# Fluorescent Hybridization Probes for Sensitive and Selective DNA and RNA Detection

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**ABSTRACT**

We outline the different approaches taken by our group in the design of fluorescent hybridization sensors. Molecular beacons (MBs) and binary probes (BPs) using two dyes (2d-MB and 2d-BP, respectively) have been synthesized; these sensors serve as switches in emission upon binding to target biomolecules, such as DNA. These sensors allow for ratiometric fluorescence detection of polynucleotides (PNs) by visualization of the probes when bound to a target PN. Additionally, three-dye MBs (3d-MB) and BPs (3d-BP) have been developed, where an energy-transfer cascade is employed to decrease the overlap between the fluorophore emission spectra, resulting in a low direct excitation of the acceptor fluorophore. Pyrene-based MB (Py-MB) and BP (Py-BP), which possess the advantage of long fluorescence lifetimes, have also been synthesized. Time-resolved fluorescence spectra (TRES) can be used to discriminate between short-lived background fluorescence and long-lived fluorescence of the pyrene probes. This technique was demonstrated by time-resolving the signal of a Py-BP from the background fluorescence in *Aplysia californica* cell extracts.

## Introduction

The development of novel, sensitive and selective sensors for the detection of DNA and RNA polynucleotides (PNs) has become a very active research field in recent years.[1–3] For example, the sequencing of the human genome provides the genetic map of the proteins and nucleic acids for a human being but lacks the information on how these biomolecules are involved in the processes within the cells. The visualization of DNA and RNA *in vivo* can provide information about the location, kinetics, and function of these biomolecules.[3] Likewise, techniques such as the polymerase chain reaction (PCR) require probes with sufficient sensitivity to detect very small amounts of samples quantitatively and also with sufficient selectively to identify a specific PN sequence.[4] Probes for the detection of PN and oligonucleotide (ON; we will use this term for relatively short nucleotide chains) sequences include the use of metal complexes,[5,6] organic dyes,[7] and pyrene-based intramolecular excimer-forming probes.[8] In general, these probes function by binding to PNs by hydrophobic or electrostatic interactions that are nonspecific, which means that they bind to PNs irrespective of their sequence. Such probes provide information about the amount of PNs available in a sample or cell or their position but are not specific to a definite target sequence.

The selective detection of specific DNA and RNA sequences can be achieved by using ON-based antisense hybridization probes.[9,10] Generally speaking, these probes are fundamentally an ON sequence complementary to the target sequence containing a reporter group that can be monitored using a spectroscopic technique, such as fluorescence spectroscopy. Because, in living systems, ONs have been engineered over millions of years to selectively hybridize to their complementary sequence, fluorescent antisense ONs provide the exquisite probe selectivity. Additionally, the chemistry of nucleic acids is well-known, and their assembly into ONs can be executed easily, employing commercially available synthesizers. A simple probe of this kind is composed of a single fluorescent dye bound to an ON chain; the probe labels its target by hybridizing with some part of its target sequence.[11]

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Fluorescence spectroscopy is widely used because it is by far the most sensitive of the available spectroscopic techniques. A significant disadvantage of a single labeled probe is that quantification and/or visualization of the target is made difficult by the strong fluorescence background because of unbound probes, which requires an extra step for the removal from the medium of the probes that are not hybridized to the target.[11] During the last 2 decades, new generations of fluorescent antisense ON probes have emerged with interesting properties and have been employed to attack important problems in fields ranging from *in vitro* PCR monitoring to *in vivo* mRNA detection.[1] We have focused a significant part of our research in trying to improve the properties of ON probes, especially molecular beacons (MBs)[12–14] and binary probes (BPs),[15,16] to develop strategies for ever-increasing higher detection limits, selectivity, and background discrimination. In this Account, we present some of the challenges and recent advances developed in our laboratory for the detection of ONs and PNs *in vitro* and *in vivo*.

## Molecular Beacons

The MB concept was introduced by Tyagi and Kramer in 1996 and was successfully applied to nucleic acid hybridization assays.[17] The simple and novel architecture of MBs prompted their widespread applications, ranging from single nucleotide polymorphism (SNP)[18] to the detection of bacterial bioterrorism agents.[19] A MB is a ON that contains a fluorophore and a quencher at different ends of the strand (Figure 1a). This ON strand is composed of a probe region (loop) complementary to a target sequence and a self-complementary region of five to six nucleotides at opposite ends (stem). In the absence of the target, the complementary parts of the probe hybridize together, forming a hairpin structure (loop–stem), bringing the fluorophore and the quencher into close proximity.[20] Because of the close proximity enforced by the stem, the quencher deactivates the fluorophore excited state, resulting in a strong quenching of the fluorescence (left in Figure 1a). However, in the presence of the target, the probe region of the MB hybridizes to it, promoting the opening of the hairpin conformation and separating the fluorophore and the quencher from each other (right in Figure 1a). Because of the enforced separation after hybridization with the target, the fluorophore fluorescence is not quenched after hybridization. This allows for the observance of fluorescence emission from the fluorophore, in principle, only after the probe has selectively hybridized to the target.

An ideal MB should not show any fluorescence emission in the "closed" hairpin conformation (absence of the target) and a strong fluorescence emission in the "open" conformation when hybridized with the target. The efficiency of target detection of a MB is measured by its signal-to-background ratio (S/B), which is the ratio of the fluorescence signal in the presence of the target with the fluorescence signal before the addition of the target.[21] The background signal arises from a number of sources. For

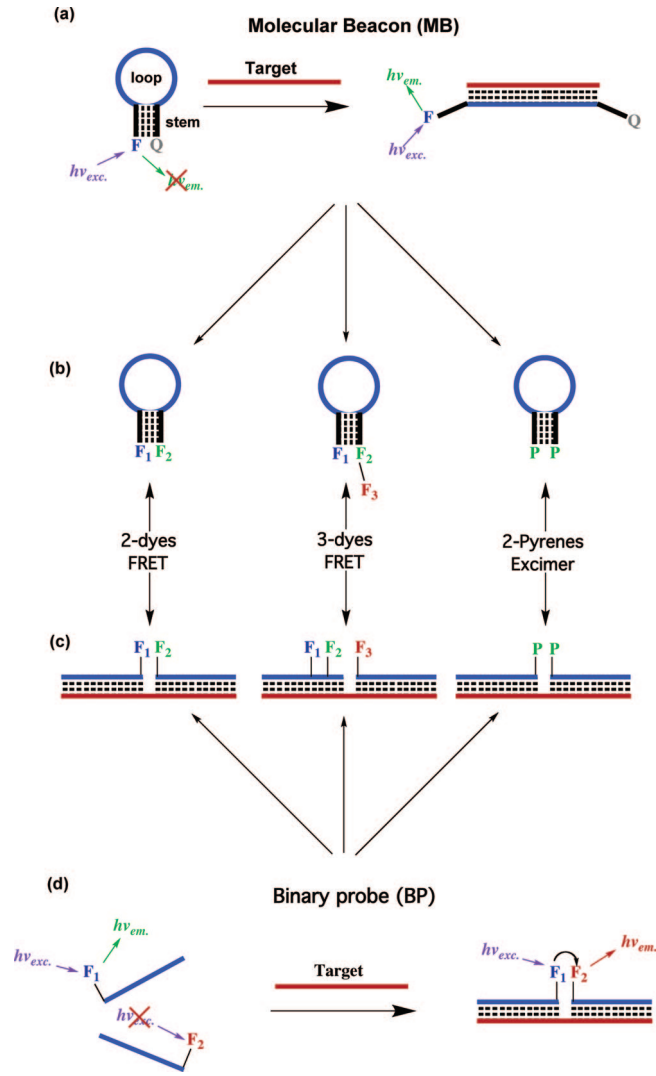


**FIGURE 1.** (a) MB in its hairpin conformation (left) and hybridized with the target (right); (b) 2d-MB (left), 3d-MB (center), and Py-MB (right) in their hairpin conformations; (c) 2d-BP (left), 3d-BP (center), and Py-BP (right) hybridized with the target; and (d) free BP in solution (left) and after hybridization with the target (right).

example, fluorescence occurs from partially labeled MB (possessing the fluorophore but not the quencher), a small amount of "open" hairpin structures in the absence of the target, and the inefficiency of the quencher to totally deactivate the dye fluorescence.[13]

Despite all of the relevant intrinsically inherent advantages of MBs, it has been observed that, when employed in complex biological environments (e.g., cells), they often give false positive signals;[22,23] possible explanations for these false signals include the "opening" of the hairpin conformation as a result of the interaction with proteins or membranes and intercalating agents in the cell cytoplasm that disrupt the hairpin stability. In addition, the detachment of the fluorophore or quencher from the MB by nucleases might also be possible. In cells where MBs suffer from spontaneous opening in the absence of the target, the use of BP may be successful.[15] Another challenge of MBs is their insertion into the cell. A classical MB should be dark in the absence of the target; therefore,

its injection into the cell is usually not "imageable". Thus, the diffusion of the MB into the cell cannot be visualized until it enters the cells and binds to the target. Later in the text, we will show how these challenges can be overcome with the use of different kinds of MBs.

## Binary Probes

BPs (parts c and d of Figure 1) are hybridization probes, which depend upon fluorescence resonance energy transfer (FRET) to report the binding to the target.[24] The occurrence of FRET depends upon various parameters, such as the distance between the donor and acceptor fluorophores and the spectral overlap between the donor emission and acceptor absorption spectra.[25] The efficiency ($E$) of FRET is given by the following expression:[26]

$$E = \frac{R_0^6}{R_0^6 + r^6} \tag{1}$$

where $R_0$ is the distance between the fluorophores at which half of the donor molecules are quenched by FRET (termed the Förster distance) and $r$ represents the actual distance between the fluorophores during the measurement. The Förster distance ($R_0$) can be calculated using the following expression:[25]

$$R_0^6 = \frac{9000(\ln 10)k^2\varphi J}{128\pi^5 n^4 N_{Av}} \tag{2}$$

where $n$ is the refraction index of the medium, $\varphi$ is the fluorophore quantum yield of the donor, $N_{Av}$ is Avogadro's number, $k^2$ is the orientation factor equal to 0.667 for free molecules in solution, and $J$ is the spectra overlap given by[27]

$$J = \int_0^\infty f_D(\lambda)\ \varepsilon_A(\lambda)\ \lambda^4\ d\lambda \tag{3}$$

where $f_D(\lambda)$ is the fluorescence spectrum of the donor and $\varepsilon_A\ (\lambda)$ is the absorption spectrum of the acceptor in extinction coefficient units. From eq 1, it can be deduced that FRET strongly depends upon the distance between the fluorophores, while eq 2 shows the quantitative dependence of $R_0$ on the overlap integral (eq 3) and the quantum yield of the donor. These factors need to be carefully adjusted to achieve an optimal FRET.

In terms of structural detail, a BP consists of two ON strands (or probe sequences) that are complementary to adjacent regions of the target (parts c and d of Figure 1).[24,28,29] One of these ON strands is labeled with a donor fluorophore, while the other is labeled with an acceptor fluorophore, forming a FRET pair. In the absence of the target, the fluorophores of the BP are randomly distributed in solution, and because the concentration of the BP is very low, intermolecular FRET is not observed. However, in the presence of the target, a hybrid is formed, bringing the dyes close to one another and favoring FRET (Figure 1d). An ideal BP allows for selective excitation of the donor fluorophore. In the absence of the target, ideally, only the fluorescence from the donor fluorophore is observed; however, in the presence of the target, ideally, only the fluorescence of the acceptor fluorophore is observed because of FRET from the donor. Because the donor fluorescence is also quenched as a result of FRET in the presence of the target, BPs experience a switch in color when changing from free in the solution state to the hybridized state, when binding to the target.

Similarly to a MB, the effectiveness of a BP is determined by its S/B ratio.[15] The latter depends upon how efficiently the fluorescence of the donor fluorophore is quenched and to what extent the intensity of the acceptor is increased when the BP pair binds to the target. The S/B ratio can be generally described by eq 4

$$S/B = \frac{\dfrac{F_{Acceptor+Target}}{F_{Donor+Target}}}{\dfrac{F_{Acceptor}}{F_{Donor}}} \tag{4}$$

where $F$ is the fluorescence intensity at $\lambda_{max}$. As in the case of the MB, an ideal BP should not display any background in the absence of the target; however, this condition possesses intrinsic limitations from any real system (see the next section).[28]

BPs have found applications in the detection of gene translocation,[28] real-time monitoring of *in vitro* transcriptional mRNA synthesis,[29] and mRNA visualization in living cells.[30] One important advantage of BPs is that they do not yield false positive or nonspecific signals. The architecture of a BP is not a hairpin structure, as in the MB, but two separated ON strands that lack secondary structures (Figure 1d). To obtain a positive signal, both parts of the BP must be in the proximity of one another, which only occurs when the BP has hybridized to the target sequence.

Despite these advantages and applications of BPs, there have been some challenges that have been the focus of intense research yielding novel and creative approaches. First, the hybridization kinetics of BPs are slower than those of MBs because BPs depend upon the binding of two different components to the target. Furthermore, the entropy of a BP decreases more when bound to the target (three independent units, two probes plus the target, becoming one hybrid) than the MBs (two independents units, MB plus the target, becoming one hybrid), which decrease the equilibrium stability of the hybrid.[31] Also, it is in practice difficult to avoid some overlap between the emission spectra of the donor and acceptor fluorophores, which can decrease the S/B ratio greatly.[16] Finally, it is also usual to observe some fluorescence of the acceptor fluorophore, even in the absence of the target; acceptor fluorescence is caused by direct excitation of the acceptor at the excitation wavelength of the donor.[28]

The first two challenges have been addressed by two independent groups. Rosmarin et al. and Yang et al. employed two-probe sequences with the BP bound together using ON chains (C probes)[31] or a polyethylene glycol (PEG) polymer chain,[32] respectively. Because, in these approaches, the probing regions are bound together, the hybridization kinetics are faster and the decrease in entropy is minimal. In addition, the probe/target ratio does not have to be near unity for these probes because of the low probability that the two probe regions of the

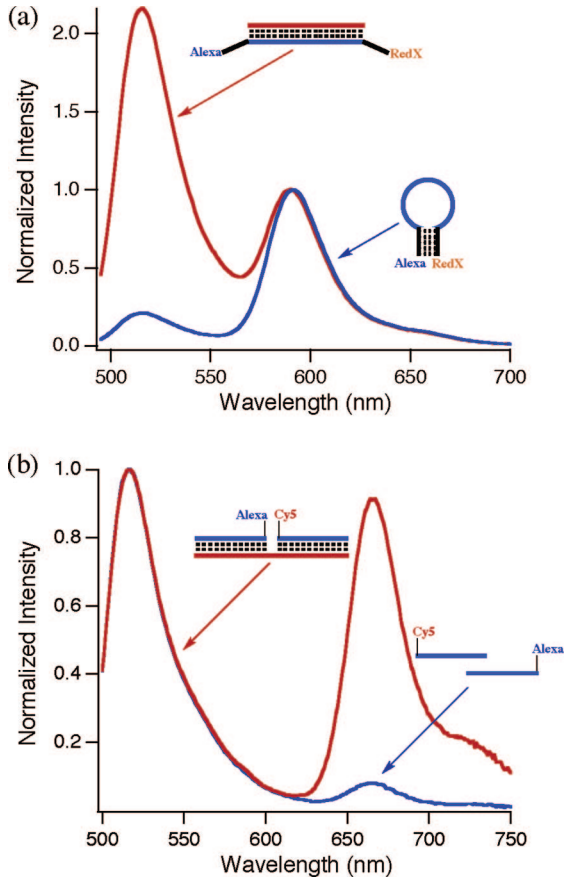


**FIGURE 2.** Fluorescence emission spectra of (a) 2d-MB and (b) 2d-BP in the presence (red) and absence (blue) of the target, modified from refs 13 and 16, respectively. The spectra were normalized to the same intensity at 590 and 665 nm for 2d-MB and 2d-BP, respectively.

same BP bind to different targets. The last two disadvantages have been the target of considerable investigation by our research group leading to systems in which low S/B ratios because of fluorescence spectra overlap and direct acceptor excitation are practically absent.[16] These systems will be explained in more detail through the following sections.

## Two-Dye Probes

Two-dye MBs (2d-MBs, left in Figure 1b) possess the advantages of better visualization of the injection in cells, the possibility of a ratiometric analysis to improve the S/B ratio,[33] and the use of two-channel detection allowing for the visualization of not only the diffusion of the MB hybridized to the target but also the MB in its hairpin structure. In 2d-MBs, the quencher is substituted by another fluorophore, which acts as an energy acceptor (Figure 2a).[13,33] In the hairpin conformation, the two fluorophores are close to one another, leading to FRET between them, whereas when hybridized with the target, the two fluorophores become separated in space and FRET is not observed. The spectra in Figure 2a show fluorescence emission mainly from the RedX acceptor in

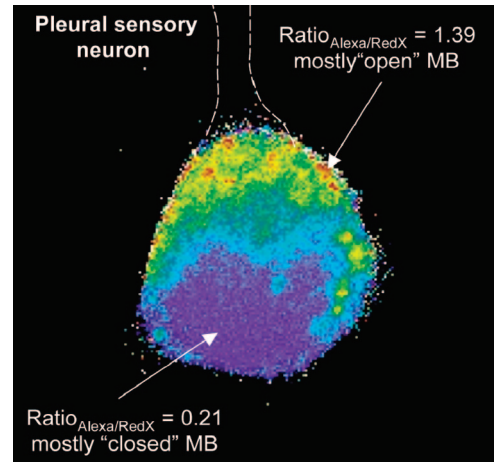


**FIGURE 3.** Fluorescence image of an *Aplysia californica* neuron injected with 2d-MB.

the absence of the target, indicating FRET from the Alexa-488 donor (chemical structures of all of the fluorescent dyes presented in this Account are included as Supporting Information). In the presence of the target, both dyes are separated as a result of hybridization, impeding FRET and leading to a strong unquenched emission of the donor. Figure 3 shows the ratiometric fluorescence image of an *Aplysia californica* neuron injected with a 2d-MB. The blue background corresponds to the visualization of the distribution of hairpin probes inside the cell, while the red spots correspond to the visualization of regions where the MB seems to be mostly "open"; thus, it is possible to visualize the MB in two different conformations.

For BPs, the two-dye ensemble is part of their design (left in Figure 1c).[24] Selection of the fluorophore pair is important for optimization of the S/B ratio.[28] As discussed above, the common procedure to select a FRET pair is based on the maximization of the overlap integral ($J$) of the pair of dyes involved in the FRET (eq 3). However, a very large $J$ produces a large overlap between the emission spectra and increases the probability of direct excitation of the acceptor fluorophore because of their overlapping transition for absorption and emission. This effect can be minimized by designing the BP with a focus on the distance between fluorophores rather than in the overlap integral ($J$).[16] The distance between the fluorophores is very important because FRET depends upon the inverse 6 power of the distance between the fluorophores.[25] In previous studies on multifluorophore-labeled DNA strands, we showed that a fluorescein–Cy5 pair separated by two nucleotides displays a FRET efficiency of 72%.[34] On the basis of this architecture, we designed a BP with the FRET pair Alexa 488–Cy5, which has a relatively poor overlap integral because of the large spectral separation, which minimizes the direct excitation of the acceptor (Cy5). This BP was also designed in such a way that, upon hybridization, the fluorophores are separated by seven nucleotides. The results, shown in Figure 2b, afforded a very low background emission before the addition of the target and a very strong FRET emission in the presence of the target.

Figure 2b also shows the small overlap between the emission spectra and the small amount of fluorescence because of direct excitation before the addition of the target. A similar design was used previously by Tsuji et al. to monitor mRNA levels *in vitro*[29] and *in vivo*.[30] An important advantage of this kind of BP is that, because the acceptor does not display any significant fluorescence in the absence of the target, it is possible to add more acceptor probe to the system, because it would not interfere with the target detection.[16] A higher concentration of the acceptor facilitates the hybridization of the probe and enhances the probability that a donor and an acceptor will be close together to enable efficient FRET.

## Three-Dye Probes (Combinatorial FRET)

The idea of three-dye probes is based on our research on trichromophore-labeled DNA.[34] In this approach, the energy of a donor dye is transferred to an acceptor dye via an intermediate chromophore that serves as an energy relay. An example of a MB using this architecture (center in Figure 1b), possessing 6-carboxyfluorescein (Fam) as the primary donor, *N,N,N′,N′*-tetramethyl-6-carboxyrhodamine (Tam) as the primary energy acceptor/secondary donor, and cyanine 5 (Cy5) as the secondary energy acceptor, has been studied.[14] In the hairpin conformation, the excitation of Fam (which is at one end of the MB) promotes an energy-transfer cascade to Tam and then to Cy5, which are at the other end of the MB. In the presence of the target, Fam and the Tam–Cy5 pair become separated, inhibiting the energy-transfer cascade and, ideally, resulting only in emission from Fam. Figure 4a shows the fluorescence signature of one of these MBs with and without the target (3d-MB). An advantage of this design is that FRET is expected to be very efficient, because it occurs between fluorophores that possess large overlap integrals that are close to one another.[35] In addition, this relayed FRET provides a good energy separation ("Stoke's shift") between the primary donor and the final acceptor, reducing the direct excitation of the final acceptor. Furthermore, the fluorescence signature of 3d-MBs can be tuned by varying the distance between the dyes, allowing for the creation of multiple FRET tags, with each possessing a unique and distinctive fluorescence pattern.[14,27,34] This approach of multiple tags has been termed combinatorial fluorescence energy transfer and can be used to develop libraries of probes with different fluorescence signatures for different applications and targeting different sequences departing from a very small set of dyes.[14]

Analogously, three-dye BPs (3d-BPs) using this architecture have also been studied (center in Figure 1c).[16] This kind of BP also contains the Fam–Tam–Cy5 triad and presents distinctive signals before and after hybridization with the target. In this architecture, one of the probes is labeled with Fam and Tam, which are separated from one another by four nucleotides. When 3d-BP is hybridized with the target, Tam, which is located at the 3′ end of the

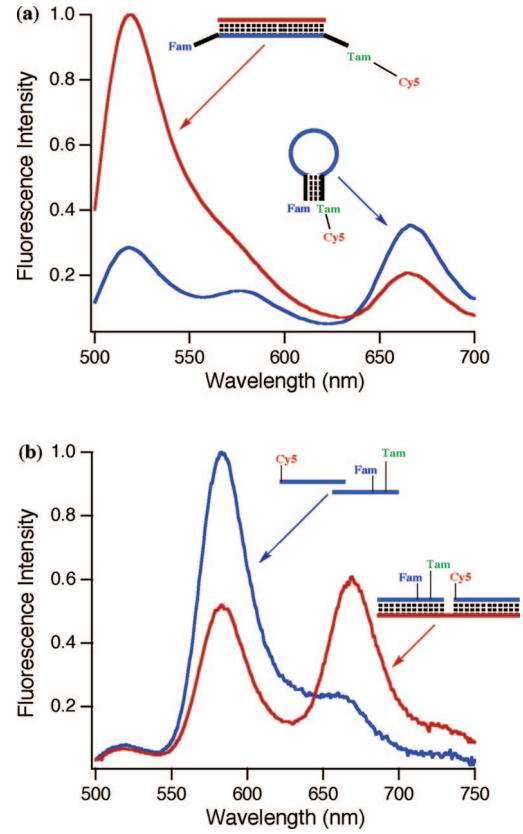


**FIGURE 4.** Fluorescence emission spectra of (a) 3d-MB and (b) 3d-BP in the presence (red) and absence (blue) of the target, modified from refs 14 and 16, respectively.

one probe strand, is brought into the proximity of Cy5 (which is at the 5′ end of the other probe strand), allowing for the FRET pathway to be optimized (center in Figure 1c). The emission spectrum of 3d-BP in the absence of the target (blue in Figure 4b) shows fluorescence mainly from Tam because of the efficient FRET from Fam; because this part of the probe is free in solution and not close enough to Tam, FRET to Cy5 is not observed. After hybridization with the target, a fraction of the probes become close to one another allowing for FRET from Fam to Tam to Cy5 to occur, producing a decrease in Tam fluorescence and an increase in Cy5 fluorescence emission (red in Figure 4b). This FRET cascade results in a unique emission pattern that provides efficient FRET and a large "Stoke's shift".

## Pyrene Excimer Probes

Probes have been designed to take advantage of the capability of pyrene to form fluorescent excited-state dimers (excimers).[25,36,37] An excimer is formed when a photoexcited pyrene molecule forms a weak association with another pyrene in its ground state. The excimer possesses a broad red-shifted emission at ~480 nm in contrast with the pyrene monomer, which displays an emission in the range of 390–410 nm. A pyrene-based MB (Py-MB) was developed by Fujimoto et al.[38] In this approach, two pyrene groups were attached at both ends

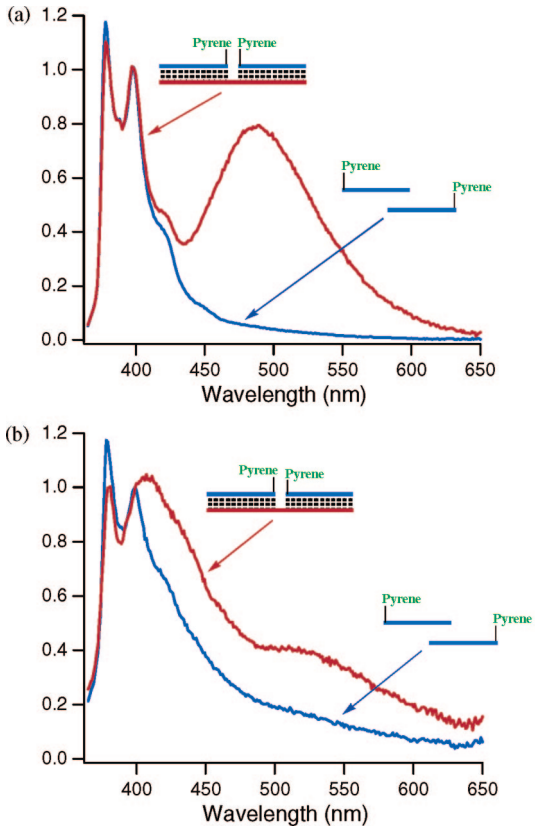


**FIGURE 5.** Fluorescence emission spectra of Py-BP in the presence (red) and absence (blue) of the target in (a) buffer solution and (b) cell extract from *Aplysia californica*, modified from ref 15 (the spectra were normalized at 400 nm).

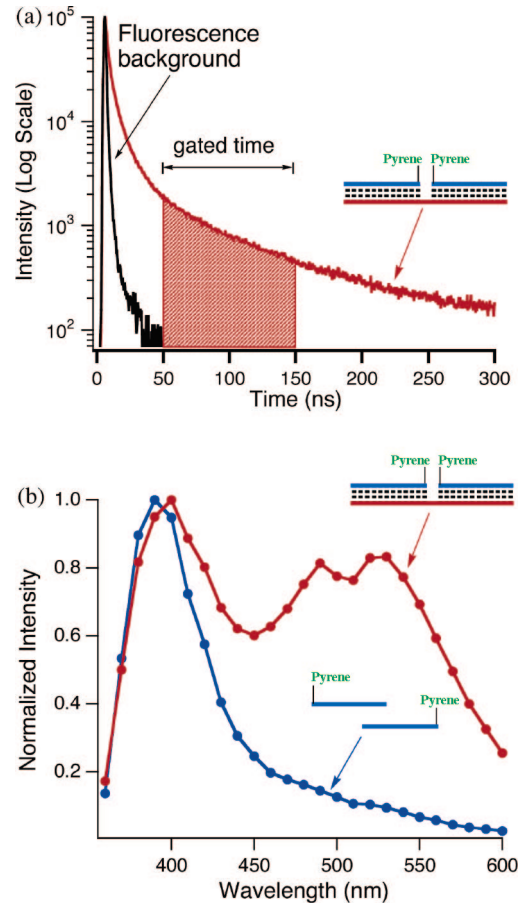


**FIGURE 6.** (a) Fluorescence decay for the *Aplysia californica* cell extract (black) and the Py-BP in the cell extract (red). The diagonal lines mark the gated time used for TRES. (b) TRES of Py-BP with (red) and without (blue) the target in the cell extract (with a gated time from 50 to 150 ns), modified from ref 15.

of the MB. When the Py-MB is in its hairpin conformation, the pyrene groups are close to one another and excimer emission can be observed (right in Figure 1b). In contrast, in the presence of the target, the pyrene groups are at opposite sites of the strand and only monomer emission is produced.

Py-BPs have also been designed with the purpose of detecting DNA and RNA strands.[15] Variation of such probes have been synthesized, differing in the chemistry used to attach pyrene to the ON strand, the length of the linker chain (between the pyrene and the ON), the strand position where the pyrene group is attached, and the number of nucleotides between the two parts of the BP when hybridized to the target.[39–41] Py-BPs have a pyrene group attached at both ends of the probe chain (right in Figure 1c). When the probes are free in solution, the pyrene groups are separated from each other and the excimer is not observed (Figure 5a). When the probe is hybridized to the target, the pyrene groups are close to one another, favoring the formation of the excimer (Figure 5a). Although the probe displays excellent S/B *in vitro*, in the presence of the cell extract (which mimics the background emission of a living cell), the "autofluorescence" is severe, leading to a poor S/B (Figure 5b).

It occurred to us that an important photoluminescence property of pyrene is its relatively long fluorescence lifetime (65 ns in aqueous solution),[15] which results from a spin-allowed but radiative-symmetry-forbidden transition from the lowest excited state ($S_1$) to the ground state ($S_0$).[25] Molecules with long lifetimes can be distinguished easily from short-lived fluorophores if the fluorescence is time-resolved. In time-resolved emission spectra (TRES), instead of collecting all of the photons that reach the detector, photons are collected at a definite time interval after pulsed excitation of the chromophores. Because the background "autofluorescence" in biological systems generally decays much faster than the emission of pyrene, TRES can be collected after the background "autofluorescence" has decayed (Figure 6a). This approach was tested on a pyrene-based hairpin aptamer probe developed to detect a platelet-derived growth factor.[42] The detection assays were performed in Dulbecco's cell-growth medium, which provides a strongly fluorescent background and obscures the detection of the probe employing standard steady-state fluorescence measurements. Because the fluorescence of the cell-growth medium has a fluorescence emission lifetime of <10 ns, the longer-lived pyrene fluorescence was easily time-resolved from the Dulbecco's cell-growth medium background.

Recently, we reported a Py-BP to detect mRNA in highly fluorescent cellular extract using time-resolved emission

spectroscopy.[15] Figure 5b shows the steady-state fluorescence spectra of the Py-BP in the cell extract, where very little signal discrimination is obtained; this is in contrast with the results in buffer solution shown in Figure 5a. To preferentially detect the Py-BP fluorescence in the cell extract, TRES were obtained. When the fluorescence detection is time-gated after the fluorescence of the background has decayed (Figure 6a), the TRES closely resembles the fluorescence spectra of the probes in buffer solution (compare Figures 5a and 6b). This technique merges the selective probe detection of Py-BP with the signal discrimination capabilities of TRES, providing new alternatives for the study of cellular processes, even in environments where the fluorescence of standard dyes is obscured by the cellular autofluorescent background.

## Summary and Outlook

In this Account, we have described some of the recent advances in the detection of DNA and RNA by fluorescent hybridization probes. Most of the research described here is based on MB and BP because they can be potentially used for both *in vivo* and *in vitro* detection. The effective use of these probes requires overcoming challenges such as spontaneous MB opening inside the cell, cell autofluorescence, injection and distribution visualization of MBs within the cell, and a low S/B ratio in BPs because of direct excitation of the acceptor fluorophore and/or overlap of the fluorescence spectra of the dyes. Modifications of the "classical" architecture of MBs have allowed for the enhancement of detection capabilities. 2d-MBs display a change in fluorescence upon binding to the target, which is advantageous in the visualization of PN in living cells and allows for a ratiometric quantization of low levels of PNs and ONs in solution. Analogously, dyes with a wide spectral separation between their fluorescence spectra have been used for the synthesis of 2d-BP, presenting a low overlap between the emission spectra of the dyes and a low direct excitation of the acceptor dye, increasing the S/B ratio. These probes can be upgraded by using three dyes, arranged in such a way that the energy is collected by a donor dye and relayed to an acceptor dye by means of an intermediate fluorophore, in a FRET cascade fashion. 3d-MBs provide the advantage of multiple signal detection and wide spectral separation between the donor and acceptor fluorophores. Furthermore, several 3d-MBs with unique signals can be synthesized using the same set of dyes by just changing spacing between them. 3d-BPs also benefit from the use of three fluorophores by diminishing the direct excitation of the final acceptor dye, increasing the S/B ratio.

Probes using pyrene have also been studied. Py-MBs and Py-BPs display distinctive signals when bound to the target or free in solution, which are based on the pyrene monomer and excimer emission. The relatively long fluorescence lifetime of pyrene allows for the use of TRES to reduce fluorescent backgrounds and increase the sensitivity of the probes. Py-BPs present very low S/B ratios in cellular extracts of *Aplysia californica* because of the strong cellular autofluorescence; however, when TRES is used, the long-lived Py-BP fluorescence is time-resolved from the short-lived autofluorescence.

Although most of the challenges described for these probes have been efficiently addressed by the research described in this Account, further improvement is necessary to achieve greater levels of sensitivity. Currently, we are working on the development of probes with improved capabilities for time resolution. Although pyrene probes present a good proof of concept, their excitation wavelength (<360 nm) might present a problem for studies in biological samples. Furthermore, molecules with longer fluorescence lifetimes may improve the time resolution capabilities of these probes even further. One of the alternatives being explored is the use of transition-metal complexes, which can possess lifetimes in the range of microseconds,[5] or lanthanide chelates, which can possess lifetimes on the order of milliseconds,[43] as energy donors. We are confident that, with the advent of new time-resolved fluorescence microscopes and with the resent developments in the field of fluorescence lifetime imaging microscopy (FLIM),[44] these kinds of probes will find their place in the study of DNA and RNA processes *in vivo*. Another approach to further increase the detection sensitivity of the ON probes is the use of strongly emissive fluorophores, such as quantum dots (QDs).[45] QDs possess relatively good quantum yields and large extinction coefficients, which result in a strong fluorescence emission.[46] Furthermore, QDs possess narrow emission spectra and moderately long emission lifetimes, which can also be advantageous for the construction of ON probes. Other techniques such as two-photon excitation[47] and spin-labeled probes[48] are also good prospects for the detection of PN and ON in different media.

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**Supporting Information Available:** Chemical structures of the fluorophores used in the synthesis of MBs and BPs. This material is available free of charge via the Internet at http://pubs.acs.org.

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