{AA} - dI_{DD}$$

Based on the “three-cube FRET” theory, a variety of methods have been developed to characterize the FRET signal and applied in biological research. The method developed by Tsuji et al. (2000) requires prior knowledge of the dye concentration and absorption coefficients. It also assumes that the acceptor is not excited at the donor excitation wavelength, and thus, there is no fluorescence contamination in the donor channel. They applied this method to directly observe a specific mRNA in a single living cell. The same assumptions were applied to the method developed by Suzuki (2000), except for the donor. They used the same approach to observe the dynamics of myosin motor by characterizing the FRET between GFP and BFP (Suzuki, 2000). These methods are most appropriate for monitoring dynamic FRET as only one sample was required during detection, although both donor and acceptor fluorophores were present.

Other methods were developed to provide FRET signal with more complete signal bleed-through (SBT). The underlying assumption is that the amount of cross-talk is independent of the absolute intensity of the fluorophore and thus can be calibrated by ratiometric analysis of donor and acceptor signals. This permits the off-line calibration of bleed-through ratios in samples containing only one of the two fluorophores at arbitrary concentrations. These methods have been applied to study cell-surface staining (FRET pair: FITC-Tritc (Tron et al., 1984)), cytoskeletal components of cell adhesion (FRET pair: FITC-rhodamine) (Kam et al., 1995), Bcl-2-Berclin interaction on chromosome (FRET pair: FITC-rhodamine) (Gordon et al., 1998), Ber-2-Bax interaction in mitochondria (FRET pair: BFP-GFP) (Mahajan et al., 1998), and functional expression analysis of protein subunits in rat neurons (FRET pair: CFP-FP) (Victor Ruiz-Velasco, 2001).

In contrast, Elangovan et al. (2003) developed a step-by-step algorithm to remove SBT contamination in FRET images collected by wide-field, confocal, and two-photon FRET microscopy, in which the bleed-through ratios were not considered constant but were determined at different fluorescent intensities. Mehta et al. (2009) compared different methods of characterizing FRET efficiency and FRET index by a self-developed Monte Carlo simulation algorithm and a surface FRET system with controlled amounts of donor and acceptor fluorophores and controlled distances between them. They obtained conclusions of optimized donor-to-acceptor ratios of higher energy transfer efficiencies. Moreover, the FRET signals have been characterized by a complex matrix of fluorescent intensities of donor, acceptor and FRET, according to the concentration of free donor and acceptor and energy transfer “linking” the donor-acceptor pair with α, β, γ and ξ, where α characterizes contaminated FRET signal (direct excitation of acceptor by the donor excitation wavelength), β characterizes spectral bleed-through (spillover of donor emission into the acceptor emission channel), γ characterizes the ratio of extinction coefficient of acceptor/donor at donor’s excitation wavelength, and ξ characterizes fluorescent intensity of the acceptor’s sensitized emission signal to the fluorescence intensity that would have arisen from the quenched donor. This complex matrix has been used to study the cellular protein binding affinities (K_d) by three-dimensional FRET microscopy images (Mehta et al., 2009) and dynamic protein interaction to insulin secretory granule behavior with total internal reflection fluorescence (TIRF) microscopy (Lam et al., 2010).

An alternative approach for quantitative characterization of FRET signal is photobleaching, which is comparatively simple to perform on a conventional fluorescence microscope. Photobleaching can be used in multiple ways. The donor bleach rate is directly related to the excited state lifetime and, thus, provides a way to detect change lifetime, which can be monitored by fluorescence lifetime imaging (FLIM). FLIM is independent of changes in probe concentration, excitation intensity and other factors that limit intensity-based steady-state measurements (Wallrabe and Periasamy, 2005). Instrumental methods for measuring fluorescence lifetimes can be divided into frequency domain and time domain, either of which can be used in one- or two-photon FRET-FLIM microscopy. FLIM can independently determine FRET efficiency without being affected by concentrations and allows the measurement of dynamic events at very high temporal resolution (ns). Whereas one- or two-photon FRET

produces an "apparent" energy transfer efficiency, the donor lifetimes obtained by FRET-FLIM usually exhibit as a combination of quenched and unquenched, which allows a more precise estimate of distance than that based on FRET donors only.

Albertazzi et al. (2009) developed a tandem construct with EGFP-mCherry FRET pair linked with a thrombin cleavage site. The advantages of this FRET pair have been quantitatively analyzed by FRET-FLIM spectroscopy. A spectral bleed-through index (CT), which is the ratio between the required correction (cross-excitation plus cross-emission contributions) and the measured fluorescence signal, was applied to characterize the FRET properties. A lower CT index is preferable when choosing FP pairs in FRET. A new concept of a minimal fraction of donor molecules involved in FRET (mf_D) introduced by Padilla-Parra et al. (2008) was applied to monitor dynamic changes in protein-protein interactions between the bromodomains of TAF_{II250} and acetylated histones H4 at high spatial and temporal resolution in living cells by fast acquisition time domain FLIM. This method quantitatively determines multi-lifetime donors in FRET-FLIM without fitting procedures. The requirements of FRET-FLIM technique are that the acceptor is photostable and the donor is photolabile. A variant is measurement of the photobleaching of the acceptor in response to excitation via FRET (Kenworthy, 2001; Valentin et al., 2005; Van Munster et al., 2005), which requires the acceptor to be photolabile and the donor is photostable.

A more straightforward approach is to use direct acceptor photobleaching, which frustrates the occurrence of FRET and monitors the reappearance of the donor fluorescence. The basic principle is the measure donor intensity before and after complete acceptor bleaching, thus providing an internal control by eliminating the occurrence of FRET. The increase in donor intensity can be directly related to the FRET efficiency and should be corrected for bleed-through of the acceptor into the donor detection channel. As its working principle, this method requires that acceptor bleaching be complete, which is difficult due to the low excitation intensities. This limitation was solved by a gradual acceptor photobleaching method, which monitored the donor and acceptor continuously during acceptor photobleaching. It requires curve fitting to determine the FRET efficiency and was relatively simple to use on a normal wide-field microscope (Van Munster et al., 2005). This method was adapted by Peter et al. (2005) to study the complex formation of sterile 2 α-factor receptor protein (Ste2p) *in vivo* with a scanning laser confocal microscope. The developed method included correction for donor bleaching, both for the "complete" and gradual acceptor bleaching. The spectra determined by fitting the measured spectrum were expressed by the normalized spectra of donor and acceptor with scaling factors, which represented the individual amounts of fluorescence emitted.

In addition to the above methods, various strategies correct FRET contamination in special cases (e.g., quantum dots used as FRET donor (Cheng et al., 2009; Prasuhn et al., 2010), single-molecule FRET spectroscopy (Merchant et al., 2007), and FRET between a flat surface and a spherical shell (Bendix et al., 2009)).

Quantitative FRET-based protein interaction affinity and enzyme kinetics determination in the SUMOylation pathway

Recently, we have been developing and applied quantitative and high-throughput FRET-based techniques with particular interests in SUMOylation and other ubiquitin-related pathways. The ubiquitin-related post-translational modification of proteins is critical for various physiological processes. SUMOylation regulates diverse cellular events, including transcription, nucleocytoplasmic translocalization, protein-DNA binding activity, protein-protein interaction, transcriptional regulation, DNA repair, and genome organization (Johnson, 2004). About 100 proteins have been identified as potential SUMO-conjugation targets.

SUMOylation is a transient modification: a member of the SUMO family of proteins covalently attaches to lysine residues in specific target proteins via an enzymatic cascade analogous to the ubiquitination pathway (Andreou and Tavernarakis, 2009). In this dynamic process, the conjugation is mediated by three different enzymes, activating (E1), conjugating (E2), and ligating (E3) enzymes, and the removal of SUMO peptide from substrates is conducted by a family of ubiquitin-like protein-specific proteases(Ulp) in yeast and sentrin/SUMO-specific proteases (SENP) in humans (Yeh, 2009) (Fig. 1). All eukaryotic SUMO proteins are translated as immature precursors. First, a preSUMO is processed by a protease to generate the mature form. In the mature form of SUMO proteins, a C-terminal diglycine motif that is required for efficient adenylation by a heterodimeric SUMO E1 enzyme in an ATP-dependent process is exposed. Second, the SUMO adenylate is attacked by a conserved Cys in E1 enzyme to form an E1-SUMO thioester, and then SUMO is transferred to a conserved Cys in a SUMO E2 enzyme, generating an E2-SUMO thioester. *In vitro*, the SUMO E2 directly recognizes SUMO substrates and transfers the SUMO to the acceptor Lys residue in the substrates. *In vivo*, E3 protein ligases often help to facilitate the sumoylation process by two mechanisms. They recruit the E2-SUMO thioester and substrate into a complex to promote specificity, or in the case of substrates that interact directly with the E2 enzyme, they enhance conjugation by stimulating the ability of the E2 enzyme to discharge SUMO to substrates. Finally, an isopeptide bond is formed between SUMO and its target lysine residue in the substrate, altering the function of the substrate. SUMO is subsequently removed from the substrate by the isopeptidase activity of the UIP/SENP family (Gareau and Lima, 2010).

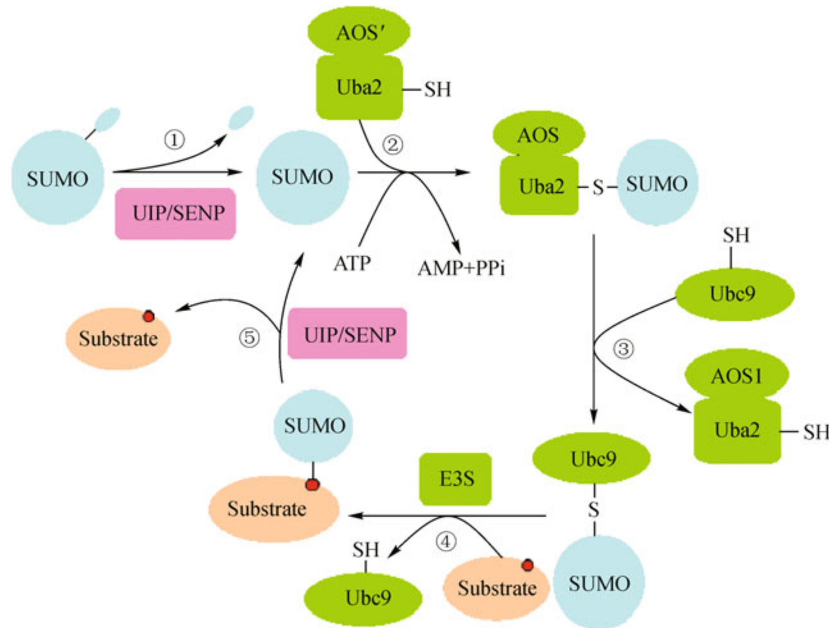

Figure 1 SUMOylation conjugation cascade. ① Maturation of SUMO by cleavage of SUMO C-terminus by SENP proteases. ② SUMO activation (linkage to E1) by heterodimer of E1 ligase. ③ SUMO is transferred to E2 ligase. ④ Conjugation of SUMO peptide to substrate(s) is mediated by E3 ligase. ⑤ Removal of SUMO peptide from substrate by SENP proteases. Please see the text for abbreviations.

We developed a FRET-based method to measure the disassociation constant (K_d) for the interaction of SUMO1 and Ubc9 and a cell-based HTS assay in the SUMOylation cascade by FRET technology (Song et al., 2011). These methods are based on the quantitative analysis of FRET spectra (Fig. 2). We reasoned that the FRET signal is correlated with the bound partner concentration. The direct emissions at 530 nm of the donor and the acceptor should be determined and excluded from the total emission at 530 nm. When the mixture of donor and acceptor is excited at 414 nm, two emission peaks can be observed at 475 nm and 530 nm (Fig. 2A), respectively. Emission at 475 nm is from the emission of unquenched donor (FL_{DD}). The emission intensity at 530 nm (FL_{DA}, Fig. 2A yellow) consists of three components: the direct emission of unquenched donor (Fig. 2A blue), the direct emission of acceptor (Fig. 2A light green), and the emission of acceptor excited by energy transferred from donor (Em_{FRET}, Fig. 2A light yellow). Excited at 475 nm, an emission peak at 530 nm (FL_{AA}) can be observed, which results from the direct excitation of acceptor but not donor (Fig. 2B).

The direct emission of donor at 530 nm is proportional to its emission at 475 nm when excited at 414 nm with a ratio factor of a (Fig. 2C), while the direct emission of acceptor at 530 nm is proportional to its emission at 530 nm when excited at 475 nm with a ratio factor of b (Fig. 2D). Therefore, the FRET emission signal of the acceptor (Em_{FRET}) can be determined by:

$$Em_{\mathrm{FRET}} = (FL_{DA}) - a * (FL_{DD}) - b * (FL_{AA})$$

Where FL_{DA} is the total fluorescence emission at the acceptor wavelength when excited at the donor excitation wavelength, FL_{DD} is the fluorescence emission at the donor wavelength when excited at the donor excitation wavelength, and FL_{AA} is the fluorescence emission at the acceptor wavelength when excited at the acceptor excitation wavelength.

An engineered FRET pair, CyPet and YPet, was used in this analysis. CyPet and YPet are developed from CFP and YFP with much higher fluorescence quantum yield and FRET efficiency (Nguyen and Daugherty, 2005). In contrast to the traditional ratiometric criteria of a FRET signal, we differentiated and quantified absolute fluorescence signals contributed by donor, acceptor and FRET, respectively, at the acceptor emission wavelength and converted them into protein concentrations with control fluorescent protein standards. This method gives much better resolution than traditional ratiometric measurement of FRET. The K_d of SUMO1 and Ubc9 has been determined by this technique and agrees very well with that determined by other methods, such as surface plasmon resonance (BIACORE). We improved the theoretical and experimental procedure to derive K_d from FRET signal by characterizing the quantitative contributions of donor and acceptor alone. Another group also demonstrated the application of the FRET assay for K_d measurement with the classical CFP and YFP pair. However, in this approach, the donor and acceptor must be calibrated in separate fluorescent measurements. The data from all assays fit into a very small range and are very tight to each other (Tatham et al., 2003; Martin et al., 2008). This shows the

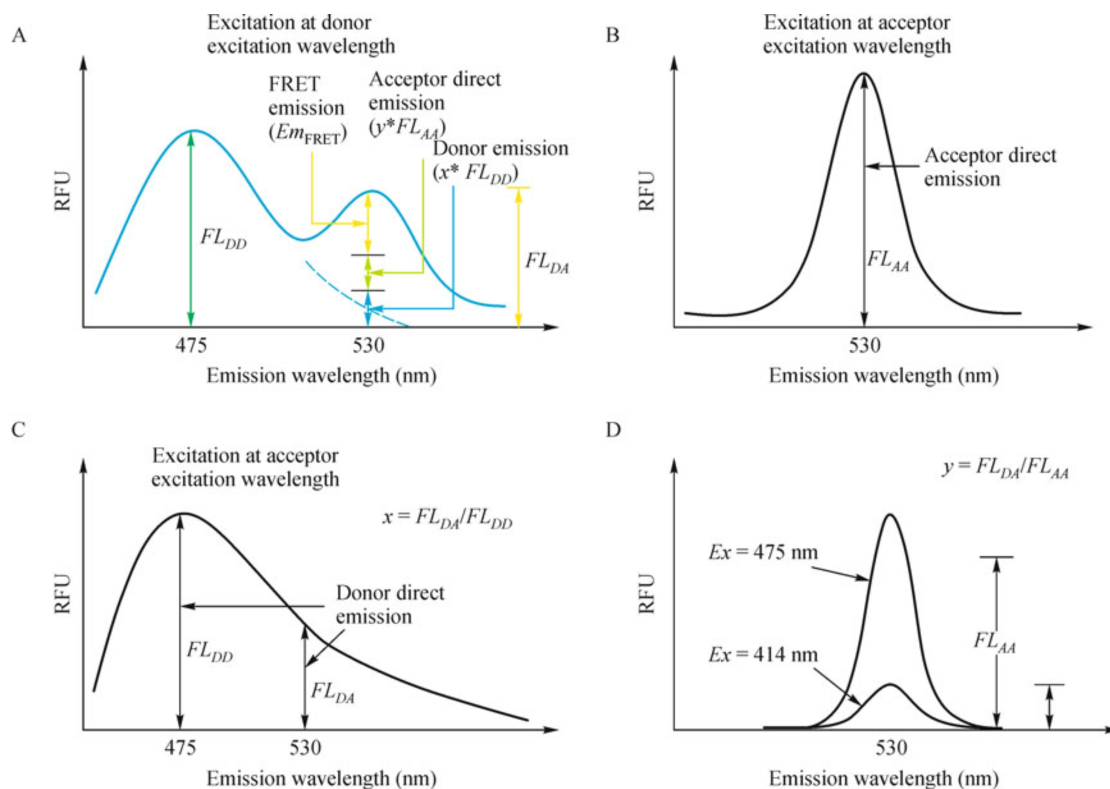

Figure 2 Quantitative analysis of fluorescence signals of FRET emission. RFU: Relative fluorescent units. (A) Fluorescent emission at acceptor wavelength (530 nm) (FL_{DA}) can be divided into three fractions—FRET emission, direct emission of donor, and direct emission of acceptor. (B) Acceptor emission when excited at 475 nm (FL_{AA}). (C) Donor emission at 475 nm (FL_{DD}) and 530 nm (FL_{DA}) when excited at 414 nm. (D) Donor emission at 530 nm when excited at 414 nm (FL_{DA}) or 475 nm (FL_{AA}).

interaction affinity measured by the FRET assay is not only feasible but also accurate.

SENP acts as an endopeptidase to process the pre-SUMO or an isopeptidase to deconjugate SUMO from its substrate. We developed another highly sensitive and quantitative FRET-based protease assay to characterize the maturation process of the preSUMOs. Similar to the protein interaction affinity study, theoretical and experimental procedures were developed to determine the catalytic efficiency (k_{cat}/K_m) of different SENPs toward various pre-SUMOs substrates. The catalytic k_{cat}/K_m ratio of catalytic domain of SENP1 toward pre-SUMO1 was obtained by this FRET-based protease assay and consistent to the results obtained from other methods (Reverter and Lima, 2006; Shen et al., 2006).

Bioactive small chemical compounds offer better spatial and temporal control of biological processes and can be used to investigate the biological function of proteins when gene knockout studies are not feasible. Therefore, small chemical compounds that disrupt the interactions between components in SUMOylation will be very useful to dissect its roles *in vivo*. Currently, there is no available small chemical compound specific for SUMOylation pathways, and this observation indicates an urgent need in developing HTS assays for small-molecule inhibitors. We developed a cell-based FRET HTS assay to identify small chemical compounds that disrupt the interaction of SUMO1 and Ubc9 (Song et al., 2011). Cell lines that stably express the two fusion proteins, CyPet-SUMO1 and YPet-Ubc9, were obtained. A library of compounds was applied to the cells to determine their effects on the FRET signal in these cells. This screening format can simultaneously determine activities and cell-permeability of compounds. We also developed another two *in vitro* FRET-based HTS assays for the SUMO1-Ubc9 interaction and the pre-SUMO's maturation inhibitor discovery with the same FRET pair. After purified recombinant proteins were mixed *in vitro*, compounds were added to determine their ability to inhibit FRET signal between two proteins. The conditions of the screening can be easily optimized by changing the amount of proteins used in the assay, and it was performed in 384-well plate format, which readily allows reproducible study and large-scale screening, such as industrial applications.

The novel developments of quantitative FRET analysis and HTS can be general platform for K_d and k_{cat}/K_m measurements, and chemical probe discoveries, as long as two interaction partners can be labeled by a FRET pair. The FRET technology, employing either fluorescent proteins or organic/inorganic fluorophores, has been widely used in various biological studies. Our methodology can be applied to general FRET platforms for K_d determination of protein-protein, protein-small molecule or small molecule-small molecule

interaction. Our FRET-based HTS is suitable for high-throughput assays. After the genomic era, large-scale and genome-wide mapping protein-protein interactions and inhibitor discoveries can provide comprehensive understanding of protein interaction networks in cells and potential new drug targets.

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