Review Article
Review of FRET biosensing and its application in biomolecular detection

Chao Fang, Yong Huang, Yongxiang Zhao

National Center for International Research of Biotargeting Theranotics, Guangxi Key Laboratory of Biotargeting Theranotics, Collaborative Innovation Center for Targeting Tumor Diagnosis and Therapy, Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, P. R. China

Abstract: Life science research is advancing rapidly in the 21st century. Many innovative technologies and methodologies are being applied in various fields of the life sciences to reveal how macromolecules interact with each other. The technology of using fluorescent molecules in biomedical research has contributed immensely to progress in this field. Fluorescence-based optical biosensors, which show high specificity, exhibit huge potential for clinical diagnosis and treatment of many of the life-changing diseases. Fluorescence resonance energy transfer (FRET), is a technique that has been widely employed in biosensing ever since its discovery. It is a classic fluorescence technique, and an important biosensing research tool extensively utilized in the fields of toxicology, pharmacology, and biomedicine; many biosensor designs are based on FRET. Radiometric imaging of biological molecules, biomolecular interactions, and cellular processes are extensively performed using FRET biosensors. This review focuses on the selection of FRET donors and acceptors used for biosensing, and presents an overview of different FRET technologies. Furthermore, it highlights the progress in the application for FRET in nucleic acid and protein biosensing, and provides a viewpoint for future developmental trends using FRET technology.

Keywords: Biosensing, fluorescence, FRET, fluorescent proteins

Introduction

Biological macromolecules are complex and varied, both in their structure and function. The internal structure of different molecules varies widely, and all their physiological functions are not entirely yet known. With advances in science and technology, encompassing various analytical techniques, it has now become possible to study the three dimensional structures of complex macromolecules. Currently, the most common techniques used to determine the three dimensional structures of molecules and their internal molecular arrangements are X-ray crystallography and the nuclear Overhauser effect (NOE) [1-8]. However, these two methods have distinct disadvantages, such as their inability to measure biomolecular dynamics. In addition, X-ray analysis cannot be used to analyze various complex biological macromolecules. For measurements of atomic distance, such as those <1 nm, the NOE effect is the best choice. However, there is no suitable technique for performing measurements of nanometer-scale distances. This limits the study of most biological macromolecules, such as proteins and nucleic acids, which are only a few nanometers in size.

The emergence of the fluorescence resonance energy transfer (FRET) methods is extremely convenient for these kinds of analytical problems. FRET is a special phenomenon of energy transfer between acceptor and donor chromophores. The transfer distance is generally in 1-10 nm range [9-16]. In 1948, Förster proposed the theory of dipole-dipole interactions for the experimental phenomenon of energy transfer. Techniques subsequently developed based on this phenomenon, like FRET, has been broadly employed in biosensing because of its extreme sensitivity to distance [9, 13]. Especially, FRET-based biomolecular probes are utilized as imaging agents, and chemosen-

sors are usually preferred as detection methods in biological applications, owing to their rapid cellular uptake. In addition, their ability for nondestructive visualization, and potential for *in situ* detection with little interference in the structure-function characteristics of key biomacromolecules, and the ease associated with which their structural characteristics can be modulated, which are also reasons for choosing FRET-based bioprobes. Thus, FRET offers a fast, simple, and sensitive approach for determining the internal structure of macromolecules and monitoring dynamic processes [11] and therefore, it has become extremely valuable in biosensing research.

With the development of imaging technology at the microscopic scale, researchers have increasing requirements for higher accuracy and sensitivity, of imaging resolution. Acquisition of low resolution images are thus, no longer accepted, as they cannot meet the needs of many researchers who study the intricate molecular details, which would need more advanced, and powerful imaging functions, and image processing functions. As an extension of fluorescence phenomenon, FRET effect realizes the "transmission" of fluorescence. After decades of research and development, FRET technology is now used to study a wide variety of complex processes in the biomedical field. Various FRET systems have been developed, and it is a good tool for biomolecular detection. In the following sections, FRET is described in more detail. We hope that this review will be helpful to researchers in this field.

FRET

Principle of fluorescence

Fluorescent technologies have been extensively used in medical diagnosis, food safety, and environmental monitoring because of their inherent sensitivity, and high selectivity [17-21]. Hence, they have attracted widespread interest from biologists, chemists, and researchers from other areas of study as well. Importantly, fluorescent sensors provide a one-of-a-kind route for detecting biologically crucial analytes, and help uncover their pathological and physiological roles [22]. When receiving external incident light, electrons in the ground state with lower energy in fluorescent substances absorb the energy of photons and transition to an excit-

ed higher energy state in approximately 10^{-15} s. Depending on whether the electrons are spin-paired or not, excited states can be divided into the single and triplet states. Electrons in the excited state are unstable and can go back to the ground state through the decay of nonradiative and radiative leaps. The decay of the radiative leap is accompanied by the emission of photons that produce fluorescence. The energy of emitted photons is generally less than that of absorbed photons owing to non-radiative energy loss [10].

Principle of FRET

FRET refers to the course of nonradiative energy transfer between an acceptor or chromophore, and a fluorescent energy donor via inter-dipole interactions, also known as long-range energy transfer. In FRET experiments, biomolecules, such as proteins and nucleic acids, are labeled by fluorescent groups [23-25]. As a result of the interaction between biomolecules, the donor-acceptor fluorescent groups come closer together. Then, if the donor is excited by an external stimulus, it transmits emitted light energy to the acceptor. The energy emitted from the acceptor and donor have different wavelengths, and can be distinguished by fluorescence detection devices to analyze the interactions between biomolecules [9]. The three main conditions for FRET are the following: (1) the donor is at a suitable distance from the acceptor, (2) an overlap exists between the acceptor absorption spectra, and the donor emission spectra, which provides a condition for energy matching, and (3) the donor-acceptor dipole has a certain spatial orientation for dipole-dipole coupling [26] (**Figure 1**).

FRET has unique advantages over conventional normal fluorescent techniques in practical applications. The first advantage is dynamic visualization. In FRET experiments, FRET occurs only if the corresponding conditions are met by the donors and acceptors. Changes in the fluorescence intensity of the donor and acceptor can be observed in real time. The second advantage is high sensitivity. FRET donors and acceptors are extremely sensitive to distance changes, in contrast to normal fluorescence techniques. The third advantage is a strong anti-interference ability. Since the FRET system is a dual fluorophore, the FRET efficien-
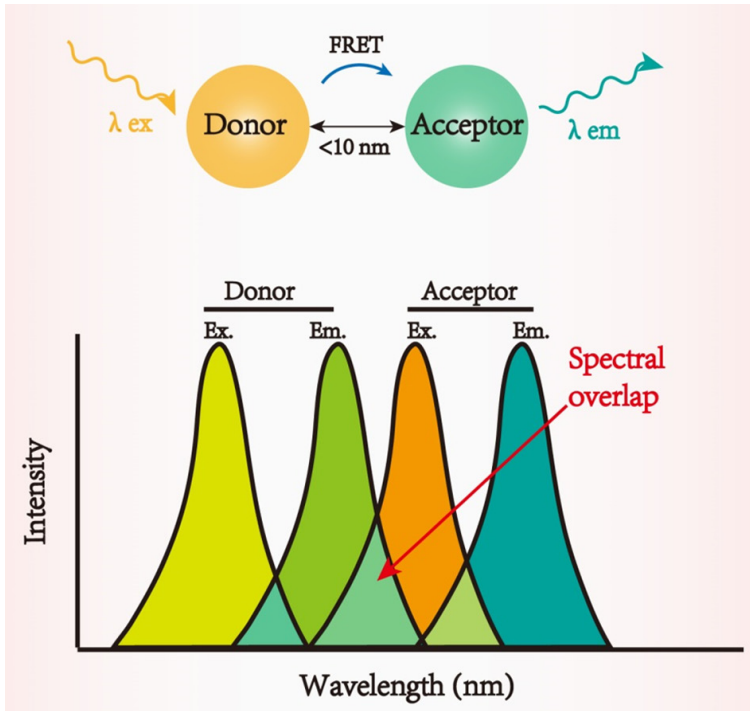
Figure 1. The conditions for generation of fluorescence resonance energy transfer.
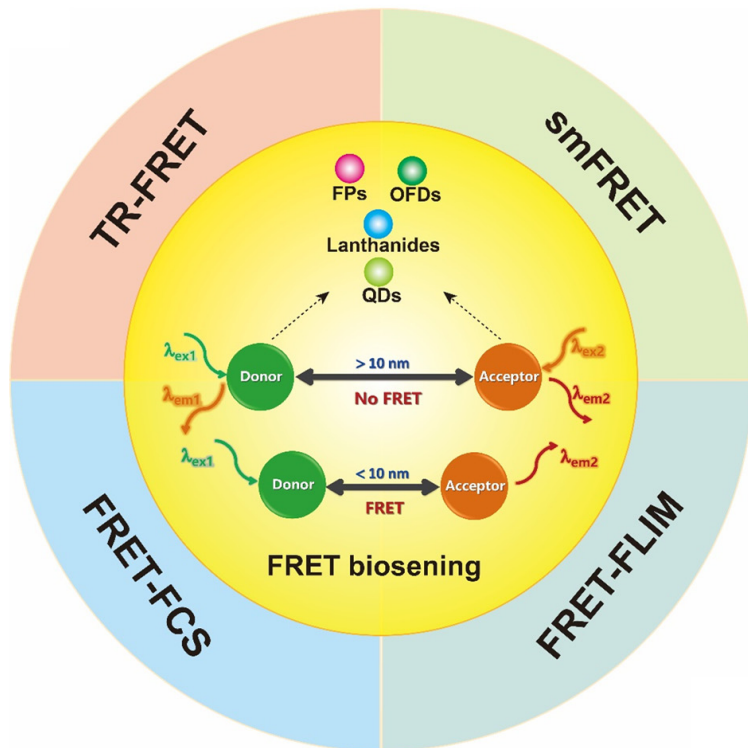

Figure 2. The application of fluorescence resonance energy transfer in biosensing.

cy is maximized only under light with a specific excitation wavelength, and the anti-interference ability is stronger than that of traditional common fluorescence. Finally, the fourth advantage is a unique magnification effect. The application of FRET in nucleic acid hybridization systems can achieve a notable amplification of the fluorescence of the acceptor. Ordinary fluorescence technologies cannot achieve this because only a single fluorescence is used.

Selection of FRET bioprobe donors and acceptors

FRET bioprobe donors and acceptors are divided into the following four main categories: fluorescent proteins (FPs) [27], organic fluorescent dyes (OFDs) [28], lanthanides [29], and quantum dots (QDs) [30] (**Figure 2**). In the following sections, we present the characteristics of each FRET bioprobe donor and acceptor separately and provide an overview of their special advantages. Importantly, we summarize the current state of development of each FRET donor and acceptor and provide an overview of their limitations.

Fluorescent proteins (FPs)

FRET biosensors based on FPs allow the monitoring of molecular dynamics in real time in time and space; this is essential for proper modulation, and functioning of complicated cellular courses [31]. According to the types of molecular events to be monitored, various sensing strategies have been adopted for the optimal design of FP-based biosensors. A series of mutant FPs, such as blue

fluorescent protein (BFP) [32], cyan fluorescent protein (CFP) [33], yellow fluorescent protein (YFP) [34], and red fluorescent protein (RFP) [35], have been obtained from the naturally occurring homologue, the green fluorescent protein (GFP). The best-known fluorescent protein (FP) pair in FRET-based biosensors is the CFP-YFP pair [36]. Because the emission and absorption spectra of the fluorescent proteins are largely continuous and readily available, they provide various optional donor-acceptor pairs for FRET experiments. FPs have the following characteristics: (1) FPs are proteins; as an indispensable molecule for biological organisms, proteins are more easily accepted by an organism and do not cause an immune rejection reaction, and (2) as a biological macromolecule, genetically encoded biosensors based on FPs are easier to be modified and have a wider range of uses. It is established that proteins are essential for the proper functioning of regulatory complex cells. Importantly, FPs have certain special advantages. They enable the real-time detection of intracellular molecular dynamics and have a wide spectral range. Furthermore, the FPs with different colors engineered from GFP, enable monitoring of multiple proteins of interest in live cells [37, 38].

Currently, FPs are widely used in FRET experiments. Zhao et al. [39] successfully performed *ex vivo* visualization of NADP using CFP and YFP as a FRET fluorescence pair, to observe the NADP concentration in individual cells. Mohsin et al. [40] successfully monitored methionine levels in bacterial and yeast cells using CFP and YFP as a FRET fluorescent pair, and methionine as a sensing element. Furthermore, Jablonski et al. [41] improved the signal-to-noise ratio of BFP by changing the laser parameters, further expanding the scope of BFP applications, and Horton et al. [42] used RFP to label and image neurons, within mouse hippocampal structures, enabling deeper biological studies of the tissue. Nevertheless, FPs have certain limitations in FRET applications. For example, since FPs are proteins, they are sensitive to pH and temperature. Hence, in complex environments, FPs are easily denatured, limiting their further development.

Organic fluorescent dyes (OFDs)

OFDs are one of the most common fluorescent indicators. They have the advantages of fast detection, good repeatability, and no radiation. Most OFDs contain reactive groups that can bind to some biomolecules through covalent bonds. The main and commonly used OFDs are fluorescein, rhodamine, and acridine. OFDs have been employed for enhancing the contrast in the imaging of biological samples for many years and have long been important in fluorescence microscopy. However, the applications for live cellular and organism imaging have expanded since their discovery, and there is a wide implementation of genetically encoded FPs. Owing to some key methodological advances, OFDs are becoming more popular for use in live imaging [43-46]. A main difficulty was overcome by the development of genetically encoded proteins, such as the self-labeling proteins with SNAP and Halo tags, which bind to modified organic dyes to label specific proteins of interest. Moreover, chemical strategies for the targeted labeling of specific structures, such as mitochondria and other cellular organelles, have also largely extended the live-cell applications of OFDs [47]. Various OFDs are available for a broad array of applications, and can be paired with other types of probes as needed [48, 49]. Recent advances in designing and fabricating organic dyes were reviewed by Cheng et al. [50]. Ji et al. [51] used rylenecarboximide (RI) as a dye to further expand the use of organic fluorescent dyes; they used RI for fluorescent, and photoacoustic cancer imaging, and cancer therapy. Cyanine dyes are widely used in FRET experiments, among which Cy3, Cy5, Cy5.5, and Cy7 are often used as donors and acceptors [52-54]. OFDs have special advantages in FRET experiments because they can be easily modified and obtained. OFDs can be obtained by chemical reactions. FRET donors and acceptors can be designed according to requirements. Furthermore, in the FRET experiments of nucleic acids and proteins, OFDs and target substances can be directly connected by chemical bonds.

Although OFDs have the above advantages, they also have certain inevitable disadvantages. They exhibit poor photochemical stability, serious photobleaching, and photodegradation. Moreover, their fluorescence lifetime is short. High-energy UV or visible light is often used as the excitation light source for OFDs, which results in lower tissue penetration. Furthermore, the interference of the organism's

own background limits its application in bio-medicine. Although several near-infrared OFDs exist in the market, the choice is still limited. The development of more near-infrared OFDs can effectively solve the above shortcomings.

Lanthanides

Lanthanides are rare earth elements, often used in combination with conventional organic dyes, as FRET donors and acceptors, respectively [29, 55]. They have the following advantages: (1) improved accuracy and signal-to-noise ratio in comparison to traditional FRET dyes, and (2) insensitivity to incompletely labeled donor and acceptor samples. In their free state, lanthanides produce only a very weak fluorescence signal and transfer only the energy of the intermolecular resonance energy level. In FRET experiments, lanthanides have the following special advantages: (1) a broader excitation band and a narrower emission band [55], and (2) long fluorescence duration, and a large Stokes shift of the fluorescence spectra. Owing to these advantages, spectral-resolution and time-resolution techniques can be used to effectively exclude the interference from light excitation, and non-specific fluorescence [56]. These characteristics render them as promising luminophores for sensing applications. The most commonly used lanthanides are samarium (Sm) [57, 58], europium (Eu) [57], terbium (Tb) [59, 60], dysprosium (Dy) [61], and actinium (Ac) [62]. At present, lanthanides are widely used in the field of biosensing. Pei et al. [63] used nanoprobes mixed with lanthanides, enabling the *in situ* monitoring of implant degradation, angiogenesis, and early inflammation during mouse skull repair. Liang et al. [64] used a strategy of combining near-infrared dyes with lanthanide elements. This method can clearly delineate the lymph, spine, and vessels of mice, and differentiate vessels showing acute vascular inflammation. It paves the way for preparing near-infrared-II emissive metal-organic frameworks, and will likely to improve their bio-applications.

Although lanthanides have their own characteristics, there are still limitations associated with their practical application. Currently, most lanthanides used are radioactive, and their long-term use can cause toxic side effects to the body [65]. Furthermore, large doses of lan-thanides can cause damage to the gastrointestinal tract [66]. At present, surface modification of lanthanide elements is commonly used to prepare lanthanides for biomedical applications. Solutions to the side effects of lanthanides still need to be further explored.

Quantum dots (QDs)

The combination of FRET with QDs, and their superior performance has allowed the design of new and advanced biosensors [67, 68]. Advancements in the application, and development of FRET biosensors utilizing QDs have rapidly developed over the past decades [69]. QDs are nanoparticles between 1 and 100 nm in diameter that can receive excitation light to produce fluorescence. QDs have several features, such as a wide range of emission and excitation spectra, along the narrow and symmetrical fluorescence peaks of individual QDs. Furthermore, the overlap between the absorption spectrum of the receptor molecule and the emission spectrum of the QDs can be adjusted by altering the size of the QDs. Moreover, QDs emit fluorescence with high intensity, and stability. Owing to these advantages, QDs are increasingly being used as new generation of fluorescent probes in biosensing applications [70]. Yang et al. [71] showed that an unfavorable urine microenvironment (UME) can be applied as the design source for constructing a UME-responsive 3D-printed hydrogel patch to achieve scarless memory repair. In this process chemically crosslinked silicon QDs are used to generate laser-excited reactive oxygen species, and mechanical strength elevation accessible. Deng et al. [72] reported the usage of fluorescent, and radioisotopic QDs for the multimodal imaging of macrophages. Macrophage specificity is achieved by clicking on conjugated glucans, a biocompatible polysaccharide targeting the cell types.

However, the poor stability and biological toxicity of QDs are problems that remain to be solved. Like OFDs, QDs usually require high-energy ultraviolet or visible light as an excitation light source. Obvious disadvantages are lower tissue penetration and background interference from biological tissue. This is also the main reason that currently hinders the further development of QDs. Therefore, to overcome the aforementioned challenges, the luminous

efficiency of the existing QDs must be improved or the surface of the QDs must be modified. Developing new materials to replace the current QDs is also a good solution.

FRET detection methods

The discovery of new probes can expand the scope of application for FRET. New test analysis methods play a catalytic role in promoting the sensitivity, and resolution of FRET technology [53, 73, 74]. In the following sections, we will introduce the detection methods and research status of FRET. The characteristics of each FRET detection method will be represented and its limitations will also be discussed.

Time-resolved FRET (TR-FRET)

The pace of biomedical research largely relies on technologies enabling the quantitative investigation of interactions among proteins and other bio-polymers or their small-molecule ligands. TR-FRET assay platforms provide high specificity, and sensitivity [60]. TR-FRET analyzes molecular interactions in biochemical processes, and is used to study cellular signal transduction pathways, protein interactions, DNA-protein interactions, cytotoxicity, and receptor-ligand binding [75-79]. Compared with conventional fluorescence detection techniques, the main advantages of TR-FRET detection include reduced background and improved signal-to-noise ratio, thus enabling increased sensitivity. In addition, the assay technology has a robust hybrid reading mode that eliminates the need for cleaning steps, making it suitable for automated and miniaturized screening-type applications. TR-FRET has unique advantages in that, it combines time-resolved fluorescence detection with FRET detection technology [80]. In TR-FRET experiments, the excitation, and emission of the donor-acceptor fluorophore may be detected after the disappearance of the short half-life background fluorescence owing to the long half-life of the donor fluorophore emitted light [81].

Since TR-FRET is flexibly constructed and can be reused, the number of TR-FRET-related reports is increasing. Zhang et al. [78] designed a luminescent nanoparticle based on the TR-FRET platform for lifetime quantification of intracellular caspase-3. This work, based on TR-FRET analysis, provides support for exploring dynamic life processes. Using the TR-FRET system, Won Lee et al. [82] developed a novel lateral flow immunoassay (LFIA) system. Their study successfully achieved quantitative analysis of cardiac troponin I (cTnI) in serum, opening a new avenue for disease diagnosis. Although TR-FRET has several applications, it still has its own unsatisfactory aspects. TR-FRET-related products are expensive, leading to higher usage costs which limit its further use. We believe that in future research, scholars will devise techniques to circumvent the drawbacks of TR-FRET and enhance its performance.

Single molecule FRET (smFRET)

Prior to the advent of smFRET technology, molecular biological studies were often limited to detecting the average behavior of many molecules over a specific period. For example, western blotting is used to detect protein expression. As molecular biological studies have progressed, scientists have begun to investigate the behavior of individual molecules. This is because even with the same genetic material and the same surrounding environment, these molecules can develop in different directions and sometimes acquire different functions. Moreover, changes in individual molecules are also associated with the development and occurrence of various conditions, such as neurological disorders and cancer. However, previous studies have tended to focus on static molecular observations, which make it more difficult to analyze the dynamics of individual molecules. Because the study of dynamics also requires the cooperation of physics, mechanics, and biology, smFRET technology is an excellent solution to this problem.

The smFRET technique has rapidly developed in the past several years, and holds the key to the structural dynamic studies of individual molecules [83-85]. When a single fluorescent group is used to label a target molecule, the target molecule can be detected so that its spatial distribution within the cell can be determined using fluorescence imaging. The smFRET technique can be used to analyze many biological mechanisms, because of its ability to monitor changes within individual molecules, or in molecular complexes, at nanoscale level in real-time [83, 86]. Currently, single-molecule detection is achieved mainly by the following:

(1) small excitation volume to eliminate background noise, (2) highly efficient optical components, and (3) high-efficiency detection. Niu et al. [87] used smFRET to study RNA dynamics. Their findings suggested that RNA dynamics is highly correlated with Mg^{2+}. Their report highlights an unusual regulatory mechanism underlying RNA dynamics, providing new insights into exploration of biological mechanisms underlying various processes. Kunal et al. [23] used smFRET to achieve a highly sensitive detection of microRNAs (miRNAs), and circulating tumor-derived DNAs (ctDNAs). Their research further optimizes existing sensor designs to maximize detection sensitivity, and support clinical diagnosis. The disadvantage of this technique is that the emitted light from the donor easily leaks onto the detector of the acceptor. Therefore, the choice of donor and acceptor is important in smFRET. This is also a common problem with FRET systems.

FRET and fluorescence lifetime imaging microscopy (FRET-FLIM)

With rapid development in optical techniques, new genetic methods have been proposed to investigate the spatiotemporal dynamics of biomolecules in living cells. The FRET-FLIM technique uses fluorescence lifetime imaging in FRET experiments [88, 89]. It features a one-of-a-kind edge in detecting and analyzing the conformational alterations and dynamic interactions of biomolecules in living cells in real time. It allows the real-time "visualization" of living cells and features the advantages of high space-time resolution, highly sensitive tests and reliable results, and simple and fast analysis procedures without complicated operations. The FRET method can provide spatial resolution at the nanoscale but is unable to provide instantaneous time-resolved information. However, FLIM can be adopted for calculating the fluorescence lifetime distribution in different cell regions, thus providing a fluorescence lifetime image. By comparing the alteration in the donor fluorescence lifetime in the absence and presence of the acceptor, it is possible to determine whether FRET occurs between the acceptor and donor. Law et al. [90] used the FRET-FLIM technology to observe cell migration efficiency. The combination of FRET with time-domain FLIM enabled detection of minute fluorescence signals by the Barber et al. and

Fruhwirth et al. [91, 92]. In contrast, FLIM has several limitations that prevent it from being the primary method for FRET imaging. First, lifetime range measurements are complex, and instruments are expensive to acquire and maintain. Furthermore, the application of such precision equipment is limited and cannot be promoted on a large scale. In addition, FLIM imaging is slow, which limits its utility in many FRET experiments. These restrictions may be lifted in the future as technology advances. Among other disadvantages, the lifetime of fluorescent proteins in living cells often shows multi-exponential decay, requiring more comprehensive data analysis for quantitative FRET analysis.

FRET and fluorescence correlation spectroscopy (FRET-FCS)

Since the early 1990s, FCS and other similar technologies have been widely employed to analyze biomolecular fluctuations, and interactions in living cells, and solutions. FCS is characterized by the observation of minute and instantaneous fluctuations in molecular fluorescence intensity. By analyzing the time-varying function of the rising and falling fluorescence signals, necessary information, such as molecular concentration and chemical kinetic parameters, is deduced. This technique has been utilized for analyzing the dynamics of film proteins, such as film transport proteins and film receptors, resulting from their superior capability of addressing spatiotemporal heterogeneity and the trace amount requirement of analytes [93-95]. FCS is sensitive to fluctuations on the microsecond time scale, and fluorescence signals can be detected with high sensitivity in low-content specimens [96]. Nonetheless, detecting these dynamics requires sensitivity to conformational changes in protein structure. Such sensitivity can produce single fluorophores under specific conditions resulting from environmental impacts; however, FRET serves only as a general method. Thus, the coupling of FRET with FCS offers sensitivity to intramolecular dynamics. Wennmalm et al. [95] proved that combining FRET with FCS features a one-of-a-kind capability of detecting small sub-populations of FRET-active molecules, and oligomers. Peng et al. [97] used FRET-FCS and discovered that alkylpurine glycosylase D (AlkD) follows an asymmetric diffu-

sion path in a slow model, i.e., rotation followed by a translation.

The limitation of the FRET-FCS method is the requirement for several pieces of detection equipment. The analysis of small fluctuations should be as precise as possible. Similar to FRET-FLIM, these shortcomings will be resolved as technologies continue to evolve. It is believed that FRET-FCS will be more widely used in the future.

Progress in applications of FRET in biomolecule detection

FRET constitutes a technology broadly utilized for unraveling the structural and conformational alterations of biomolecules, which are crucial to all living organisms. The most basic principle of FRET is to reflect the distance information of two molecular groups, using easily detectable information on the efficiency of FRET. A distance of approximately 10 nm is robust evidence of two interacting molecules, or two structural domains of a molecule coming close to each other due to conformational changes. Intermolecular interactions or conformational changes in proximity can be determined by the increase in FRET signals. Conversely, the decrease in a FRET signal can be used to demonstrate the moving away of two molecules, that lose their interaction, or separation of two portions of a molecule from each other due to, either cleavage, or conformation changes in them. Theoretically, any biological mechanism can be studied at the molecular level using FRET technology. The key is to find the appropriate fluorescent probe and detection equipment. In the following section, we introduce the application of FRET, mainly from structural and functional studies of biological macromolecules and nucleic acid hybridization.

Applications of FRET in the structure and function of biomolecules

FRET is a fast and sensitive technique that analyzes the structure of biological macromolecules in solution without complicated sample processing steps, such as crystallization. Significantly, the results are more reflective of the structure-function relationship between biomolecules than X-ray crystal diffraction. In addition, FRET technology can easily analyze the three-dimensional structure of biological mac-

romolecules, and the distance between specific sites, such as the study of enzyme catalytic sites, and protein-protein interactions [98-100]. The broad range of FRET applications results from the match between its viable scope, and the length scale of bio-macromolecules. FRET is best known for its nanometric distance determinations, as a 'molecular ruler' to probe structures and function of biomolecules, it can be explored for different applications ranging from monitoring the dynamics of intracellular protein-protein interactions and function of biomolecules, to providing signal transduction within countless bioassays. Alterations in local environment, conformations, and bio-macromolecular relativity and dissociation, constitute some of the target courses typically lending themselves to FRET, as they can induce switching between, off and on FRET states. Benaissa et al. [101] engineered a fluorescent chemogenetic reporter, featuring adjustable spectral and optical performance. Fluorogenic chromophores featuring different electronic performance allows the generation of bimolecular fluorescent assemblies covering the visible spectrum to red from blue, employing a single protein tag upgraded, and engineered by rational design and directed evolution. The capability of tuning the fluorescence color and performance via simple molecular regulation offers wide experimental versatility to image proteins in live cells, comprising neurons, and in multicellular organisms. Furthermore, it opens up a way to optimize FRET biosensors in live cells. Schubert et al. [102] developed two-color fluorescence microscopy, combined with photo-induced electron transfer probes, as an approach for simultaneously detecting two structural coordinates in single protein molecules: one color per coordinate. Their results revealed conformational motions synchronicity at remote sites during ATPase-driven closure of the Heat Shock Protein 90 (Hsp90) molecular clamp, offering evidence for a cooperative mechanism in the chaperone's catalytic cycle. Ucar et al. [80] showed that transient, and fine pushing of presynaptic boutons with a glass pipette, remarkably promoted the assembly of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins and the evoked release of glutamate, as determined by fluorescence lifetime imaging, and FRET in rat slice incubation fabrications.
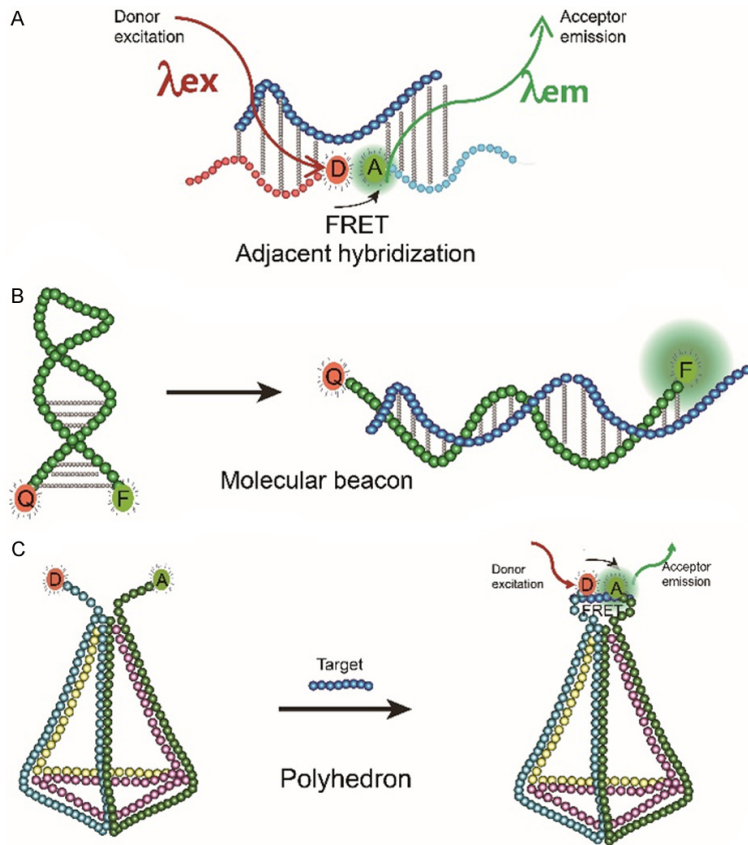
Figure 3. The application of fluorescence resonance energy transfer in nucleic acid hybridization analysis. A. The principle of adjacent hybridization. B. The principle of molecular beacon. C. The principle of polyhedron DNA.

Applications of FRET in nucleic acid hybridization analysis

Nucleic acids, comprising RNA and DNA, have sparked great attention as potential biomarkers for early and precise diagnoses of cancers. Nonetheless, resulting from their instability, and small quantity in body fluids, sensitive and precise detection is crucial. FRET technology can sensitively and rapidly detect nucleic acid hybridization using a conventional fluorometer [48, 103-105]. Alterations in the fluorescent signals by the biological events, like DNA hybridization, can be quickly discovered by fluorescence detection equipment. This technique can also be used for solid-phase analysis, or single-cell analysis using special fluorescence microscopes in which it requires special auxiliary equipment. The principle of FRET nucleic acid hybridization analysis is simple. When single strands of nucleic acid probes with acceptor, and donor markers are hybridized, the distance between the acceptor and donor changes, and whether hybridization occurs, is determined by the change in the FRET fluorescence intensity [49]. Currently, there are three modes of probe design for detecting nucleic acid hybridization using FRET techniques: (1) adjacent hybridization (AH) (**Figure 3A**); (2) molecular beacons (MB) (**Figure 3B**); and (3) polyhedral DNA (**Figure 3C**). Imaging technologies with genetically encoded FRET biosensors may constitute an effective method of dynamically tracking signal transduction in tissues featuring high spatiotemporal resolutions and live cells. Recent cases of applying the FRET principle to detect nucleic acid hybridization in cancer are summarized in **Table 1**. Wei et al. [106] designed 3D DNA origami structures using the FRET principle. They monitored the temperature-dependent alterations of FRET efficiency occurring when the dye-labeled structure was annealed and melted, and then extracted information about the binding, and dissociative behaviors of the origami. This work emphasizes the significance in understanding the basic aspects of DNA nanostructure self-assembly, and can be employed for guiding the designing of more complex DNA nanostructures, optimizing annealing protocols, and manipulating functionalized DNA nanostructures. Preus et al. [107] reported a general, versatile methodology to simulate, and analyze FRET in nucleic acids, and demonstrated its ability to model FRET between probes featuring restricted rotational, and diffusional freedom.

Conclusion and outlook

FRET technology has been effectively used on an optical scale, to study the structural aspects of biomolecules. Because of its high spatiotemporal resolution, FRET technology can rapidly track, and record distance changes over ultra-

Table 1. Application of FRET technology to detect biomarkers in cancer

Type	Biomarker	Tumor	Donor	Acceptor	Detection limit	Ref.
Adjacent hybridization	C-MYC	Breast cancer	Cy3	Cy5	0.6 pM	[108]
Adjacent hybridization	MicroRNAs	Lung cancer	QD	Cy5	0.54 fM	[109]
Adjacent hybridization	ncRNA	Breast cancer	UCNP	AuNP	0.316 fM	[110]
Tetrahedral	TK1 mRNA	Liver cancer	Cy3	Cy5	0.33 nM	[111]
Adjacent hybridization	MicroRNAs	Cervical cancer	Cy5	BHQ2	0.8 pM	[112]
Adjacent hybridization	MicroRNAs	Breast cancer	FAM	TAMRA	2 nM	[113]
Molecular beacons	TK1 mRNA	Liver cancer	FAM	TAMRA	2 nM	[114]
Adjacent hybridization	MicroRNAs	Breast cancer	QD	Gold NPs	4.56 pM	[115]
Molecular beacons	MicroRNAs	Breast cancer	FAM	ROX	0.18 pM	[116]
Adjacent hybridization	MicroRNAs	Lung cancer	Cy3	BHQ2	0.27 pM	[117]
Molecular beacons	MicroRNAs	Breast cancer	Cy5	Cy5	30 pM	[118]
Adjacent hybridization	MicroRNAs	Breast cancer	FAM	BHQ2	0.74 fM	[119]
Molecular beacons	MicroRNAs	Breast cancer	Cy3	Cy5	0.11 fM	[120]
Molecular beacons	MicroRNAs	Breast cancer	FAM Cy3	Cy5	100 pM	[121]
Molecular beacons	MicroRNA	Cervical cancer	Cy5	Gold NPs	1.5 fM	[122]
Adjacent hybridization	MicroRNAs	Cervical cancer	FAM	Cy5	0.9 nM	[123]
Tetrahedral	MicroRNA-21	Cervical cancer	Cy5	Cy5	2.14 pM	[124]
Tetrahedral	Survivin Mrna	Breast cancer	Cy3	Cy5	1.5 nM	[125]

short periods of time, making it suitable for studies on enzyme catalysis, muscle contraction, signaling, and spatial ranging. The spatial resolution is influenced by the uncertainty of the distance, and size of the donor-acceptor moiety. However, the current level of resolution is sufficient for FRET technology to be used to study conformational changes, and other aspects in biological macromolecules. The advantages of FRET include high sensitivity, with an average sample volume of nmol being sufficient for analysis. This sensitivity can be further improved by improving the instrumentation. Furthermore, if suitable donor-acceptor pairs and labeling methods are available, FRET technology can be used for analysis, as well as in complex environments, such as intracellular signaling pathway analysis. According to its characteristics, FRET technology has prospects for wide applications in homogeneous fluorescence immunoassay, and nucleic acid molecular hybridization. Despite the rapid progress in the development of fluorescent substances, currently, there is no perfect FRET donor and acceptor pair. Likewise, although the aforementioned methods have their own advantages, there is no perfect FRET measurement method. In addition, FRET is not suitable for all biosensors due to certain limitations. Clearly, FRET is not a good approach for detection for spatial distances >10 nm. The development of biosensors based on new technologies helps to thoroughly elucidate, the intracellular biochemical mechanisms underlying various process, a major focus of future research.

In conclusion, FRET technology, with all its unique characteristics and advantages, is likely to be further combined with other fluorescence detection equipment for scientific research in the future. This will enable researchers to explore various new aspects of life processes. Nowadays, human diseases, especially cancer, have a high incidence, and more diversified detection methods are needed for early detection and treatment. Routine detection methods in hospitals, such as imaging examinations, tissue biopsies, and blood tests, can no longer meet the needs of rapid social development. FRET technology combined with nanomaterials, is a promising and welcome option for cancer detection. The application of FRET and related technologies is growing every year. However, at present, it is still challenging to achieve large-scale applications of FRET technology. With the increasing convergence of methodological techniques in various fields, and the emergence of new instruments, analytical testing capabilities in biosensing using FRET technology will continue to be enhanced.

Disclosure of conflict of interest

None.

Abbreviations

FRET, Fluorescence Resonance Energy Transfer; NOE, Nuclear Overhauser Effect; FPs, Fluorescent Proteins; OFDs, Organic Fluorescent Dyes; QDs, Quantum Dots; BFP, Blue Fluorescent Protein; CFP, Cyan Fluorescent Protein; YFP, Yellow Fluorescent Protein; RFP, Red Fluorescent Protein; GFP, Green Fluorescent Protein; Sm, Samarium; Eu, Europium; Tb, Terbium; Dy, Dysprosium; Ac, Actinium; TR-FRET, Time-Resolved FRET; LFIA, Lateral Flow Immunoassay; cTnI, Cardiac Troponin I; smFRET, single molecule FRET; miRNAs, MicroRNAs; ctDNAs, circulating tumor-Derived DNAs; FRET-FLIM, FRET and Fluorescence Lifetime Imaging Microscopy; FRET-FCS, FRET and Fluorescence Correlation Spectroscopy; AlkD, Alkylpurine glycosylase D; Hsp90, Heat shock protein 90.

Address correspondence to: Yong Huang and Yongxiang Zhao, National Center for International Research of Biotargeting Theranotics, Guangxi Key Laboratory of Biotargeting Theranotics, Collaborative Innovation Center for Targeting Tumor Diagnosis and Therapy, Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, P. R. China. E-mail: huangyong503@126.com (YH); yongxiang_zhao@126.com (YXZ)

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