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Recent applications of FRET-based multiplexed techniques

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ABSTRACT

Fluorescence resonance energy transfer (FRET) has become a powerful tool for visualizing molecular interactions, single-molecule conformational dynamics, binding, and other molecular signaling events. However, until recently, FRET spectroscopy/microscopy has been mainly used for monitoring single events. Recent pursuits on simultaneous detection and analysis of multiple analytes have led to the development of various FRET-based multiplexed techniques. The concomitant increase in the experimental complexity and data analysis due to the use of multiple donors/acceptor pairs and multiple excitation sources have proven to be the major challenges for rapid expansion of these techniques. In this review, we will focus on recent applications of FRET-based multiplexed techniques using coupled chromophore platforms composed of organic dyes, quantum dots (QDs), lanthanides, and fluorescent proteins, and discuss some of the current challenges that need to be addressed to further expand their applications in this new exciting area.

1. Introduction

Förster resonance energy transfer (FRET), also known as fluorescence resonance energy transfer, is a process by which an excited state fluorophore (termed donor) non-radiatively transfers energy to another fluorophore (termed acceptor) via distance-dependent long-range dipole-dipole interactions [1–5]. FRET occurs only when the emission spectrum of the donor overlaps with the excitation spectrum of the acceptor. The distance over which energy transfer can occur is limited to ~10 nm, and the efficiency of transfer is extremely sensitive to small changes in the donor-acceptor distance, which makes the FRET measurement a powerful analytical tool for visualizing conformational dynamics at the molecular level, probing inter-molecular interactions, and binding/unbinding events [1–5]. FRET measurements can be used as a molecular ruler for determining conformations and dynamics of biomolecules by real-time monitoring of the distances between the donor-acceptor pair via changes in their fluorescence signals or FRET efficiencies. Since the FRET process is not mediated by photon emission and thus the acceptor dye does not have to be fluorescent, the technique is broadly applicable to diverse systems. However, FRET is sensitive to the surrounding environment, such as solvent pH, polarity, viscosity, etc. Therefore, environmental factors should be considered when designing the experiments and interpreting the FRET data [6–8]. FRET measurement can be performed either with a single FRET or multiplexed FRET technique. Compared to techniques using single FRET, multiplexed FRET techniques have several advantages. For example, such methods can be used to investigate coinciding biomolecular events, probe inter- and intramolecular interactions, and simultaneously analyze multiple analytes [4–6]. While the basics of FRET and the multiplexed FRET phenomena have been covered by previous reviews [1–5], in this review, we will focus on recent applications of FRET-based multiplexed techniques.

During the past decade, the development of FRET-based multiplexed techniques has attracted a great deal of attention [4,5,9–12] with the primary motivation to enhance the applications and throughput of FRET-based assays/measurements. Multiplexed detection allows multiple processes or targets to be studied simultaneously, conserving time, cost, and resources. Multiplexing is of particular interest in fields such as clinical diagnostics as the reliability of disease diagnosis can be significantly improved by simultaneous detection of multiple biomarkers. It is equally important in biotechnology as the simultaneous analysis of biomarkers can provide a better understanding of the interplay of interacting biomolecules. Of the various techniques used to develop multiplexed platforms (surface enhanced Raman spectroscopy, nanopores, electrochemical sensors, etc.), photoluminescence-based approaches are common [13]. Photoluminescence-based multiplexed platforms range from commercially available

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fluorescent microarrays for nucleic acid and protein detection [14,15] and multiplex polymerase chain reactions [16] to recently developed ratiometric fluorescence sensors [17,18], temporal barcodes [19–21], and fluorescent nanoparticles [22–25]. For example, Liu et al. developed a DNA triangular-prism sensor and successfully applied it for simultaneous detection of three different DNA sequences based on ratiometric fluorescence [18]. Similarly, multi-fluorophore FRET has been used to study protein-protein interactions and to determine stoichiometry in protein complexes [5] using various microscopy methods [4]. Additionally, intracellular imaging/sensing has been advanced with multiplexed FRET platforms geared toward monitoring multiple biomolecular events in an effort to provide a clearer picture of intra-cellular pathways [26]. Since FRET efficiency is a ratiometric quantity dependent on the fluorescence emission intensity of the donor and/or acceptor which correlates to the distance between the two fluorophores, it has been utilized for multiplexed sensing of nucleic acids, proteins, and small molecules. Several methods have been adopted, such as using multiple orthogonal FRET pairs, two-step FRET cascades where excitation of one fluorophore results in sequential FRET to a second and third fluorophore, and antenna systems made up of only one donor and many acceptors [5,27]. FRET has also been harnessed for multiplexed detection using only a single FRET pair as multiple FRET efficiencies can be achieved by tuning the donor-acceptor distances [28–30].

A variety of materials have been used to create these multiplexed approaches [13,31–33]. This review will focus on the most prominent techniques developed for multiplexed-FRET using coupled chromophore platforms composed of organic dyes, quantum dots (QDs), lanthanide-based composites, and fluorescent proteins (Fig. 1). With their ease of chemical modification and labeling onto other substrates, these techniques are suitable for imaging and sensing of a variety of molecular events and spectroscopic measurements. Below, we have discussed these prominent FRET-based multiplexed techniques and highlighted their recent applications spanning a wide range of fields such as analytical sensing and cellular imaging.

2. Multiplexed techniques

2.1. Organic dyes, cationic polymers, and carbon-based quenchers

Organic dyes are the most commonly used fluorophores in FRET experiments today. This is due to their ease of labeling onto various substrates, such as proteins and nucleic acids, and recent development of a large variety of dye families that are commercially available. With recent advances in the production of photostable dyes with high quantum yields, the number of applications using organic dyes have soared for both single- and multiplexed-FRET approaches. One way of multiplexing using organic dyes involves a sequential FRET between donor and acceptor fluorophores where excitation of one or more donors results in a cascade of energy transfer [34–36]. For example, Li et al. demonstrated a proof-of-concept multiplex cascade sensing strategy by utilizing four spectrally overlapping dyes to simultaneously detect three different DNA targets [34]. This cascade technique has also been applied to create potential photonic circuit board devices [35]. For example, using these linear FRET cascades, Stein et al. showed how energy transfer paths can be disrupted or switched to generate different FRET signals [35]. By arranging three different fluorophores in a linear fashion on a DNA origami platform, they were able to switch the position of an intermediary dye to produce two different FRET cascade paths with potential applications in photonic devices as well as in sensing (Fig. 2a).

Similarly, organic fluorophores have aided advances in multicolor FRET barcodes with applications in FRET imaging. These barcodes consist of multiple organic fluorophores labeled onto or encapsulated within nano- or microparticles [37–39]. Wagh et al. developed polymeric nanoparticles that encapsulated varying combinations and concentrations of four different carbocyanine-based dyes to enable two-, three-, and four-fluorophore FRET [38]. The key advantage of these polymeric nanoparticles is that they can be employed for multiplexed imaging both *in vitro* and *in vivo* with a single wavelength excitation. In addition to multiplexed imaging, these dye-loaded nanoparticles can be used in traceable drug delivery systems by encapsulating aptamer-bound anti-cancer drugs [39]. Therefore, they bear tremendous potential for targeted delivery and multiplexing by using multiple aptamer-drug complexes and various combinations and concentrations of fluorophores.

Apart from multiplex FRET barcoding for imaging and sensing applications, multi-fluorophore FRET systems have also been emerging for studying multi-component dynamics of biomolecules, such as proteins and nucleic acids [41]. For example, using a single donor and two (or more) acceptors that are labeled onto different regions of biomolecules (or different biomolecules), complex biomolecular interactions have been determined [42–44]. For instance, using alternating laser excitation and lifetime measurements, Yoo et al. studied the fast folding dynamics of a protein, a_3D, by labeling its different domains with a single donor and two acceptors [42]. Similarly, Barth et al. demonstrated a three-color photon distribution analysis to extract distances between

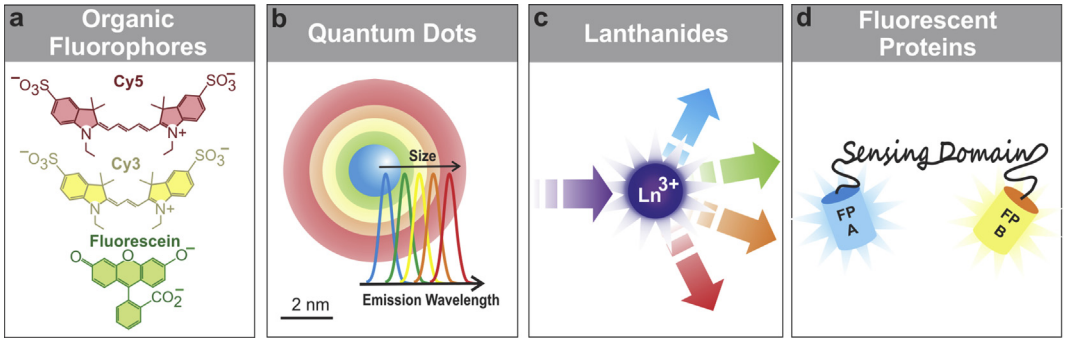

Fig. 1. Schematic representation of four common materials used in multiplex FRET-based techniques. (**a**) Example of organic fluorophores with distinct emission wavelengths, (**b**) quantum dots with size tunable emission, (**c**) lanthanides producing multiple emission wavelengths with single wavelength excitation, and (**d**) fluorescent proteins tethered by a sensing domain.

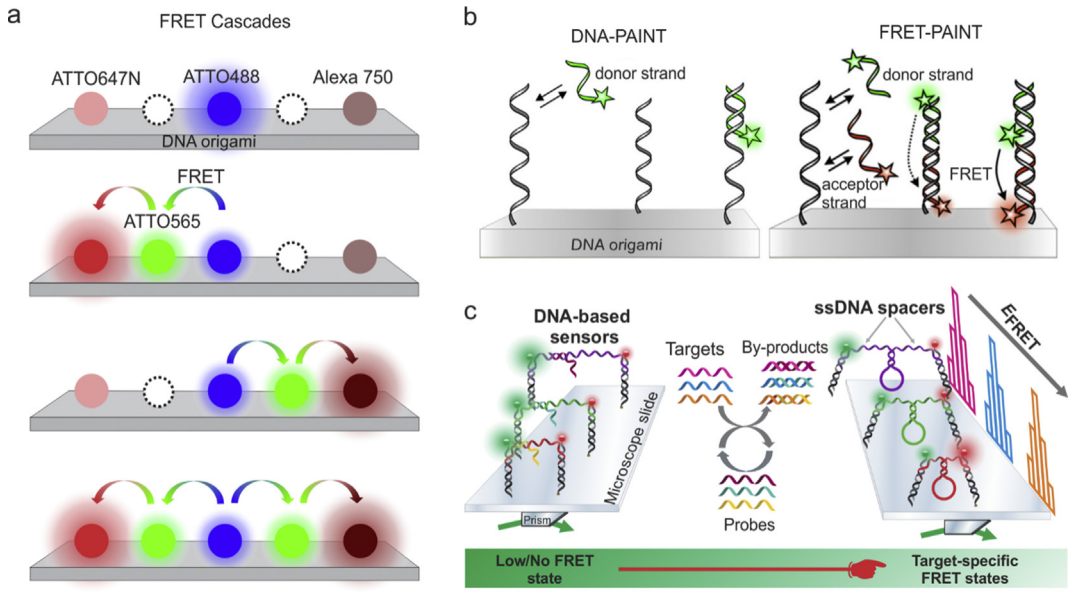

Fig. 2. Multiplexed FRET using organic dyes. (**a**) FRET cascades showing how energy transfer paths can be changed depending on the location of the intermediary dye which acts as an acceptor for the blue dye and a donor for both the red and infra-red (IR) dyes. Open circles represent the absence of the intermediary dye (green, ATTO565), blue refers to the primary donor fluorophore (ATTO488), red and maroon represent red (ATTO647N) and IR (Alexa 750) emission, respectively. Adapted from Stein et al. [35] with permission from the American Chemical Society. (**b**) Comparison of traditional DNA-PAINT (uses a singly labeled reporter) and FRET-PAINT (utilizes donor and acceptor labeled strands). Two different inter-dye distances provide two different FRET efficiencies allowing multiplex detection. Adapted from Deußner-Helfmann et al. [40] with permission from the American Chemical Society. (**c**) Working principle of DNA-based multiplexed sensors using a single donor-acceptor pair. Toehold-mediated strand displacement (TMSD) of a probe by a target sequence initiates hairpin formation, which allows energy transfer from donor to acceptor. Single-stranded DNA (ssDNA) spacers of varying lengths tune the distance between the two fluorophores providing distance-dependent FRET efficiencies for each sensor. Adapted from Kaur et al. [28] with permission from the American Chemical Society.

different domains of Hsp70 chaperone BiP in diffusion-based three-color FRET experiments, successfully visualizing its conformational changes [43].

Although multi-color FRET systems have several advantages, as described above, one major drawback of these systems, particularly for sensing and imaging applications, is the instrument cost and complexity of the data analysis. This is because these systems often require multiple excitation sources and processing of multiple emission wavelengths. Multiple excitation sources are possible only with high-end instrumentation that are technically involved and costly. Some alternative approaches have been emerging in recent years to tackle these limitations. For example, tuning the FRET distance between a single donor and acceptor has been shown to provide varying FRET efficiency values, which can be used to distinguish different events occurring within the same system under study. For instance, multiple excitation sources are required for the original DNA-PAINT [45,46] and Exchange-PAINT [47] techniques as they utilize multiple fluorophore labels and sequential imaging which hinder the throughput of the experiment. Deußner-Helfmann et al. offered an alternative approach by taking advantage of the distance-dependent nature of FRET [40]. They expanded correlative single-molecule FRET to DNA-PAINT with the use of two imaging strands each labeled with either a donor or acceptor fluorophore (Fig. 2b). Each imaging strand was designed to produce a different FRET efficiency upon binding to the docking strand depending on the sequence and labeling position of the fluorophore. In this study, it was possible to assign FRET values to specific targets which provided a means to multiplex DNA-PAINT with the use of a single donor-acceptor pair. Similarly, multiplexed sensing using multi-fluorophore platforms such as antenna or surplus systems are promising but these techniques involve complicated data analysis procedures [5]. Kaur et al. has recently developed a FRET-based multiplexing platform for the detection of unlabeled DNA sequences with the use of only a single donor-acceptor pair (Fig. 2c) [28]. Simultaneous detection of three different DNA targets was possible by tuning the inter-dye distance of interconvertible Hairpin-based sensors (iHabSs). In this strategy, three iHabSs, each containing a hairpin moiety flanked with different lengths of ssDNA spacers, were used to tune iHabS-specific FRET efficiencies. A significant portion of each hairpin sequence was designed to partially hybridize with a specific DNA probe to keep the iHabSs in a low-FRET state in the absence of targets. The presence of targets induce the formation of DNA hairpins through toehold-mediated displacement of corresponding probes, bringing the donor-acceptor pair closer together, providing iHabS-specific FRET values. Three spectrally resolvable FRET efficiencies were demonstrated with potential to detect up to six targets simultaneously. The key advantage of this detection platform is that multiplex detection can be achieved by sensor-specific FRET efficiency regardless of the position of the sensors on the slide surface [28].

Aside from organic dyes, there are other organic materials which have also been shown to exhibit FRET. By coupling with multiple organic dye acceptors, cationic conjugated polymers (CCPs) have been used as donors to create multiplex FRET for detection of DNA mutations [50,51], microRNAs [48], and protein activity [52]. The general principle has been to employ CCPs exhibiting excitation and emission in the UV–Vis range with fluorophore-labeled DNA probes. The negatively charged backbone of DNA readily binds to the positively charged CCP allowing energy transfer from CCP to the fluorophore (Fig. 3a). In this strategy, multiple fluorescence signals can be generated for different targets. For example, Zhou et al. used FAM- and Cy3-labeled DNA probes to detect two different microRNA sequences (Fig. 3a) [48]. Here, the DNA probes electrostatically interact with the CCP producing a FRET cascade as CCP transfers energy to both FAM and Cy3 in the absence of miRNA targets. The presence of a specific microRNA (target) results in the formation of a DNA-miRNA duplex and

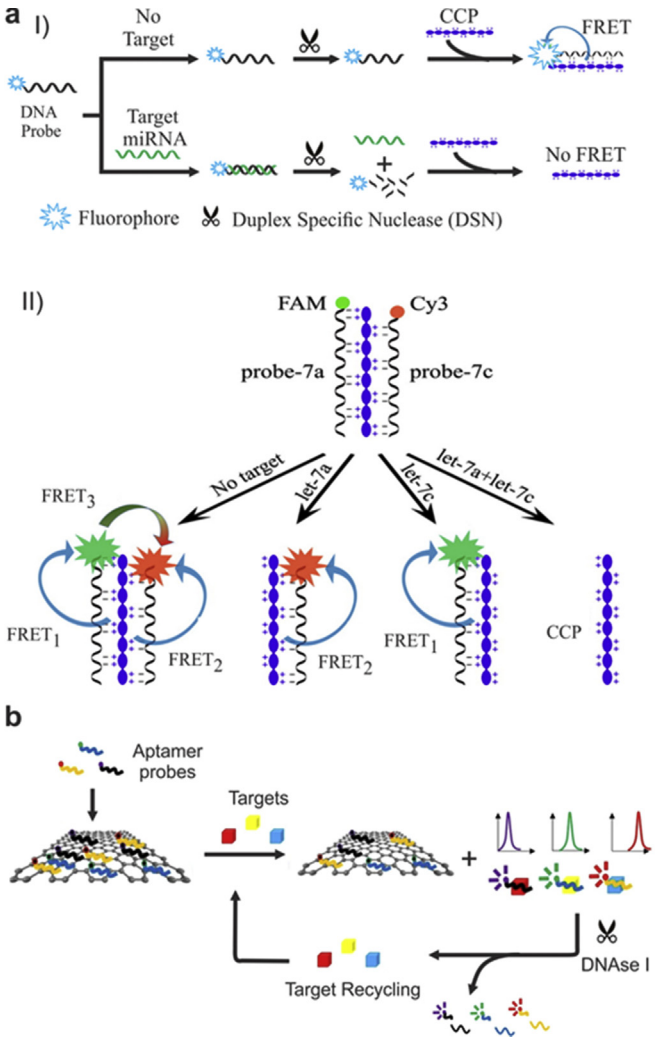

Fig. 3. Multiplexed FRET using organic dyes. (**a**) i) Working principle of microRNA detection using fluorophore-labeled DNA probes and a cationic conjugated polymer (CCP). ii) Schematic representation of multiplexed miRNA analysis. Changes in fluorescence spectra due to one or more FRET pathways reveal the presence or absence of targets. Adapted from Zhou et al. [48] with permission from the American Chemical Society. (**b**) Stacking interactions between fluorophore-labeled DNA aptamers and graphene oxide effectively quenches fluorescence. The binding of small molecule targets to specific aptamers disrupts the stacking interaction, allowing digestion of aptamers by DNAse I and ultimately increasing the fluorescence. The release of targets upon digestion of aptamer allows for multiple rounds of sensing. Adapted from Youn et al. [49] with permission from Creative Commons License.

nuclease degradation of the DNA probe by duplex-specific nuclease (DSN), leading to a loss of FRET [48]. In contrast, while CCPs act as donors to amplify the acceptor signal, other materials such as organic quenchers act as acceptors to quench the fluorescence. Such quenchers have similarly been used to create multiplex FRET platforms based on signal on-off strategies [53–56]. Graphene oxide is one such example (Fig. 3b) where fluorescence of a labeled-DNA probe is quenched by the stacking interaction between graphene oxide and DNA [49,57,58]. Using this principle, multiplexed detection of small molecules has been successfully demonstrated employing fluorophore-labeled DNA aptamers and graphene oxide [49]. Because the binding of small molecule targets to specific aptamers disrupts the stacking interaction, the aptamers are released from the graphene oxide surface, producing fluorescence. Another advantage of this platform is that the release of targets due to the digestion of aptamer by DNAse I allows multiple rounds of sensing.

2.2. Quantum dots (QDs)

Since there have been several review articles which highlight the various applications of QD-based FRET systems [59–67], we will focus on a few recent applications of QD-based multiplexed FRET. Quantum dots are inorganic semiconductor nanocrystals (some common examples being those made with CdSe or PbSe) [33,65,66]. They exhibit unique photophysical properties, such as broad absorption bands and narrow emission spectra that can be tuned by varying the size of the nanocrystal [65]. This size-dependent emission, high chemical and thermal stability, high surface area, and well-established approaches for coupling QDs with a variety of chemicals and biomolecules have made them a great choice for multiplexed FRET applications [61,62,65].

Although QDs have been used as donors, acceptors, or both, most QD-FRET techniques take advantage of their donor properties, coupling them with multiple acceptors. Such systems have been applied to DNA hybridization reactions [68–70], cellular imaging [71], and monitoring of enzymatic activity [72–76]. In the simplest form, Zhang et al. used a streptavidin coated QD as a substrate to capture and detect two different DNA sequences simultaneously [68]. The target DNA was captured onto the surface of the QD by hybridization between a biotin-immobilized capture probe and a fluorophore-labeled reporter probe (Alexa Fluor 488 or Alexa Fluor 647). In this approach, upon excitation at 488 nm, both Alexa Fluor 488 and the QD produce spectrally resolved emissions while Alexa Fluor 647 acts as an acceptor for the QD. Therefore, it allows multiplexing by using both coincidence fluorescence and FRET emission. Similarly, Hu et al. showed a similar process coupled with isothermal amplification of a circular DNA template for the detection of low abundance microRNAs [70]. In both examples, the only donor is the QD and both acceptor dyes are orthogonal to one another. Alternatively, Wang et al. created a multiplexed aptasensor for detection of aflatoxin B1 and fumonisin B1 using green or red quantum dots labeled onto aptamers specific to each mycotoxin [77]. A graphene oxide (GO)/Fe_3O_4 nanocomposite was used as the acceptor for both quantum dots where the Fe_3O_4 facilitates magnetic separation and removal of the scaffold. In the absence of mycotoxins, the QD fluorescence is quenched due to stacking interactions between the aptamer and the GO/Fe_3O_4. However, the aptamers are released upon binding to their respective mycotoxin targets, resulting in the recovery of fluorescence signals.

More complex QD-FRET systems have also been developed where QDs act as a donor for multiple acceptors, which in turn act as donors themselves. Like FRET barcodes and cascade systems using organic dyes [34,36,78], these concentric FRET (cFRET) systems yield multiplexed detection by monitoring the changes in photoluminescence of all possible emitting species [73,79,80]. Most commonly, the cFRET studies have been applied to detect and monitor enzymatic activity [71–75]. For example, the simultaneous sensing of the proteolytic activity of trypsin and chymotrypsin was successfully achieved by tethering two different dye-labeled peptides that are specific to digestion by one of the two proteases to the QD surface [75]. This design enables cFRET upon excitation of the central donor QD which can donate energy to both fluorophores, one of which can subsequently act as second donor, thus producing three FRET pathways. Digestion of one or both peptides results in a change in the photoluminescence and number of FRET pathways available resulting in detection of the proteolytic activity. Massey et al. further expanded this system by adding a third dye-labeled peptide specific for another protease (Fig. 4a). In this design, all three dyes act as acceptors for the central QD, while some dyes also act as donors for dye-to-dye FRET [76]. Similarly, cFRET has also been applied to detect enzymatic activity of multiple endonucleases using a defined DNA tetrahedral nanostructure tethered to the

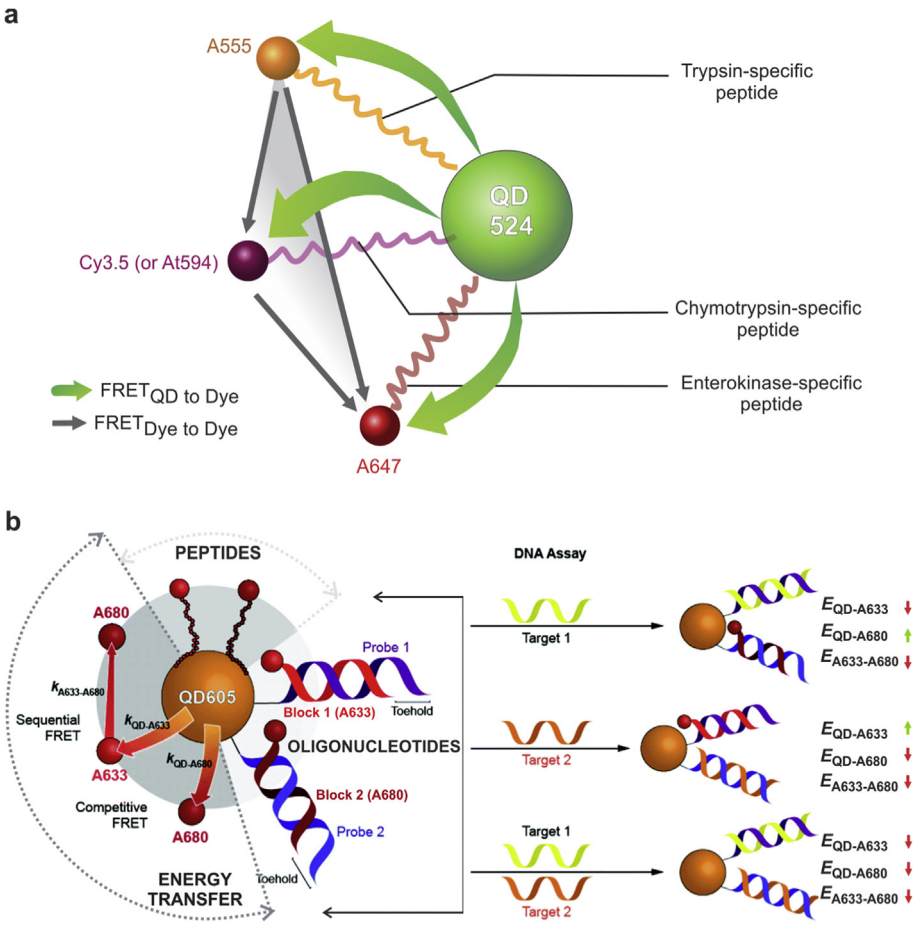

Fig. 4. Multiplexed FRET using surface-modified QDs. (**a**) Design of the concentric FRET (cFRET) relay system for sensing proteolytic activity of chymotrypsin (ChT), trypsin (TrP), and enterokinase (EK). Protease specific peptides labeled with either Alexa Fluor 555 (A555), Alexa Fluor 647 (A647), and Cy3.5 (or At594) bind to the QD (524 nm emission) via a His-tag. A647 acts as an acceptor for QD, Cy3.5, and A647. Further, Cy3.5 acts as an acceptor for both QD and A555 whereas A555 acts as an acceptor for QD only. Adapted from Massey et al. [76] with permission from the American Chemical Society. (**b**) Working principle of cFRET between two dye acceptors, Alexa Fluor 633 (A633) and Alexa Fluor 680 (A680), and a central QD (605 nm emission) where A633 can act as a sequential donor. These fluorophores can either be labeled onto peptides for protease activity or onto oligonucleotides for nucleic acid detection. DNA hybridization via TMSD of fluorophore labeled strands (Block 1 or 2) by sequence specific targets result in varying energy transfer ratios. Adapted from Li et al. [69] with permission from the Royal Society of Chemistry.

surface of a QD where the tetrahedral nanostructure serves to maintain a well-defined inter-dye spacing among three different dyes and the QD [74]. Cleavage of a specific sequence results in the loss of a FRET pathway, enabling the detection of specific enzymatic activity. Apart from enzymatic activity, Li et al. were able to show that cFRET can be used to detect unlabeled nucleic acid sequences by displacing dye-labeled oligonucleotides (via TMSD) from sequence-specific probes tethered to a QD (Fig. 4b), thus expanding the application of the technique [69].

2.3. Lanthanides

In recent years, lanthanide complexes have been emerging as promising fluorescent materials in sensing and other applications [81–83]. Lanthanide complexes made up of a lanthanide center (usually Tb^{3+} or Eu^{3+}) coordinated to aromatic ligands have become popular donors in FRET systems with the use of time-gated FRET [82–85]. Although they have narrower UV-range absorptions than QDs, lanthanide complexes are excellent donors due to their long photoluminescent decays, particularly for organic acceptors or QDs. Further, with the use of time-gated fluorescence detection, background fluorescence from directly excited, short-lived acceptors, and auto-fluorescence can be minimized improving the signal-to-noise (S/N) ratio for solution-based sensing [85].

Although there are emerging applications of Eu^{3+}-based FRET techniques, they are not as common as Tb^{3+}. In this review, we will focus on Tb^{3+}-based applications that have shown great promise as multiplexed FRET platforms due to their distinct and well-resolved emission spectra. To enable multiplexing, lanthanide complexes are often coupled with multiple fluorophores [29,86–90], QDs [91–93], or a combination of both [30,94] as acceptors [83–85]. In the case of a single Tb^{3+} donor and multiple dye acceptors, Geißler et al. developed a sandwich type immunoassay using terbium and dye-labeled antibodies [95] that can detect up to five tumor markers simultaneously. Excitation of terbium labels by UV light resulted in FRET emission of the dye, which could be monitored by time-gated photoluminescence decay measurements. The dye has a longer decay lifetime upon excitation via FRET from a Tb^{3+} donor than non-specific excitation due to the long-lived photoluminescence of lanthanide complexes, which makes such solution-based multiplexed detection possible. Instead of multi-color FRET detection, Qiu et al. and Guo et al. took advantage of FRET by tuning the distance between Tb^{3+} donors and dye acceptors to achieve multiplexing [29,87]. Qiu et al., in particular, coupled this distance-tuning method and time-gated FRET with rolling circle

amplification (RCA) [96,97] to achieve a high sensitivity detection of nucleic acids (Fig. 5a) [98]. They were able to simultaneously and quantitatively determine the concentrations of four different targets with attomolar (10^{-18} M) detection limits using only two dyes and a single excitation wavelength.

Like QDs, lanthanides are generally used as donors in FRET-based multiplexed systems. When coupled with Tb^{3+}, however, QDs can be efficiently used as acceptors with narrow emission spectra which can be tuned to emit between the gaps of the terbium emission spectra. Geißler et al. showed how Tb-QD FRET systems could be easily multiplexed by utilizing the size-tunable emission of QDs [93]. As a proof-of-concept, using five different sizes of biotin-coated QDs that produced spectrally separated emissions falling between the gaps of terbium emissions, excitation of terbium-labeled streptavidin resulted in simultaneous FRET emission of the QDs upon the formation of a complex via biotin-streptavidin interaction. Qiu et al. utilized this technique and applied it to detect three different microRNAs using DNA probes immobilized on the surface of three QDs producing three different luminescent maxima (Fig. 5b) [91]. In this system, the detection was facilitated by the hybridization of target microRNA to a reporter DNA labeled with a Tb^{3+} complex that could only be stably hybridized to a QD in the presence of target microRNA. This strategy allowed 3-fold multiplexing in solution with low nanomolar (10^{-9} M) detection limits even in the presence of up to 10% serum.

Aside from sensing applications, lanthanide complexes have also been utilized for FRET-based imaging applications. In particular, lanthanides coupled with QDs can be used as multicolor barcodes for imaging. For example, Chen et al. utilized core-shell nanoparticles with a QD core and a Tb^{3+} (or Eu^{3+}) loaded shell of varying thicknesses to produce multiplexed time-gated FRET barcodes (Fig. 5c) [30]. Emission was monitored at three different time points to determine the ratio of red/green/blue (RGB) emission upon excitation of the lanthanide complexes in the shell. By

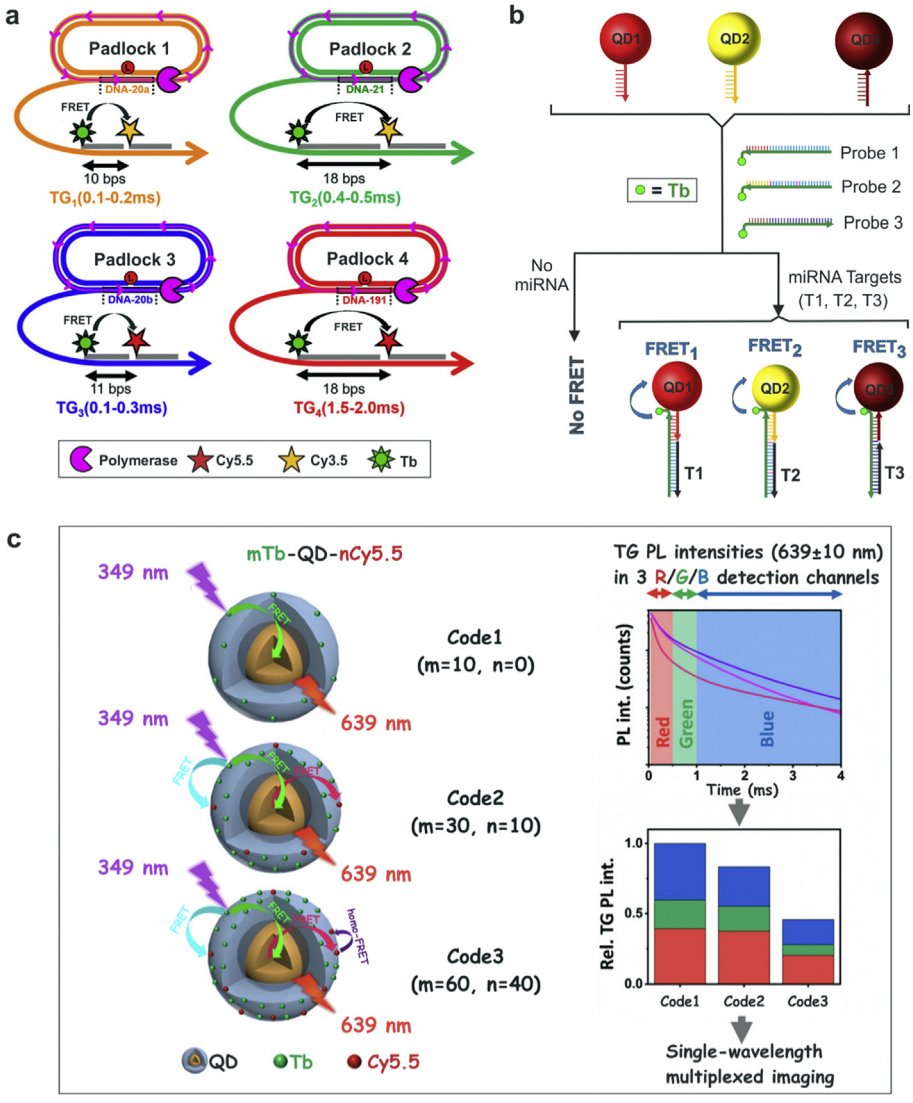

Fig. 5. Multiplexed FRET using QDs along with terbium and organic dye. (**a**) Rolling circle amplification (RCA)-mediated detection of 4 different DNA targets. Binding of target facilitates ligation of padlocks and allows RCA to commence. Distance tuned probes, one labeled with a terbium donor and another with one of two acceptor dyes, provides two time-gated (TG) FRET signals in two detection channels (Cy5.5 and Cy3.5). Adapted from Qiu et al. [98] with permission from the American Chemical Society. (**b**) Multiplex detection of three microRNA sequences is facilitated by complex formation between microRNA target, terbium-labeled probe, and short DNA strands surface-conjugated to three different QDs. The energy is transferred from terbium to QD ($FRET_1$, $FRET_2$, and $FRET_3$). Sequence specific probes labeled with terbium complexes remain unbound in the absence of their microRNA targets due to poor hybridization efficiency of short DNA strands conjugated to the three QDs. Adapted from Qiu et al. [91] with permission from the American Chemical Society. (**c**) Design and working principle of multi-hybrid core-shell nanoparticles for single wavelength multiplex barcoding. Ratios of TG photoluminescence intensities in the red, green, and blue detection channels produce distinct barcodes for application in imaging. Adapted from Chen et al. [94] with permission from the American Chemical Society.

using two different lanthanides which produce distinct emission spectra and tuning the distance between lanthanide complexes in the shell from the QD core provided four different FRET barcodes. They further optimized this system by introducing an organic dye into the shell at varying concentrations, which produced different RGB ratios [94]. Three different barcodes were prepared, one of which contained only Tb^{3+} complex in its shell with a QD core and two multi-hybrid particles with varied concentrations of either Tb^{3+} complex or Cy5.5 (Fig. 5c). These particles created a FRET cascade where direct excitation of Tb^{3+} resulted in energy transfer to the QD core, which further transferred energy to Cy5.5. In order to test the applicability of their multiplexed barcode system, Chen et al. prepared multi-hybrid nanoparticle-doped microbeads and demonstrated that the microbeads can be distinguished by their RGB ratios [94]. Taken together, these studies demonstrated that depending on the loading concentration of Cy5.5 or Tb^{3+}, different RGB ratios can be achieved producing multiplexed FRET barcodes for potential cellular imaging.

2.4. Fluorescent proteins

Fluorescent proteins are popular FRET pairs for applications in sensing and imaging directly within cells as they can be genetically encoded and labeled onto specific protein domains [5,99]. Since cells contain complex signaling networks which are often an accumulation of multiple inter- and intra-biomolecular interactions, such as conformational switching of single proteins, or interactions between biomolecules, genetically encoded sensors allow a means to look at these biomolecular interactions *in situ*. Several reviews have focused on the preparation of fluorescent-protein FRET-pairs and how to design and implement them as biosensors [100–103]. In this part of the review, we will focus on some recent applications of multiplexed FRET using fluorescent proteins for cellular imaging of signaling events.

The most obvious method for simultaneous imaging of multiple cellular events is multiplexed FRET utilizing spectrally distinguishable FRET pairs. With the development of a wide range of fluorescent protein variants with spectrally distinct emission properties, optimizing distinct FRET pairs for application in multiplexed imaging of Ca^{2+} signaling and enzymatic activity has become a field of great interest [104–110]. For example, Ding et al. developed two spectrally distinguishable FRET pairs, mAmetrine-tdTomato and mTFP1-mCitrine, which they termed “Yin” and “Yang” respectively (Fig. 6a) [104]. By sandwiching two different sensing domains (either sensitive to Ca^{2+} or capsase-3 activity) between the FRET pair, they were able to spectrally distinguish events associated with Ca^{2+} signaling, which induced a high FRET response. However, a low FRET was observed upon cleavage of one of the fluorescent protein labels indicating caspase-3 proteolytic activity. Similarly, Grant et al. used two spectrally distinguishable FRET pairs, ECFP-Venus and the red-shifted TagRFP-mPlum to investigate spatiotemporal aspects of Ca^{2+} concentration with respect to Ras activation [111]. Due to the low quantum yield of mPlum, fluorescence lifetime imaging microscopy (FLIM) was used to monitor FRET between TagRFP and mPlum. In this way, Ras activation was observed upon binding of mPlum-labeled Raf-RBD to an activated TagRFP-labeled Ras binding domain while change in the intracellular concentration of Ca^{2+} was monitored by an ECFP-Venus biosensor. Additionally, Hodgson et al. developed a near-infrared (NIR) fluorescent protein FRET pair (miRFP670-miRFP720) and coupled it with an existing biosensor (CFP-YFP) for application in spectrally resolved multiplexed imaging [112]. Using the miRFP670-miRFP720 pair, they were able to simultaneously image RacI activity with that of its antagonist RhoA or its regulation by G-nucleotide dissociation inhibitors (GDIs) in the same cell. Their results demonstrated the antagonistic dynamic of these two GTPases (RacI and RhoA) and, separately, the binding of GDI to an active RacI.

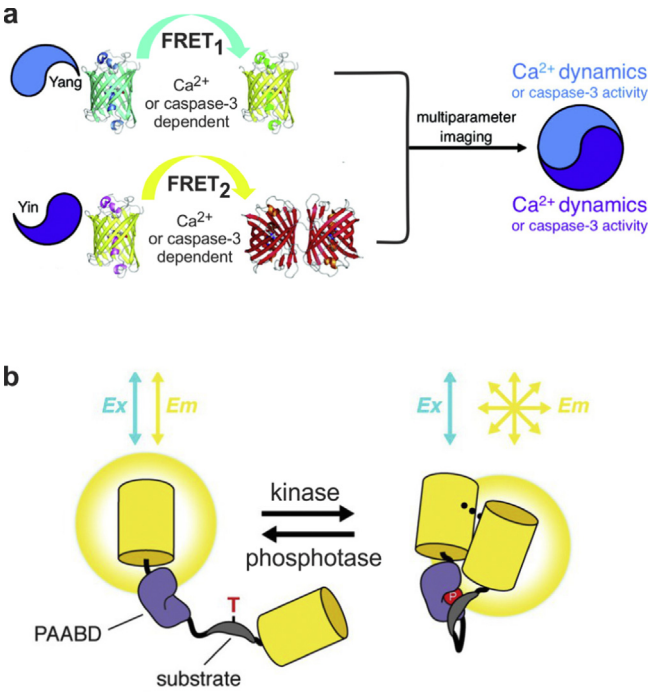

Fig. 6. Multiplexed FRET using fluorescent proteins. (**a**) Design of two spectrally distinct FRET pairs, Yang and Yin (450 nm and 400 nm excitation respectively) for simultaneous imaging of Ca^{2+} or caspase-3 proteolytic activity. Adapted from Ding et al. [104] with permission from the American Chemical Society. (**b**) Design and working principle of a kinase activity FLARE based on homo-FRET detected via fluorescence anisotropy. FLARE: fluorescence anisotropy reporters. PAABD: phospho-amino acid binding domain, T: threonine residue, and P: phosphorylated site. Adapted from Ross et al. [108] with permission from Creative Commons License.

In order to simplify the experimental procedure and data analysis, effort has been made to develop single excitation, multi-readout FRET pairs [109,113]. These systems, however, still require very precise choices of fluorescent protein pairs as well as specific dichroic mirrors and filters to minimize crosstalk. One interesting example of such a readout was found by Ringer et al. who developed multiplexed tension sensors to study the tension applied across talin-1 [113]. By using mechanically responsive peptides that are sensitive to specific force ranges as the sensing domains and tethering them between FRET pairs, they were able to visualize mechanically-engaged molecules within cells. In this case, a single excitation system was adopted for dual-color fluorescence lifetime imaging by using mTFP1 and LSSmOrange as donors. Both of these donors can be excited at a single wavelength and by coupling them with a “dark” fluorescent protein acceptor ShadowG and red-shifted emission protein mKate2, spectral interference could be minimized.

There are some inherent limitations of fluorescent proteins. For example, the need for spectrally resolvable hetero-FRET pairs (FRET between different fluorophores) often limits the multiplexing utility of fluorescent proteins. For this reason, homo-FRET (FRET between identical fluorophores) with fluorescence polarization has recently been used to minimize spectral crosstalk and the need for multiple overlapping emission wavelengths. Detecting FRET using fluorescence polarization or anisotropy relies on monitoring the loss in polarization of emission upon energy transfer from donor to acceptor (Fig. 6b) [107,108]. Therefore, homo-FRET pairs allow detection with only a single color channel and thus multiplexed

sensing can be achieved by using different (either hetero- or homo-) fluorescent protein pairs. Ross et al. recently developed fluorescence anisotropy reporters (FLAREs) which utilize fluorescent protein homo-FRET pairs instead of the traditional hetero-FRET pairs for simultaneous cellular imaging of protein kinase A activity, Erk activity, and intracellular concentration of Ca^{2+} [108].

3. Conclusion and future perspectives

This review summarizes the emerging applications of FRET-based multiplexed techniques and provides an overview of recent advances in using these techniques in chemical and biomolecular sensing. It is clear that these methods are found incredibly valuable and are being rapidly promoted. However, some challenges remain in each of these approaches in terms of making them more amenable to different research environments and accessible to more researchers. The primary challenges associated with current techniques include the need for sophisticated materials, complicated experimental design, high-end instrumentation, limited data-acquisition time due to rapid photobleaching of fluorophores, the number of different fluorophores that can be combined in a given experiment, and complicated data analysis procedures. As such, areas for improvement include minimizing the crosstalk between multiple fluorophores and enhancing the S/N ratio when using these multiplexed platforms both *in vitro* and *in vivo*. Although different research groups have encouraged other researchers by making the data analysis codes and protocols freely available [19,20], the real impact of these technologies will only be fully realized when the technologies are simplified in a way that these approaches can be utilized by more researchers across the globe. Given that these techniques have been developed with a specific application in mind, another challenge that remains to be addressed is the versatility of these approaches. It is hoped that these challenges will be addressed with the development of smarter materials, simplified fluorophore labeling schemes, development of photostable dyes, miniaturized instruments with multiple excitation sources with simple operation, and other versatile advances in the near future.

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