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REVIEW

Fluorescent hybridization probes for nucleic acid detection

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Abstract Due to their high sensitivity and selectivity, minimum interference with living biological systems, and ease of design and synthesis, fluorescent hybridization probes have been widely used to detect nucleic acids both in vivo and in vitro. Molecular beacons (MBs) and binary probes (BPs) are two very important hybridization probes that are designed based on well-established photophysical principles. These probes have shown particular applicability in a variety of studies, such as mRNA tracking, single nucleotide polymorphism (SNP) detection, polymerase chain reaction (PCR) monitoring, and microorganism identification. Molecular beacons are hairpin oligonucleotide probes that present distinctive fluorescent signatures in the presence and absence of their target. Binary probes consist of two fluorescently labeled oligonucleotide strands that can hybridize to adjacent regions of their target and generate distinctive fluorescence signals. These probes have been extensively studied and modified for different applications by modulating their structures or using various combinations of fluorophores, excimer-forming molecules, and metal complexes. This review describes the applicability and advantages of various hybridization probes that utilize novel and creative design to enhance their target detection sensitivity and specificity.

Keywords Molecular beacon · Binary probes · Fluorescence · FRET · Oligonucleotide · Signal-to-noise ratio

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Introduction

In the post-genomic era, sensitive, selective, and inexpensive methods of identifying nucleic acid sequences are critical to biomedical research, disease diagnosis, and drug discovery [1]. Although the development of next-generation DNA sequencing technologies [2–5] has dramatically reduced the cost of sequencing, there is still a continuously increasing demand for oligonucleotide-based fluorescent probes. In the general approach, these probes are designed to have a fluorescence reporter group covalently attached to an oligonucleotide sequence that is complementary to a nucleic acid target. In the absence of the target, the fluorescence of the reporter group is either quenched or generates a unique signal. When the target is added, the oligonucleotide-based probes hybridize to their target and produce a distinctive fluorescence signal.

Fluorescent hybridization probes have a wide variety of applications in chemistry, biology, and biomedical studies [6, 7], many of which cannot be fulfilled using other nucleic acid analysis methods. For example, due to their minimal interference with living biological systems, fluorescent hybridization probes enable the visualization of DNA or RNA molecules in vivo [8, 9], providing information on the location, transportation, and kinetics of these nucleic acids. This information is usually not

available from DNA sequencing. In addition, fluorescent hybridization probes do not require target purification or the removal of unhybridized probes. Therefore, these probes can monitor dynamic systems such as polymerase chain reactions (PCRs) in real time [10]. Furthermore, fluorescent hybridization probes can distinguish two DNA sequences that differ by only one nucleotide, which permits the detection of single nucleotide polymorphisms (SNPs) [11, 12]. Finally, due to their ease of synthesis, robustness, and low cost, fluorescent hybridization probes have a wide varety of applications in the identification of microorganisms [13, 14]. This review will describe the design and advantages of various fluorescent hybridization probes, in particular molecular beacons (MBs) and binary probes (BPs). Hybridization probes based on chemical reactions [15] will not be covered here. We will present the challenges of nucleic acid detection with fluorescent hybridization probes and recent advances that enhance probe sensitivity and selectivity by tailoring their structures and reporter groups to different applications.

Molecular beacons (MBs)

Molecular beacons, introduced by Tyagi and Kramer in 1996 [16], have become valuable tools for nucleic acid detection both in vivo and in vitro [1]. A standard MB contains a single-stranded oligonucleotide with a fluorophore (F) and a quencher (Q) attached at its opposite ends (Fig. 1a, left). The central sequence of this oligonucleotide (loop) is complementary to a specific target. Five or six bases are attached at each end of the oligonucleotide (stem) in order to form Watson–Crick base pairs that force the fluorophore and a strong fluorescence quencher to be in close proximity in the absence of the target. In this closed conformation of the MB, excitation of the fluorophore does not lead to fluorescence emission (Fig. 1b) because of the efficient quenching performed by the neighboring quencher. However, the presence of the target prompts the hybridization of the MB to the target, leading to the open conformation of the MB. Once the MB hybridizes to its target, the fluorophore and the quencher are spatially separated (Fig. 1a, right), which produces a strong fluorescence signal upon photoexcitation of the fluorophore (Fig. 1b).

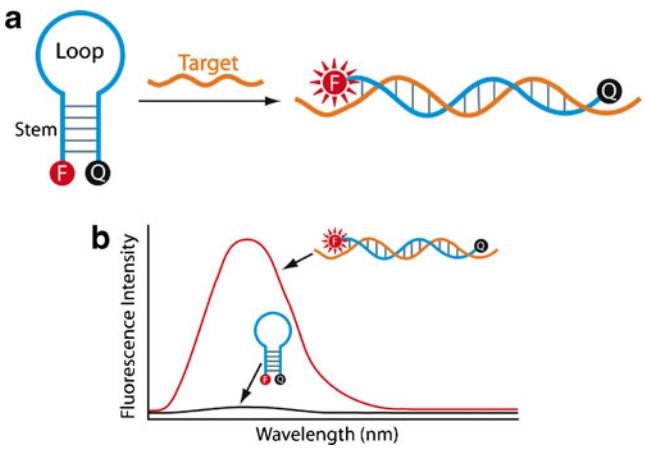

Fig. 1 **a** Standard MB in its closed (*left*) and open (*right*) conformations. **b** Representative fluorescence emission spectra of the closed MB (*black*) and the open MB (*red*) in the absence and presence of the target, respectively

An ideal MB should generate strong emission and absolutely no signal in the presence and absence of its target, respectively. However, the achievement of this objective is usually limited by a number of factors in real applications, as discussed below. To quantify target detection efficiency, the signal-to-background ratio of an MB is defined simply and conveniently as the fluorescence signal in the presence of the target over that in the absence of the target.

Despite its many inherent advantages, the standard MB shown in Fig. 1 has limitations. For example, it is difficult to differentiate the absence of the target from the unsuccessful delivery of the probe in cells; i.e., the delivery of the probe to the cell may be undetectable. In addition, the ubiquitous autofluorescence from living cells often generates a high background signal and thereby reduces the detection sensitivity. More importantly, the standard MB may produce false-positive signals, especially when applied in living cells [17, 18]. For example, in a cell, false-positive signals could result from the nonspecific opening of the MB, as induced by proteins or nucleic acids with a similar sequence to the target. Another possibility is that nucleases in the cytoplasm can break the backbone of the MB and release the fluorophore. Finally, incomplete quenching of the fluorophore may also generate spontaneous signals. To overcome these challenges, MBs with different structures and reporter groups have been developed, and will be described in the following sections.

Wavelength-shifted molecular beacons

To address the cellular delivery issue of the standard MB, wavelength-shifted MBs with distinctive emission wavelengths in the presence and absence of the target have been developed. Among these novel MBs, two-dye MB [19, 20] possesses a donor fluorophore (F_1) and an acceptor fluorophore (F_2) tethered to different ends of the probe (Fig. 2a). In its closed conformation, the stem region of the MB brings the two fluorophores into close proximity so that there will be an interaction between the fluorophore and a neighboring group after the fluorophore has absorbed a photon. Fluorescence resonance energy transfer (FRET) between two fluorophores in close proximity leads to strong

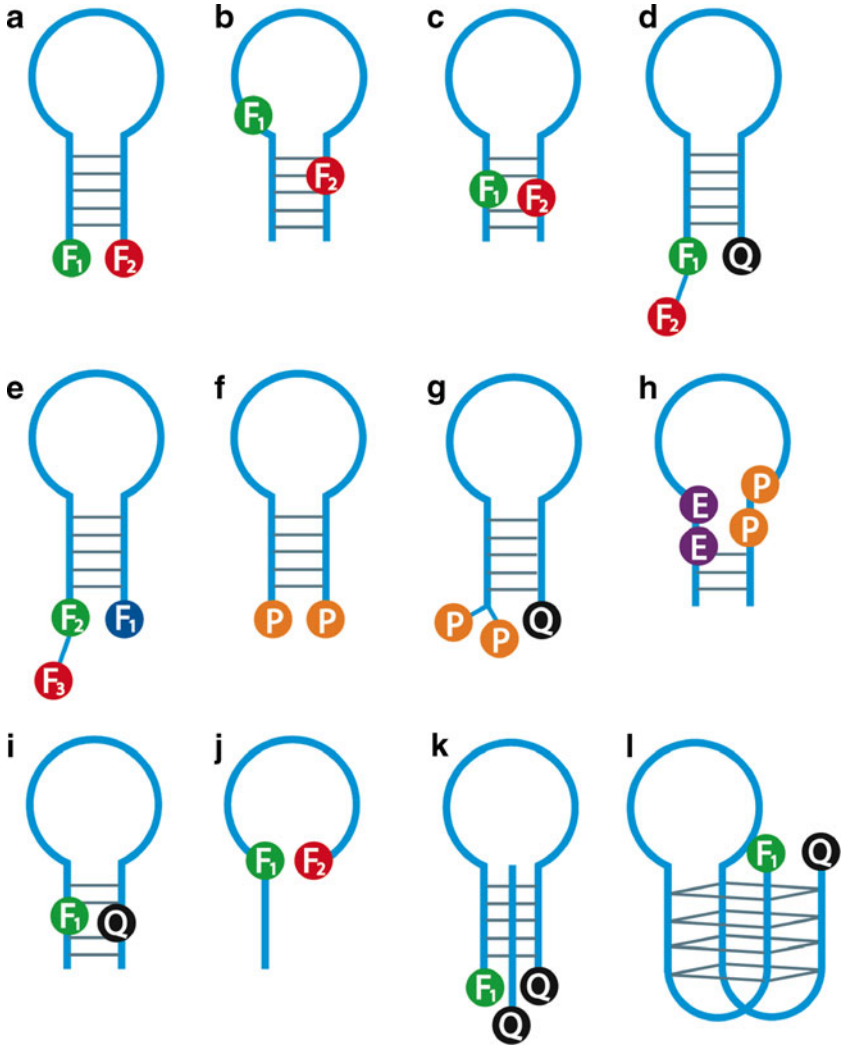

Fig. 2 Examples of different MBs. **a** Two-dye MB with a donor fluorophore and an acceptor fluorophore tethered to its ends [19, 20]. **b** MB with fluorescent nucleotides within its sequence [22]. **c** MB with a FRET dye pair as artificial nucleobases in its stem region [24]. **d** Standard multiplex MB containing a FRET dye pair and a quencher attached to its ends [26]. **e** Three-dye MB with a single fluorophore and a FRET dye pair linked to its opposite ends [27]. **f** Excimer–monomer switching MB with a pyrene moiety tethered to each end [31]. **g** Pyrene excimer MB possessing a pyrene excimer and a quencher attached to its ends [32]. **h** Excimer-controlled MB containing two pyrenes and two perylene diimides in its backbone [33]. **i** In-stem MB with a threoninol-tethered fluorophore and quencher in its stem region [35]. **j** Stemless MB with a fluorescence donor within its sequence and a fluorescence acceptor at its end [36]. **k** MB with a triplex stem possessing a single fluorophore and multiple quenchers attached to different ends of the triplex structure [37]. **l** MB with a quadruplex stem possessing a fluorophore and a quencher tethered to opposite ends of the quadruplex structure [39]

emission from the acceptor fluorophore (Fig. 3). Upon the addition of the target, the two fluorophores are spatially separated, resulting in dramatically reduced FRET efficiency, which decays as a function of the inverse sixth power of the distance between the two dyes [21]. Therefore, the most intense emission from the donor fluorophore is observed (Fig. 3) in the presence of the target following hybridization. Since a two-dye MB can be tracked in not only its open conformation but also its closed conformation, this enables the cellular delivery process to be monitored. In addition, the fluorescence intensity of the donor increases upon hybridization to the target, while that of the acceptor decreases simultaneously. This indicates that two-dye MB has great potential to achieve a higher signal-to-background ratio than the standard MB, which relies on the "on" or "off" status of a single fluorophore [20].

MBs possessing fluorescent nucleotides [22] have been investigated as an alternative to two-dye MBs. In this approach, two intrinsically fluorescent nucleotides, 2-aminopurine and pyrrolo-dC, are incorporated into the sequence of the probe. These fluorescent nucleotides in the double-stranded duplex configuration yield a reduced fluorescence intensity compared to those in the single-stranded oligonucleotide chain, owing to the more efficient

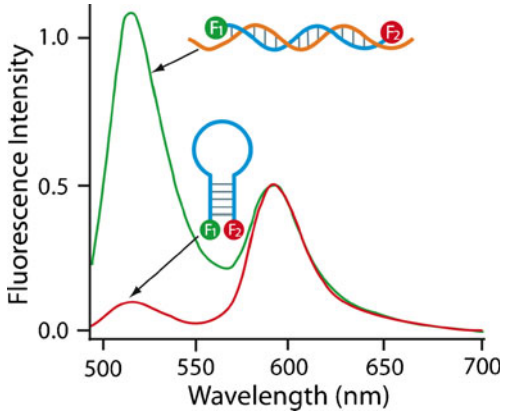

Fig. 3 Fluorescence emission spectra of a two-dye MB in the absence (*red*) and presence (*green*) of the target (modified from [19])

base-stacking interaction, which leads to fluorescence quenching [23]. In the absence of the target, pyrrolo-dC (F_2) and 2-aminopurine (F_1) are maintained in the double-stranded stem and the single-stranded loop, respectively (Fig. 2b). This leads to major fluorescence emission from 2-aminopurine. In the presence of the target, conformational reorganization of the probe results in pyrrolo-dC in the single-stranded overhang and 2-aminopurine in the double-stranded hybridization duplex. Thus, significant fluorescence emission from pyrrolo-dC is detected. Recently, MBs containing two thiazole orange (TO) fluorophores as artificial nucleobases in their stem regions have also been explored [24]. These two TO fluorophores form a hydrophobically interacting dimer in the closed conformation of the MB (Fig. 2c). Upon the hybridization to the target, the spatially separated fluorophores generate a distinctive fluorescence signal. Compared with the standard MB, an MB with fluorescent nucleotides displays enhanced sensitivity for the target. Furthermore, the elimination of the fluorophore and the quencher moieties reduces the size of the probe, which has a similar structure to natural DNA, allowing more efficient diffusion and recognition of this novel MB in a variety of media [22].

Multiplex molecular beacons

To understand the complexity and dynamics of a biological system, parallel analysis of multiple species is desirable and often required [25]. Multiplex MBs have been developed to achieve this goal. The standard multiplex MB possesses a FRET dye pair (F_1 and F_2) and a quencher (Q) attached to its opposite ends (Fig. 2d) [26]. The same donor (F_1) and different acceptors (F_2) are employed in a set of multiplex MBs. These MBs are nonfluorescent in the absence of their targets due to the quenched fluorescence donor in their closed conformation. Upon hybridization to their targets, the donor fluorophore transfers its energy to different acceptor fluorophores, which results in distinctive emission colors. Each detectable color indicates the presence of a specific target. This standard multiplex MB enables the visualization of multiple targets simultaneously with the same excitation light source. Additionally, the large Stokes shifts of the FRET dye pairs facilitate more effective filtering of the excitation light, which reduces false-positive signals.

To integrate the advantages of two-dye MB and the standard multiplex MB, a three-dye MB has been investigated [27]. In this approach, a fluorescence donor (F_1) and a FRET dye pair (F_2 and F_3) are linked to different ends of the MB (Fig. 2e). In the absence of the target, the fluorescence donor (F_1) transfers its energy to the intermediate fluorophore (F_2), and subsequently to the fluorescence acceptor (F_3), which generates the major emission. However, exposure to the target triggers a conformational reorganization of the probe that spatially separates the fluorescence donor and the FRET dye pair. This results in the most intense emission from the fluorescence donor. An advantage of the three-dye MB is that the MB is detectable in both its closed and open conformations, which enables the cellular delivery process to be monitored. Furthermore, the intermediate fluorophore makes the energy transfer from the donor to the acceptor more efficient by maximizing the spectral overlap. In addition, the well-separated excitation and emission wavelengths between the fluorescence donor and the fluorescence acceptor reduce the contribution of the direct excitation of the acceptor to the observed signal. Finally, unique and distinctive fluorescence signatures of a set of three-dye MBs can be generated by tuning the distance between the fluorophores. This approach, termed combinational fluorescence energy transfer, has been applied to develop a variety of tags with a very small number of dyes for multiplex genetic analysis [28, 29].

Molecular beacons using the pyrene excimer

The pyrene excimer, which is formed by an excited state of pyrene and ground state pyrene that are within collisional distance of one another, possesses a longer fluorescence emission wavelength than the monomer [30]. To take advantage of this property, an excimer–monomer switching MB with a pyrene moiety at its each end (Fig. 2f) has been developed [31]. In its closed conformation, two pyrene moieties are in close proximity and pyrene excimer dominates the fluorescence emission (Fig. 4). In contrast, the presence and binding of the target separate the two pyrene moieties, resulting in monomer emission (Fig. 4). This new class of MB also overcomes the undetectable cellular delivery issue of the standard MB. Moreover, the preparation of this MB is simplified by attaching the same pyrene group at both ends of the probe.

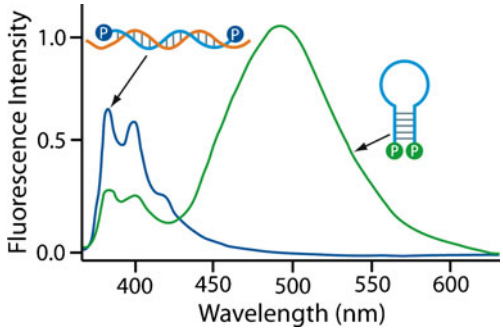

Fig. 4 Fluorescence emission spectra of excimer–monomer switching MB in the absence (*green*) and presence (*blue*) of the target (modified from [31])

The pyrene excimer possesses some desirable properties, such as a long fluorescence lifetime (~40 ns) and a large Stokes shift (130 nm); each of these properties has been applied to develop a pyrene excimer MB [32]. This MB contains multiple pyrene moieties and a quencher at different ends of the probe (Fig. 2g). Pyrene excimer fluorescence emission is expected to be observed or quenched in the presence or absence of the target, respectively. The large Stokes shift of the pyrene excimer allows more efficient filtering of the excitation light. In addition, the fluorescence emission intensity of this MB can be tuned by varying the number of pyrene moieties. Finally, the long fluorescence lifetime of the pyrene excimer facilitates the monitoring of the target in highly fluorescent cell media through time-resolved detection of the signal. A detection window of 60–100 ns can efficiently eliminate the short-lived autofluorescence background (less than 40 ns) and increase the signal-to-background ratio [32].

To effectively quench the pyrene excimer, an excimer-controlled MB containing two adjacent pyrenes and two adjacent perylene diimides in its backbone (Fig. 2h) has been developed [33]. In the absence of the target, the two pyrenes and the two perylenediimides form a donor–acceptor complex [34] that quenches the pyrene excimer emission. In its open conformation, the two pyrenes are spatially separated from the two perylenediimides, leading to pyrene excimer emission. In addition to the advantages of the pyrene excimer, such as the long fluorescence lifetime and large Stokes shift, this MB also possesses enhanced quenching efficiency as a result of donor–acceptor complex formation. This decreases false-positive signals and enables the detection of the target at low concentrations [33].

Molecular beacons with enhanced quenching efficiency

To more efficiently suppress background fluorescence in the absence of the target, an in-stem MB with a threoninol-tethered fluorophore and quencher in its stem region (Fig. 2i) has been developed [35]. Fluorescence emission is expected to be observed or quenched in the presence or absence of the target, respectively. In the closed conformation of the in-stem MB, the fluorophore and the quencher are stacked together. This close stacking interaction causes the in-stem MB to possess enhanced quenching efficiency and increased detection sensitivity.

As an alternative strategy, stemless MB based on peptide nucleic acid (PNA) has also been investigated [36]. In this approach, an intercalator dye used as a fluorescence donor is incorporated into the sequence of the probe, and a fluorescence acceptor is attached terminally (Fig. 6j). With the probe in its single-stranded conformation, the excited state of the fluorescence donor is rapidly depleted as a result of the unstacked fluorophore. In addition, the fluorescence acceptor is quenched by collisions with nucleobases. Furthermore, the uncharged PNA-based probe tends to adopt a collapsed structure in water, which facilitates the formation of dye–dye dimers or short-lived collision complexes. Thus, the emission from the fluorescence acceptor is greatly suppressed in the absence of the target. Upon hybridization to the target, the rigidity of the double helix keeps the fluorescence donor stacked and hampers quenching of the fluorescence acceptor, which restores the emission from the fluorescence acceptor. Compared with the standard MB, the efficient suppression of the fluorescence donor and acceptor emission in the absence of the target leads to an enhanced signal-to-background ratio for stemless MB.

Molecular beacons with a triplex or quadruplex stem

The sequence and length of the stem region determine the melting temperature of the MB [16]. A short stem region with few G-C base pairs leads to the rapid and nonspecific opening of the MB, which sacrifices selectivity towards its target. On the other hand, a long stem region with a large number of G-C base pairs leads to a slow opening that may inhibit hybridization to the target, resulting in decreased sensitivity. To develop MBs with the desired selectivity and sensitivity, a library of MBs with varying sequences and lengths in the stem regions are often designed, synthesized, and tested.

An alternative strategy for modulating the melting temperature of the MB is to incorporate a triplex stem into it [37]. An MB with a triplex stem is constructed by hybridizing an additional single-stranded oligonucleotide to the stem region of the standard MB. The resulting triplex stem possesses increased stability due to the hydrogen bonds between the single-stranded oligonucleotide and the original duplex [38]. Instead of synthesizing a whole library of MBs with varying sequences and lengths in their stem

regions, the length of the single-stranded oligonucleotide can be tailored to achieve a high degree of selectivity and sensitivity. Furthermore, the additional oligonucleotide allows various functionalities to be introduced. For example, a second quencher can be covalently linked to the end of this single-stranded oligonucleotide (Fig. 2k). The MB with two quenchers possesses an enhanced signal-to-background ratio compared with the standard MB [37].

An MB with a quadruplex stem has also been investigated to tune its melting temperature [39]. The quadruplex stem consists of four intrastranded oligonucleotides (Fig. 2l) held together by hydrogen bonds. Central monovalent cations can further stabilize this quadruplex structure [40]. By varying the concentrations of different cations in the medium, the melting temperature of this MB can be modulated more effectively in a less time-consuming manner. Additionally, the quadruplex stem also causes the MB to have a larger effective temperature range within which the target can be detected with high selectivity and sensitivity [39].

Binary probes (BPs)

Binary probes (BPs), another important member of the family of hybridization probes, also have widespread applications in nucleic acid identification [7]. In standard BPs, a fluorescence donor and a fluorescence acceptor are tethered to the ends of two single-stranded oligonucleotides, which are complementary to adjacent regions of a target (Fig. 5a, left). When they are not exposed to the target, the two oligonucleotide probes are distributed randomly and separated by large distances in solution, leading to the most intense emission from the fluorescence donor (Fig. 5b). Adding the target brings the two oligonucleotide strands together and forces the two fluorophores into close proximity (Fig. 5a right). Upon exciting the energy donor group, energy is transferred from the donor to the acceptor through the FRET mechanism, which results in significant emission from the acceptor (Fig. 5b).

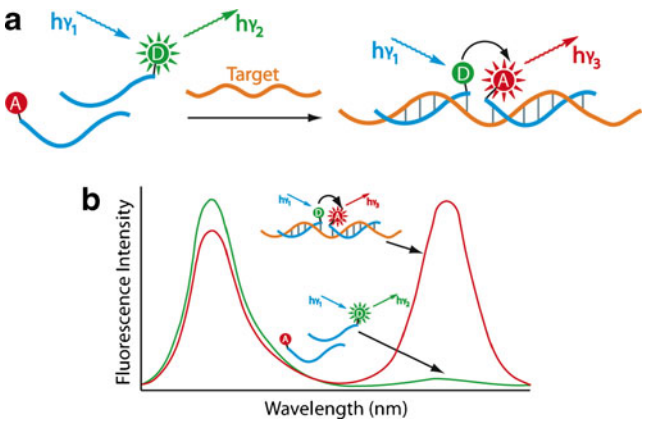

Fig. 5 **a** Standard BPs before (*left*) and after (*right*) the addition of the target. **b** Representative fluorescence emission spectra of BPs in the absence (*green*) and presence (*red*) of the target

To determine the effectiveness of the BPs, the signal-to-background ratio is defined as the ratio of the acceptor emission to the donor emission in the presence of the target over that in the absence of the target. This indicates that ideal BPs should only generate the donor emission or the acceptor emission before or after the addition of the target, respectively. However, the realization of this desired condition is limited by a number of factors, which will be discussed below.

Compared with MBs, one important advantage of BPs is that they can avoid the false-positive signals generated by the nonspecific binding of the probes to other cellular components [6]. To produce a positive signal, the two independent oligonucleotide strands of the BPs must be close to each other and in the correct orientation, which only happens when they hybridize to their target. Owing to the low concentration of the probes in solution, intermolecular FRET between the two randomly distributed probes is prevented. Furthermore, the possibility that both strands bind to the same nonspecific target is extremely low. Therefore, BPs have been widely applied to monitor nucleic acids in complex cellular systems [41, 42].

Despite the intrinsic advantages of standard BPs, further improvements in some of their properties are still required to achieve the desired signal-to-background ratio. For instance, when attempting to monitor nucleic acids in living cells, the cellular fluorescence background may obscure the signal. Additionally, the direct excitation of the acceptor and the overlap between the donor and the acceptor emission spectra can lead to acceptor emission even in the absence of the target. Finally, hybridization to the target is less favored for BPs than for MBs, both kinetically and thermodynamically. The slow hybridization kinetics of BPs results from the requirement of that two independent strands must bind to the target, while only one strand is required to hybridize for MBs. Also, the entropy decreases more when BPs bind to their target than when MBs bind to their target, since for BPs, three independent units (two probe strands and the target) become one unit after the hybridization, compared with two units (one probe strand and the target) in the case of MBs. In the following sections, we will introduce creative and novel approaches that have been developed to tackle these issues.

Binary probes based on the time-resolved method

To eliminate the cellular fluorescence background and enhance the signal-to-background ratios of standard BPs,

time-resolved methods have been investigated. Among the various examples of these novel BPs, spin-forbidden resonance energy transfer (SF-RET) BPs have been successfully applied to monitor nucleic acids in a highly fluorescent cell medium. In this approach, a ruthenium (Ru) complex acting as the energy donor and an organic fluorophore (F) acting as the energy acceptor are attached to the ends of two oligonucleotide strands (Fig. 6a) [43]. The strongest emission from the organic fluorophore and the Ru complex is expected to be observed in the presence and absence of the target, respectively. Energy from the triplet metal to ligand charge transfer (MLCT) state of the Ru complex to the singlet state of the organic fluorophore is spin forbidden. This spin forbiddenness leads to a long fluorescence lifetime for SF-RET BPs. The lifetime difference between the long-lived SF-RET BPs and the short-lived cell medium allow the time-gated detection of the probe signal after the cell medium signal has decayed away (Fig. 7). Figure 8a shows that SF-RET BPs can sensitively detect their target in nonfluorescent buffer with steady-state luminescence emission spectra. Nevertheless, their signal-to-background ratio is significantly reduced in a cell medium with strong fluorescence (Fig. 8b). With the time-resolved method, the emission spectra in Fig. 8c closely match those in Fig. 8a, suggesting that the cellular fluorescence background is efficiently eliminated.

A similar strategy that takes advantage of the long-lived pyrene excimer has also been developed [44]. Pyrene-excimer BPs contain a pyrene moiety covalently attached to each oligonucleotide strand (Fig. 6b). In the absence of the target, the two randomly distributed strands generate the pyrene-monomer emission. The addition of the target brings the two stands together and forces the two pyrenes into close proximity, resulting in the pyrene-excimer emission. This pyrene excimer possesses a longer fluorescence lifetime than the inherent fluorescence of the cell medium [44]. Therefore, the time-resolved method can be applied to enhance detection sensitivity when employing the pyrene excimer.

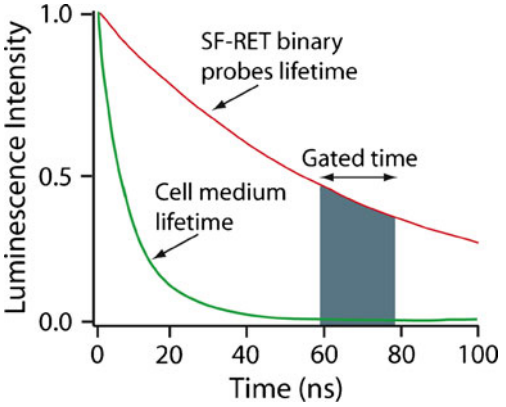

Fig. 7 Fluorescence decay traces of strongly fluorescent cell medium (*green*) and SF-RET BPs (*red*). Long-lived SF-RET BPs allow time-gated detection and increase the signal-to-background ratio (modified from [43])

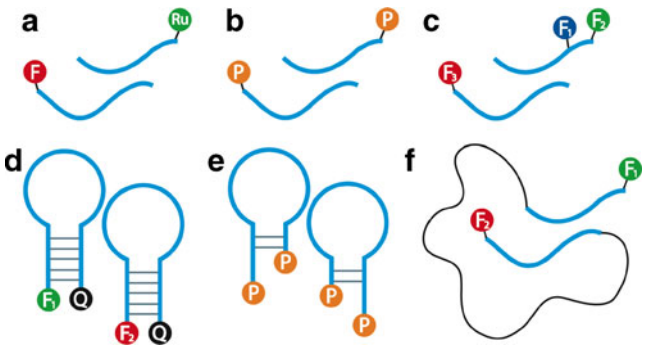

Fig. 6 Examples of different BPs. **a** SF-RET BPs possessing a Ru complex as the energy donor and an organic fluorophore as the energy acceptor [43]. **b** Pyrene excimer BPs with a pyrene moiety attached to each probe [44]. **c** Three-dye BPs with a FRET dye pair and a third fluorophore tethered to different probes [45]. **d** MB-based BPs possessing a fluorescence donor in one MB-based probe and a fluorescence acceptor in the other MB-based probe [9]. **e** Pyrene-excimer MB-based BPs with a pyrene moiety attached to each end of the probe [47]. **f** Linked BPs with the two probes covalently tethered together [48, 49]

Three-dye binary probes

To increase the signal-to-background ratio of BPs by minimizing the direct excitation of the donor and the overlap between the donor and the acceptor emission spectra, three-dye BPs have been developed [45]. In this approach, a FRET dye pair (F_1 and F_2) and a fluorescence acceptor (F_3) are tethered to the ends of two oligonucleotide strands (Fig. 6c). In the absence of the target, the two probe strands are randomly distributed in solution, leading to the strongest fluorescence emission from the intermediate fluorophore (F_2). In the presence of the target, the FRET dye pair and the fluorescence acceptor are held together. Energy is transferred from the donor (F_1) through the intermediate fluorophore (F_2) to the acceptor (F_3), resulting in the most intense emission from the acceptor (F_3). The additional intermediate fluorophore (F_2) facilitates the further separation of the absorption and emission spectra between the donor (F_1) and the acceptor (F_3). This increases the signal-to-background ratio of three-dye BPs by reducing the acceptor emission in the absence of the target. Furthermore, like three-dye MBs, a set of fluorescent tags can be developed by tuning the number of nucleotides between the fluorophores, which enables the simultaneous monitoring of multiple targets.

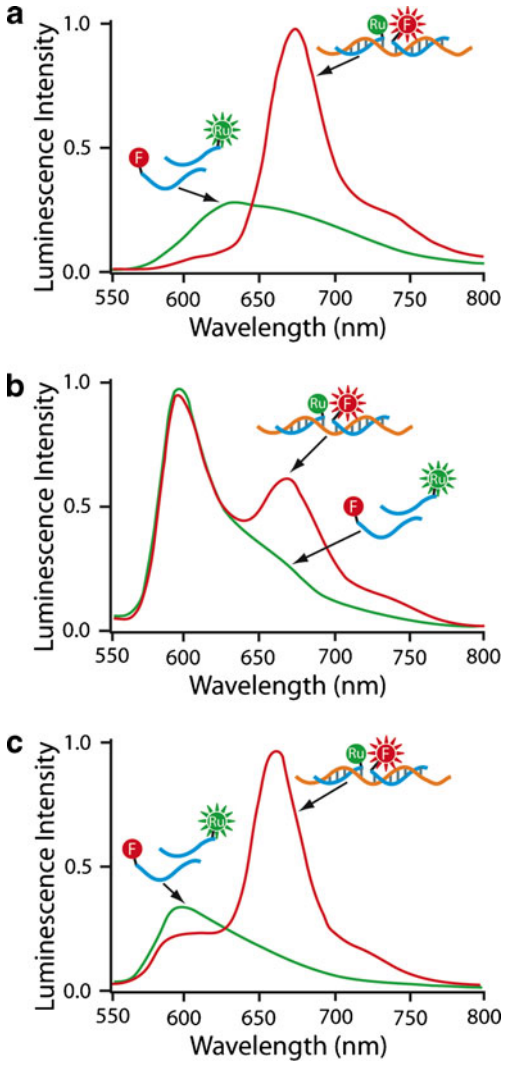

Fig. 8 **a**) Steady-state luminescence emission spectra of SF-RET BPs with (*red*) and without (*green*) the target in buffer. **b** Steady-state luminescence emission spectra of SF-RET BPs with (*red*) and without (*green*) the target in highly fluorescent cell medium. **c** Time-resolved luminescence emission spectra (59–77 ns after the excitation pulse) of SF-RET BPs with (*red*) and without (*green*) target in highly fluorescent cell medium (modified from [43])

Molecular beacon based binary probes

To integrate the advantages of MBs and BPs, Bratu et al. developed MB-based BPs [9]. In this approach, a donor fluorophore (F_1) and an acceptor fluorophore (F_2) are incorporated into two standard MBs (Fig. 6d). Before adding the target, the probes do not emit, since the two MBs are in their closed conformation. The presence of the target prompts a conformational reorganization of the two MBs, which brings the two fluorophores close to each other and spatially separated from the quenchers. This leads to fluorescence emission from the acceptor. Unlike the standard MB, MB-based BPs do not produce false-positive signals (which are generated by the released fluorophores or binding to a nonspecific target in the case of standard MBs). The fluorophores released by cellular nucleases are not close enough to each other to yield a FRET signal, due to their low concentrations. The chance that both MB-based probes will bind to the same nonspecific target in the correct orientation is extremely low. Therefore, MB-based BPs maintain the advantage of standard BPs by preventing false-positive signals. Additionally, the spectral overlap between the donor and the acceptor of MB-based BPs does not lead to acceptor emission in the absence of the target, which is one of the major challenges for standard BPs. All of these advantages of MB-based BPs make them a valuable tool to monitor nucleic acids in complex biological systems [9].

To further enhance detection sensitivity by amplifying the fluorescence signal with hybridization chain reaction [46], pyrene-excimer MB-based BPs have been investigated [47]. The two MB-based probes employed in this method possess pyrene moieties attached at their sticky ends (Fig. 6e). In the closed conformation of these probes, the pyrene moieties are spatially separated by six nucleotides at the sticky ends and the pyrene monomer dominates the fluorescence emission. The presence of the target triggers a cascade of hybridization events to yield nicked double helices composed of the two alternating MB-based probes. In these generated double helices, the pyrene moiety on one probe is brought into close proximity to the pyrene moiety on the other probe, resulting in the strongest emission from a large number of pyrene excimers. Pyrene-excimer MB-based BPs integrate many advantages of standard BPs and MBs using the pyrene excimer, including the elimination of false-positive signals generated by released pyrenes, more efficient filtering of the excitation light, and reduced cellular autofluorescence background with time-resolved analysis. Additionally, the detection sensitivity of this approach is dramatically enhanced by the numerous pyrene excimers formed in the presence of a single copy of the target.

Linked binary probes

To maximize the hybridization rate by minimizing the decrease in entropy with standard BPs, linked BPs have been designed and developed [48, 49]. In this approach, a donor fluorophore and an acceptor fluorophore are attached to one end of each oligonucleotide strand, while the other end of each strand is covalently linked to either polyethyleneglycol (PEG) polymer chains [48] or oligonucleotide chains [49] (Fig. 6f). In the absence of the target, the linked BPs possess a random coil structure,

where the donor and the acceptor are spatially separated. Excitation of the donor only leads to donor emission. On the other hand, the presence of the target brings the two probes together and keeps the donor and the acceptor close to each other. This results in the strongest acceptor emission upon donor excitation. In a similar manner to standard BPs, the linked BPs do not generate false-positive signals, since the released fluorophore cannot cause the acceptor to emit, and it is unlikely that both probes will bind to the same nonspecific target. Furthermore, by tethering the two probes together, the hybridization rate is enhanced and the decrease in entropy is reduced [6, 48]. Because of these advantages, linked BPs have a wide range of applications in specific nucleic acid detection in real time.

Conclusion

In this review, we have described the principles of MBs and BPs as fluorescent hybridization probes for nucleic acid detection and some recent advances that have improved different properties of the probes for various applications. The standard MB identifies the target by opening its closed conformation upon binding to the target and separating the fluorophore from the quencher. To address the issue of the undetectable cellular delivery process, wavelength-shifting MBs that produce distinctive emissions in the presence and the absence of the target have been investigated. Multiplex MBs can monitor a number of different targets simultaneously by using FRET to transfer energy from the same donor to different acceptors. The long fluorescence lifetime and large Stokes shift of the pyrene excimer have been used to eliminate the cellular autofluorescence signal and to allow the excitation light to be filtered, respectively. MBs with a triplex or quadruplex stem have also been developed to modulate their melting temperature. On the other hand, BPs bring their two independent oligonucleotide strands together upon hybridizing to the target. The time-resolved method has been applied to overcome the issue of intrinsic cellular fluorescence, by detecting signals after the autofluorescence has decayed. Three-dye BPs with well-separated absorption and emission spectra between the donor and the acceptor can enhance the signal-to-background ratio. MB-based BPs avoid acceptor emission in the absence of the target by introducing a quencher close to the fluorophore. To enhance the speed of target response, linked BPs with two covalently tethered strands have been developed. Generally, MBs with good sensitivities and fast response times have a wide range of applications in the field of identifying nucleic acids in dynamic systems, such as real-time PCR. In contrast, highly specific BPs are desired to monitor DNA or RNA in complex systems, including living cells and organisms.

Despite their widespread application, further improvements to fluorescent hybridization probes are still required to achieve their full potential. For example, the ideal sensitivity of the probes should enable the identification of each individual nucleic acid molecule inside a cell, so that digital gene expression [50] can be performed in living cells. Additionally, probes of the desired selectivity are expected to routinely distinguish two sequences that differ by only one nucleotide, owing to the importance of single nucleotide polymorphisms (SNPs) in human diseases [51]. Furthermore, the ideal response time of the probe should allow the immediate detection of the target when it is present. This allows the kinetics of highly dynamic systems, such as transcription initiation and elongation [52], to be studied. Finally, an ideal probe should not interfere with the transportation and the function of its target, so that the target can be studied under its native conditions.

The recent advances in fluorescence imaging technologies and novel fluorophores will contribute to the development of the desired hybridization probes. Single-molecule fluorescence methods allow the detection of each individual fluorophore [53], which satisfies the sensitivity requirement of ideal probes. Fluorescence lifetime imaging microscopy (FLIM) [54] can easily differentiate the long-lived probe signal from the short-lived cellular autofluorescence signal. Using super-resolution fluorescence microscopy, dynamic processes in living biological systems can be recorded at the nanometer scale [55]. Two-photon excitation microscopy enables the detection of a target in living tissue with high depth [56]. Also, the development of novel fluorophores, including quantum dots (QDs) [57], can facilitate the construction of the desired hybridization probes. QDs possess high quantum yields and large extinction coefficients, so they exhibit strong emission. In addition, the narrow line width of the emission spectra of QDs is advantageous for filtering the excitation light. Because of their good photostability, QDs can be applied to trace biological targets for a fairly long time. Upon single wavelength excitation, QDs of different sizes can generate various emission colors, which enables multiplex detection. All of these advances, in combination with the creative probe design discussed in this review, will lead to more widespread applications of hybridization probes in biomedical studies, disease diagnosis, and drug development.

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