Published in final edited form as:

Chem Soc Rev. 2011 December ; 40(12): 5756–5770. doi:10.1039/c0cs00162g.

Fluorescent DNA-based enzyme sensors[†]

Nan Dai and Eric T. Kool*

Department of Chemistry, Stanford University, Stanford, CA 94305, USA

Abstract

Fluorescent sensors that make use of DNA structures have become widely useful in monitoring enzymatic activities. Early studies focused primarily on enzymes that naturally use DNA or RNA as the substrate. However, recent advances in molecular design have enabled the development of nucleic acid sensors for a wider range of functions, including enzymes that do not normally bind DNA or RNA. Nucleic acid sensors present some potential advantages over classical small-molecule sensors, including water solubility and ease of synthesis. An overview of the multiple strategies under recent development is presented in this *critical review*, and expected future developments in microarrays, single molecule analysis, and *in vivo* sensing are discussed (160 references).

1. Introduction

Enzymes are involved in nearly all biological activities, and control biological pathways by modulating rates of reactions in the cell. Enzymatic studies help us to understand substrate properties and catalytic mechanisms, thus revealing details of biological processes and networks. Abnormal enzyme activities often lead to disease, and thus enzyme studies are critical in disease diagnosis and drug discovery for the pharmaceutical industry. Moreover, enzymes are also good targets as biomarkers in the studies of biological pathways.

Many different techniques have been used in enzymatic studies. Of all available methods for monitoring enzyme activity, optical detection is the most widely used approach due to its low cost and ease of performance. Among optical methods, fluorescence in particular offers high sensitivity, low background, simplicity, and can often be extended from *in vitro* to in-cell and even *in vivo* use. Because of such special advantages, fluorescence techniques for enzyme analysis are widely used in monitoring specific molecular components within complex backgrounds.

Fluorescent enzyme probes are one of the most popular tools for detecting enzyme activities. They are widely used in enzyme identification, classification, activity assays, mechanism studies, substrate/inhibitor screening, drug discovery and cellular imaging.^{1–5} In the design of a fluorescent enzyme probe, there are many factors to consider, including optical requirements, substrate structure, and the testing environment. Depending on the requirements of the experiment, the properties of the target enzyme and environment of the

[†]Part of a themed issue on the advances in DNA-based nanotechnology.

Eric T. Kool kool@stanford.edu; Fax: +1 650 725-0259; Tel: +1 650 724-4741.

measurements, researchers often need to make some compromises among fluorophore and target molecule choice, and in probe and experimental design, in order to achieve overall optimal results in accuracy, activity and sensitivity. It is clear that there is no single probe design good for all enzymes, or even for one enzyme class in different research applications. As a result, new probes and new sensor designs are needed in many applications. Here we review the recent progress made in designing fluorescent enzyme probes built around the structure of DNA.

1.1 Using DNA structure to monitor enzyme activities

The majority of existing fluorescent enzyme probes are organic small molecules, and a large array of them are now available for monitoring a wide range of enzymatic activities. However, because of the large number of possible enzyme targets, and the broad range of settings in which one would like to monitor them, there remains a need for new classes of fluorescent probe architectures. In particular, the DNA structure offers a number of potential advantages for use in this setting. One is water solubility: the negative charges associated with DNA render it highly polar and soluble in biological settings, while achieving water solubility is often a challenge with classical small molecules. A second advantage of DNA is its ease of synthesis: it can be assembled in an automated fashion on a DNA synthesizer, and many modified components and fluorescent tags and quenchers are commercially available. Indeed, a substantial number of DNA sensors such as the ones in this review can simply be ordered by the researcher, allowing scientists with little organic chemistry expertise to access useful sensors and tailor their sequence and structure to the enzymatic system at hand. A third advantage is the ease of synthesis of combinatorial libraries, which is made simple because of the modular nature of DNA synthesis.

Of course, using the DNA structure as part of sensor design also brings up some possible limitations. Chief among these is the limited cellular permeability of DNAs resulting from their multiple negative charges and larger size. For solution-based sensing this is not an issue, but for cellular assays this is a factor that must be confronted. Fortunately there is a burgeoning array of methods available for delivering DNAs into cells and tissues, including many DNA delivery agents (cationic lipids *etc.*), as well as pore-forming peptides, cationic peptide conjugates, and physical methods (electroporation and microinjection).^{6,7} In fact, in intracellular use there is an added benefit of DNA/RNA sensors, which is that the sensor can potentially be encoded genetically. Nucleic acid-based enzyme sensors are benefiting from all of these approaches for delivery, and methods of intracellular delivery will be pointed out below in specific cases.

1.2 Fluorescent system design for DNA

Once the researcher decides to employ a DNA scaffold for an enzyme sensor, there remains the design of the fluorescent reporting system. Based on the enzyme properties and requirement of the assay, fluorescent probes can be designed as single-fluorophore, bi-chromophore or multi-fluorophore systems.

For single fluorophore systems, two types of fluorophores are widely used: caged fluorophores and environmentally sensitive fluorophores. A caged fluorophore is not

fluorescent in its original form, while its fluorescence can be restored upon reaction, such as caged coumarin derivatives used in many protease assays, and fluorescein diacetate (FDA), a widely used esterase sensor. Environment-sensitive fluorophores have emission properties that depend on the local environment (for example, polarity or ionic strength). This type of fluorophore can be used to monitor local interactions from binding or substrate conformation changes, such as for 2-aminopurine (2-AP) in helicase and alkyltransferase assays.

Bi-chromophore systems can contain two identical fluorophore molecules, or different fluorophores, or one fluorophore and one quencher. The stable helical structure of DNA allows fluorescence resonance energy transfer (FRET) to be passed along the chain over relatively long distances, which makes the DNA backbone an excellent support for such multi-chromophore probes. Homogeneous fluorophore pairs that can form distinct excimer fluorescence when in close proximity (pyrene is the mostly widely used example) are favored in many probe designs. Heterogeneous fluorophore pairs are most often FRET pairs, and the formation and disruption of FRET signals are both used in enzymatic assays. Fluorophore–quencher pairs can be designed (like FRET pairs) with favorable spectral overlap, while collisional and static quenching mechanisms can also be utilized. Each of these has its own advantages and disadvantages. Fluorophore–quencher pairs usually offer lower background, and give higher fluorescence change if the fluorescence can be restored such as in molecular beacons or enzymatic assays with bond-cleavage activities. FRET pairs offer dual-color monitoring, which can yield extra signal-to-noise advantages and yield physically useful information such as distance. A challenge in bi-chromophore and multi-chromophore designs is optimizing the physical arrangement of the chromophores to yield optimal photophysical interactions between them.

Multi-fluorophore systems contain multiple interacting dyes and/or quenchers. Although more complex, such systems are designed to improve the sensitivity and accuracy, or to detect multiple targets simultaneously.

1.3 Target molecule

Fluorescent probe design is usually driven initially by the choice of target enzyme, and this is the case for DNA-based enzyme sensors as well. An enzymatic reaction commonly involves reactants, products, enzyme(s), and some cofactors. In principle, one can track any biomolecule involved in an enzymatic process. For example, protein kinases consume ATP and produce phosphorylated peptide/protein and ADP. All three biomolecules can be monitored in kinase kinetic studies. However, it is difficult to design a fluorescent probe to selectively detect the phosphorylation process of a peptide. Moreover, due to the high concentration of ATP in cells, low sensitivity is expected for monitoring the decrease of ATP concentration. As a result, for this enzyme class, monitoring the increase of ADP concentration can be the easiest and most sensitive method. In contrast to this is the example of DNA ligase-catalyzed reactions. Although AMP is also released as a product with this family of enzymes, tracking the concentration increase of AMP may not be as convenient and sensitive as directly monitoring the ligation process with labeled short oligonucleotide sequences.

Enzyme probes are commonly designed around the substrate structure of their target enzymes. For example, sensors with short substrate peptide sequences are used to detect protease activities, while compounds with cleavable ester bonds are used as esterase/lipase sensors. With nucleic acid-based probes, there is a natural utility in the design of sensors for enzymes that process nucleic acids. However, the ability to modify and conjugate DNAs has led to sensors for a broader range of enzymes as well. This review will focus on the development and application of DNA-based fluorescent probes with single or multiple organic fluorophores in the studies of many enzyme activities and related processes. Separate sections are devoted to varied classes of enzyme activity, and the related DNA sensors designs that respond to them.

2. DNA backbone as substrate

The DNA backbone is an obvious choice in the design of fluorescent probes for DNA/RNA-targeting enzymes. DNA/RNA targeting enzymes include several enzyme families, and can be classified into four categories based on their activities: enzymes with cleavage activities, enzymes with elongation activities, enzymes with ligation activities, and enzymes that change nucleic acid conformation. There are numerous probe designs and applications that have been reported, and it is nearly impossible to cover all examples in detail. Here we will only review some of the most prominent probe designs in each enzyme category.

2.1 Probes for enzymes with cleavage activities

Enzymes that cleave certain parts of a DNA/RNA structure, such as the backbone, base or a phosphate group, are important in nucleic acid metabolism and repair. The probe design can be very flexible, and mainly depends on the specific activity of the target enzyme.

For non-specific nuclease enzymes, many probe designs can be functional. Since these nucleases can totally degrade respective DNA or RNA chains, any probe can give a significant fluorescence change whether it is an environmentally sensitive single fluorophore system, or a bi-chromophore system. These non-specific nucleases are often used as a tool to degrade undesired nucleic acids in the studies of other types of enzymes. In a second example, it is reported that one can use a DNA-based molecular beacon which is complementary to an RNA sequence in real-time kinetic studies of ribonuclease H, a sequence-nonspecific endonuclease that catalyzes the degradation of RNA (Fig. 1A).⁸

For enzymes that break down the DNA/RNA backbone at specific positions, a bi-chromophore system can be effective for monitoring the cleavage activity. The major concern is where to place the fluorophores so that the interacting pair can be within an efficient FRET distance without affecting the enzyme activity. For restriction enzymes with DNA or DNA/RNA duplexes as the target, fluorophores can be placed on either chain outside the target sequence.^{9,10} For some ribonucleases that target single stranded RNA, fluorophores need to be placed on both sides outside the recognition sequence.^{11,12} If the distance is too far or the experiments have special requirements, the fluorophores can be fused onto bases outside the recognition site.^{13–15}

For glycosylases, which recognize mismatched or modified bases and release them from deoxyribose in DNA repair processes, a single fluorophore system with environmentally sensitive natural base analogs is useful. In the study of *E. coli* formamidopyrimidine-DNA glycosylase, the fluorescent nucleobase analog 2AP was placed at the position next to the lesion for enzymatic kinetics studies. Along with Trp fluorescence from the enzyme, details about the catalytic mechanism were revealed.¹⁶ A fluorophore–quencher pair was also used to study a DNA glycosylase-related base excision repair pathway.¹⁷

2.2 Probes for enzymes with elongation activities

Enzymes with elongation activities catalyze the synthesis of long-chain DNA/RNA strands, such as DNA/RNA polymerase, or short oligonucleotides, like primase, terminal deoxynucleotidyl (TDT) transferase and telomerase. Fluorescence techniques have been used widely in studies of these enzymes, and widely varied probe designs have been reported. Like probes for other DNA target enzymes, single fluorophore, bi-chromophore and multi-fluorophore systems are all used as probes to detect the enzymatic activities for different purposes, including both basic science as well as for technological uses.

Single fluorophore probes for elongation enzymes can employ an environmentally sensitive fluorescent natural base analog as the reporter. There are two major designs: the first uses the 5′-triphosphate form of a fluorescent natural base analog (dNTP) and monitors the fluorescence change after incorporating the analog into the duplex. This method is useful for probing the activity around the specific incorporation site during DNA/RNA synthesis, and makes use of enzymatic tolerance for the altered structures of natural base analogs. The second design usually places the fluorescent natural base analog in either the primer or the template strands at the positions close to the incorporating site. This design is used to monitor the incorporation efficiency and kinetics at certain positions. Examples are discussed in Section 4.

A bi-chromophore system is the most widely used design to monitor the elongation of a DNA or RNA chain. Some probes, such as molecular beacons and TaqMan probes, have already been used as the standard protocol in monitoring realtime PCR. A TaqMan probe relies on the nuclease activity that occurs simultaneously with polymerization (Fig. 1B), so its application on enzyme studies is limited to enzymes with 5′-nuclease activity.¹⁸ The molecular beacon is one of the most widely used fluorescent probe designs for polymerase assays. In the stem–loop structure, the loop part usually has the sequence complementary to the target strand, and the stem part has a short complementary sequence to bring 3′ and 5′-ends into close proximity. High binding affinity of the loop sequence will cause the structure to open, separating the quencher and fluorophore, and a fluorescence change can be then observed (Fig. 1C). This molecular beacon design has been intensively studied and systematically reviewed, and more designs and applications can be found in these articles.^{19–21}

Fluorophore-labeled DNA duplexes with FRET or fluorophore–quencher pairs represent another popular design for bi-chromophore systems. In one design, a DNA duplex has two interacting fluorophores close to each other, usually one on the 3′-end of one strand, and another one on the 5′-end of the complementary strand, such as with duplex scorpion

primers^{22,23} and “Yin-Yang” probes.²⁴ Like molecular beacons, upon binding to the target sequence, the duplex dissociates and results in a fluorescence signal change (Fig. 1D). In this design, the sequence length of the binding strand and helper strand is very important for efficient duplex dissociation and fluorescence signal turn-on.

Multi-fluorophore designs have been used as a supplementary tool for molecular beacons to lower the background and minimize false signals, resulting in better sensitivity and accuracy,²⁵ which can be easily modified for real-time PCR application.

Additional fluorescent probe designs have been reported for monitoring oligonucleotide chain elongation processes. These probes have been reviewed,^{26,27} and will not be discussed here in detail.

2.3 Probes for enzymes with DNA/RNA ligation activities

Like polymerases, DNA/RNA ligases also result in chain elongation, while the reactants are two nucleotide chains, which enables more options in probe design. Ligases are very important tools in bioengineering, and are widely used in biological research and diagnosis. Like polymerases, fluorescent probes for ligases have been used far beyond enzymatic studies, and they are widely employed in sequencing, single DNA/RNA mismatch detection, DNA–DNA interaction, protein analysis, rolling circle amplification, and microarray fabrication.²⁸

Single fluorophore systems have been used as probes in ligase activity assays. For example, the fluorescent natural base analog 2AP was incorporated into a double-stranded DNA to study the conformation change during a T4 DNA ligase catalyzed cyclization assay,²⁹ and its triphosphate form was used in the studies of RNA/DNA 5'-end single nucleotide incorporation efficiency and labeling by T4 RNA ligase.^{30,31}

Bi-chromophore systems are the most widely used probe designs in DNA/RNA ligase assays. Two fluorophores can be placed on the two target nucleotide chains, which will show a fluorescence change after ligation by a FRET mechanism or by formation of a molecular beacon-like stem–loop structure.³² Molecular beacons with a template sequence in the loop structure can also be used as a probe. After ligation, the joined strand has a higher affinity to the template, and opens molecular beacon stem–loop structure to show a significant fluorescence change (Fig. 1E).¹⁹ There are many other interesting probe designs to monitor ligase-involved activities such as DNA sequencing, rolling circle amplification, *in situ* proximity ligation assay, among others. These have been reviewed^{28,33,34} and will not be discussed here.

2.4 Probes for enzymes with conformation-changing activities

Some nucleic acid-targeting enzymes do not change the length of the oligonucleotide chains, but rather make small modifications to the target DNA/RNA sequence. Examples include alkyl-transferases, which modify single bases, or topoisomerases, which alter DNA/RNA conformation. Both these classes of enzymes often cause bases to “flip” out of substrate DNA/RNA, and this transient change can be utilized as the target in fluorescence probing studies.

The most popular probe design for base-flipping enzymatic activities is to place an environmentally sensitive fluorescent natural base analog in the middle of the substrate sequence.³⁵ During the binding process, the fluorescent natural base analog can flip out of the DNA duplex, and a significant fluorescence change can be observed (Fig. 1F). The application of this type of probes will be discussed in Section 4.

Another important conformation-changing activity is that of the helicases, which unwind duplex structures. One probe design for this activity is to place a FRET fluorophore pair or fluorophore–quencher pair on opposite strands of a DNA duplex or dsRNA at close proximity. A FRET signal change or fluorescence turn-on can be observed during the unwinding process. Probes in this class were used in measuring activity and unwinding kinetics of DNA/RNA helicases^{36–38} and were employed in inhibitor screening for hepatitis C virus helicase.³⁹ It is also the most popular design for tracking small conformation changes over a short time scale for binding, mechanism, kinetics and dynamics studies in single molecule enzymatic analysis.^{40–42}

Molecular beacons and multi-fluorophore systems can also be used in assays of helicase activity.⁴³ This is in the proof-of-principle stage, but low background and high reproducibility were reported with these more complex systems.

3. DNA backbone as binding agent

DNA/RNA chains can non-covalently bind to various biomolecules, such as complementary DNA/RNA sequences, proteins, metal ions, and small biomolecules. Many DNA-based fluorescent probes are designed based on their binding ability, and used in different areas, such as single mismatch base-pair detection, protein detection, metal ion sensing, and gas sensing. In this part, we will focus on DNA-based fluorescent affinity probes for enzymes.

3.1 Probes that bind to enzymes

Many proteins can bind to oligonucleotide chains non-covalently. Since most enzymes are proteins, DNA-based fluorescent probes are also used as reporters to detect the presence of the enzymes that can bind to DNA without direct catalytic activity on DNA structures. In this case, the molecular beacon is the most popular design for probes that use DNA backbone as binding agents. As is the case for molecular beacons in realtime PCR or DNA/RNA detection, the designed molecular beacon needs to bind more tightly to the target than the short stem sequence binds itself. In the presence of the target enzyme, the hairpin structure of the probe opens, and a fluorescence signal can then be observed.

Thrombin, a serine protease, is known to have good binding affinity (K_d around 100 nM) at a non-catalytic site to single-stranded DNA with the sequence 5'-GGTTGGTGTGG-TTGG-3' (G15D),⁴⁴ which forms a unimolecular quadruplex structure (Fig. 2A and B).⁴⁵ By adding a short CCAAC sequence at the 5'-end, and labeling 5' and 3'-ends with a fluorophore and quencher, respectively, a molecular beaconlike thrombin sensor was designed (Fig. 3A).⁴⁶ This sensor showed a 2.5-fold fluorescence enhancement upon binding to thrombin with an apparent K_d value around 10 nM when using a fluorescein–dabcyl pair.⁴⁶ Polyethylene glycol was inserted between the recognition sequence and short

complementary sequence to facilitate the refolding process. The new sensor showed a 17.6-fold fluorescence signal enhancement with a Chlorin e6-Black Hole Quencher 2 (BHQ2) pair in the presence of 300 nM thrombin, and it also exhibited good selectivity against some negative controls such as IgG, IgM and BSA.⁴⁷

A similar sensor design was also used to differentiate lactate dehydrogenase-5 (LDH-5) from other LDH isozymes. LDH-5 has higher binding affinity to single-stranded DNA and showed 10–12-fold fluorescence increase after binding a beacon with a TMR–dabcyl pair, while LDH-1 only showed a 1.2–1.5-fold increase.⁵¹ In recent studies on myotonic dystrophykinase-related Cdc42-binding kinase (MRCK), a molecular beaconlike sensor showed a 2.3-fold fluorescence enhancement with a detection limit of 0.5 μ M by using a FAM–dabcyl pair.⁵²

When the G15D DNA sequence binds to thrombin, the unimolecular quadruplex structure brings 3' and 5'-end into proximity, which results in a fluorescence turn-off for a fluorophore–quencher pair or turn-on signal if a pair of FRET fluorophores are used (Fig. 3B).⁵³ The turn-off sensor usually shows a smaller fluorescence change compared to a FRET turn-on sensor. For example, a sensor with 6-FAM and dabcyl directly linked to 5' and 3'-ends of the binding sequence G15D showed a 2-fold fluorescence turn-off with a detection limit of 373 ± 30 pM when binding to thrombin. For a coumarin–fluorescein FRET pair, a 14-fold FRET fluorescence signal increase was observed and the detection limit was determined to be 429 ± 63 pM.⁵³ In another study, fluorophore and quencher (or another fluorophore) were constructed on two different aptamers (Fig. 3C), one (G15D) which binds to the fibrinogen binding exosite,⁴⁴ and a second 60–18[29] aptamer (Fig. 2C) which binds to the heparin binding exosite.⁵⁰ These two aptamers together can greatly increase the binding efficiency (about 36-fold) compared to the single aptamer G15D.⁵⁴ This new sensor lowered the detection limit for thrombin to less than 50 pM.

The DNA duplex structure has also been used in the design of fluorescent aptamer affinity probes. In this approach, a complementary competitor DNA strand forms a DNA duplex with the probe DNA strand. The fluorophore–quencher pair or FRET fluorophores are either both on the competitor strand, or one on each strand of the duplex. When the binding strand associates with the target enzyme, the duplex dissociates, and a fluorescence change can be observed. The binding affinity competition between the competitor strand and target enzyme is key in the design of successful sensors of this type. This design has minimal modification on the binding strand, which can maintain the affinity and specificity to the target enzymes and proteins. For example, a turn-off duplex sensor with 6-FAM and Iowa Black FQ Quencher on the complementary competitor sequence was reported.⁵⁵ After binding to thrombin, the competitor chain was released, and the short complementary stem sequences at the 5' and 3'-ends hybridized to form a stem–loop molecular beacon structure, and brought the fluorophore–quencher pair in proximity to quench the fluorescence signal. A *ca.* 4.5-fold fluorescence signal change was observed.⁵⁵

Another DNA duplex fluorescent sensor was designed by using DNA-interacting dyes, such as YOYO, TOTO and ethidium bromide (EB). In this design, the fluorophores are not covalently attached to the DNA strand, while they can intercalate into the DNA duplex

which contains the binding strand. These fluorophores have low fluorescence emission in aqueous buffer, while yielding strong fluorescence enhancement upon intercalating with the DNA duplexes. When the binding strand binds a target enzyme, the fluorophores are released and a significant fluorescence decrease can be observed. A thrombin detection limit of 2.8 nM with 8-fold fluorescence decrease was observed when using EB as the fluorophore.⁵⁶

A more complex duplex-type thrombin sensor was also reported. In this design, the binding sequence was elongated at the 5'-end to form two stem sequences. The stem sequence at 5'-end is fully complementary and hybridized with a short sequence with fluorophore (FDNA), and another short sequence with quencher (QDNA) is partially complementary to the other stem sequence and complementary to a small part of the binding sequence. When the binding sequence associates with the enzyme, the conformation change causes the dissociation of QDNA from the duplex, and the fluorescence signal is turned on (Fig. 3D). In this way, a 12-fold fluorescence enhancement with a K_d value of 400 nM was reported for thrombin.⁵⁷ Moreover, since the FDNA sequence does not participate in the binding process, it can be easily modified and attached to a solid surface to form a DNA microarray for enzyme/protein identification and drug screening.^{58,59}

In order to obtain more information about an enzyme by this affinity probe, other DNA-targeting enzymes can be used. For example, by using λ exonuclease (λ exo), the activity and kinetics of T4 polynucleotide kinase (PNK) can be monitored by a DNA-hairpin probe with a single fluorophore-labeled 3-end, and a GGG-triplet at the 5-end as the quencher. After phosphorylation by T4 PNK at the 5-hydroxyl of the probe in the presence of ATP, the product was cleaved by λ exo, which led to a significant fluorescence enhancement.⁶⁰

Because the DNA itself is the substrate, affinity probes of this type do not require chemical modification to add substrate structures, thus having the benefit of facile probe synthesis. They are useful for detecting an enzyme that does not have distinguishable activity over other similar enzymes, but has specific binding affinity to a certain DNA sequence. This design is also a useful tool to track some important DNA-binding proteins that do not have catalytic activities, such as platelet-derived growth factor (PDGF).^{61–63} But since the fluorescence signals of these probes are not directly related to the activity of enzymes, they cannot be used to measure the activity of target enzymes. The binding sites often are not the active sites of the enzymes, so they cannot illustrate the enzyme's substrate properties.

3.2 Probes that bind to small biomolecules

Certain DNA sequences (aptamers) can also bind to some biologically important small molecules, such as ADP, ATP, *etc.*, which can be monitored to measure the activity of enzymes that use them. Unlike the affinity probes that directly bind to enzymes, it is possible to measure enzyme activities indirectly by monitoring the concentration change of these small biomolecules as the reaction proceeds, if the binding process is fast enough. Moreover, since the probe does not directly interact with the target enzyme, the influence of fluorophore on the enzyme activity is minimal.

The aforementioned designs for enzyme binding affinity sensors can also be adopted for small molecules simply by changing the binding sequence.^{47,55,57} The major problem to be faced is that the binding affinity for small molecules is usually much lower than enzymes and other proteins. For example, the binding affinity of an ATP aptamer is usually 2–3 orders of magnitude lower than the binding affinity to thrombin with the same sensor design.^{47,55,57} For sensors depending on affinity competition between a target and a complementary strand, the detection limit may not be high enough for practical application.

As mentioned above, protein kinases consume ATP and generate ADP during the reaction. The presence and activity of target kinases can be obtained by monitoring either the decrease of ATP or the increase of ADP in the system. For protein kinase DNA-based affinity sensors, most are designed to bind ADP.⁶⁴ In this case, the selectivity of the aptamer among different nucleotides is key for accurate and sensitive measurements.

A previously described three-strand FDNA–QDNA affinity probe was also modified to bind small biomolecules. A binding strand which has higher affinity to adenosine than AMP was used to monitor the activity of alkaline phosphatase (ALP).⁶⁵ A similar sensor design was used to monitor the catalytic process, activity and inhibition of adenosine deaminase (ADA),^{59,66} by utilizing the binding affinity preference for adenosine over inosine, the product of the ADA-catalyzed deamination reaction. It has been employed in high-throughput drug screening as well.⁵⁸

3.3 Other affinity probes

3.3.1 RNA-based affinity probes—Like DNA, RNA sequences also can fold and bind to enzymes and proteins such as thrombin.⁶⁷ RNA aptamer sensors were designed by using the DNA-interacting luminescence dye $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ to detect α -thrombin. This RNA aptamer sensor contained a long complementary duplex region and a short loop. Upon binding to thrombin, the duplex region opened and the intercalating dye was released, which caused a decrease in luminescence (Fig. 4). A detection limit of 10 pM was reported for α -thrombin,⁶⁸ which was lower than most reported DNA-aptamer based affinity sensors. The intercalating fluorescence dye TOTO was also used with an aptamer sensor for immunoglobulin E (IgE), but a lower sensitivity compared to $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ was observed.⁶⁸

3.3.2 Amplification affinity sensors—A DNA-based affinity sensor applied with rolling circle amplification (RCA) was designed for thrombin detection. In this design, the heparin exosite binding aptamer was extended at both ends and circularized. Another fibrinogen exosite binding aptamer was extended with a polyT spacer and a short primer complementary to the circular aptamer at the 3'-end. Both aptamers can bind to the same thrombin and initiate RCA by the primer sequence in the presence of DNA polymerase (Fig. 5A). The reaction was monitored fluorescently by PCR and electrochemically by incorporated ferrocene-labeled nucleotide simultaneously; a detection limit of 30 pM for thrombin was reported.⁶⁹ Multi-valent circular aptamers with the two ends of the aptamers ligated were also reported, and the stability of aptamers in biological fluids was improved by the circular structure.⁷⁰

Wang *et al.* reported a more complex method involving PCR amplification for ultra-sensitive thrombin detection (Fig. 5B).⁷¹ In this design, exonuclease I was used to digest unbound anti-thrombin aptamers, while the bound aptamers were protected from degradation. Then the bound anti-thrombin aptamer was released, and formed a duplex with two connector sequences which were complementary to half of the anti-thrombin aptamer. The two connectors were linked by T4 ligase. The ligation product (which reflected the identity and amount of thrombin) was quantified by fluorescent quantitative PCR.⁷¹ A detection limit of several hundred thrombin molecules was reported.⁷¹

3.3.3 DNAzyme/ribozyme-based affinity sensors—Another sensor design for ATP-hydrolyzing enzymes is based on small biomolecule-binding DNAzymes or ribozymes. Some DNAzymes and ribozymes have been designed to bind small biomolecules such as ATP, ADP, or AMP.^{72–75} In one sensing approach, a fluorophore-labeled ribozyme is hybridized with a short oligonucleotide chain to make a quenched probe. When this ribozyme binds to biomolecules, it undergoes self-cleavage, and releases the fluorophore, thus giving a fluorescence signal.

In this vein, an ADP-dependent aptamer-based sensor (RiboReporter) was designed to monitor protein kinase activities.⁷² This sensor contains a 3' fluorescein-labeled allosteric ribozyme and a 5' dabcyI-labeled short oligonucleotide hybridized close to the fluorophore as a quencher (Fig. 6). The sensor binds specifically to ADP even in the presence of a 100-fold excess of ATP. Upon binding to ADP, this sensor undergoes self-cleavage to release the fluorophore signal. The rate constant of the self-cleavage was shown to have a linear correlation with ADP concentration with a detection limit of 1–5 μM ADP. By monitoring the concentration of ADP generated by the kinase reaction, the kinetics of pERK2 protein kinase activity was then obtained.⁷²

3.3.4 Fluorescence anisotropy—Fluorescence anisotropy is a technique that measures the rotational diffusion between excitation and emission photons of polarized fluorescence, and can provide information about shape changes of a large biomolecule. This method can be used to monitor the binding between two molecules, and determine the binding constant. It works best when a fluorophore-labeled molecule binds to a larger biomolecule, which is perfect for the detection of aptamer–protein interactions. This technique has been used for the detection of human neutrophil elastase in solution by using a 5'-fluorescein-labeled aptamer.⁷⁶ Later, 5'-labeled anti-thrombin aptamers were immobilized on a silica glass surface, and fluorescence anisotropy experiments were performed to measure the binding activity to thrombin.^{77,78} This solid-phase method has a good detection limit (5 nM)⁷⁷ and similar binding affinity compared to solution phase measurements.⁷⁸ A small bio-chip array containing 4 different immobilized aptamers was used to detect 4 different proteins/enzymes with reasonable sensitivity and specificity.⁷⁸

One limitation of these affinity DNA-based probes is that they monitor enzyme activities indirectly; as a result, they cannot differentiate between different enzymes that produce the same molecules as the product. Most of the DNA-based fluorescent affinity probes are still in the proof-of-principle stage, and more studies are needed to investigate their utility.

4. DNA backbone as fluorophore carrier

In some DNA-based fluorescence probes, fluorophores are directly attached to a deoxyribose or ribose backbone in the place of natural bases. In this case, the DNA backbone acts as a fluorophore carrier, and a fluorescence signal can be observed as a result of different enzymatic activities, such as conformation changes, chain elongation or shortening, or binding. The target enzymes can be DNA/RNA related enzymes, such as helicases and alkyltransferases, DNA-binding enzymes such as thrombin, or even non-DNA-related enzymes such as esterases and proteases.

Such fluorescent nucleosides have some unique advantages. Automated solid phase oligonucleoside synthesis is well developed and widely used. Thus fluorescent nucleosides can be easily incorporated at any position in the sequence by using this well-established DMT/phosphoramidite chemistry, and removing the need for post-synthesis conjugation. Compared to fluorophore-appended nucleosides, this design has a smaller size, and can minimize the perturbation to the DNA/RNA conformations. Some fluorescent nucleosides even retain the hydrogen bonding pattern with natural bases. By now, many dozens of fluorescent nucleoside analogs have been reported, and a large number of them have been used in various biological studies, such as nucleic acid structure, function and microenvironment, single nucleotide polymorphism detection, DNA/RNA binding position and mechanism and more.⁷⁹ Because this topic has been reviewed recently,⁷⁹ we will limit the discussion here to a brief summary, with a focus on the enzymes being probed.

4.1 Fluorescent structural natural base analogs

Some fluorescent nucleosides are designed to maintain the size, shape and existing hydrogen bonding patterns between purines and pyrimidines (Fig. 7A). This type of conservative analog mimics the electronic properties of natural base, and can be useful in probing the catalytic activity and mechanism of enzymes. The fluorescence signals of these natural base analogs, such as 2-aminopurine (2AP, adenine analog), are often highly dependent on the local environment, especially the extent of neighboring base stacking. These analogs can be used to study enzymes that change the conformation or structure of DNA/RNA, such as topoisomerases and alkyltransferases.

For example, 2AP is the most widely used fluorescent nucleoside, which is a constitutional isomer of adenine (Fig. 7A). 2AP alone (outside of DNA) has fluorescence emission at 370 nm with a quantum yield of 0.68 in water. Its fluorescence properties are not sensitive to pH changes in the medium to high ranges, but are very sensitive to local environments.⁸⁰ 2AP can also pair with T/U without disturbing the secondary structure of either A- or B-form of DNA and RNA, while it also can pair with C with reasonable stability.⁸¹ The fluorescence of 2AP is moderately to strongly quenched when incorporated into an oligonucleotide sequence, and the quenching efficiency is sequence dependent. Theoretically, the fluorescence signal of 2AP depends on the local environment change, and thus any binding process that can change the geometry around 2AP may be observed.

2AP has already been widely used in enzymatic studies. For enzymes related to DNA synthesis, editing and repair, the catalytic process often involves base flipping. 2AP is only

weakly fluorescent in DNA duplexes. When it flips out of the duplex, a significant fluorescence enhancement can be observed. For the studies of these enzymes, 2AP is usually placed in the middle of the sequence. By using this kind of probe, the details of catalytic processes, such as amino acid residue preference of enzyme binding and catalytic sites, substrate properties, catalytic mechanism, and kinetics, can be observed by monitoring the fluorescence change. Multiple DNA-repair enzymes have been studied with this method, including methyltransferases,^{82–91} uracil DNA glycosylase,^{92,93} helicase,³⁵ T4 endonuclease,⁹⁴ and UvrABC endonucleases.⁹⁵

For DNA/RNA replication enzymes, a different design is often preferred. In this design, 2AP placed near the templating position in the primer–template duplex provides the most information about environmental changes caused by enzyme or substrate interactions. For high fidelity polymerases like Pol I(KF), 2AP at the template (T0) position shows a rapid fluorescence decrease when adding dTTP in stopped-flow experiments.^{96–98} With 2AP at the template (+1) position, the fluorescence signal shows a significant increase when a complementary dNTP binds to a non-extendable primer^{99,100} due to the base at the (T + 1) position flipping out from the primer-template duplex.¹⁰¹ With an extendable primer, the fluorescence intensity of 2-AP shows an increase followed by a subsequent decrease due to the formation of a duplex. For low fidelity polymerases such as the Y-family DNA polymerase Dbh, 2AP at the (T – 1) position can be used to track single-base deletion errors during the chain elongation process.^{102,103} 2AP can also be placed at the primer end to monitor proofreading pathways such as polymerase–exonuclease active site switch dynamics, kinetics and mechanism.^{104–108}

In studies of the DNA helicase-mediated duplex DNA unwinding process, 2AP was incorporated into the substrate at regular intervals. Using a continuous fluorescence assay, the kinetics data were obtained by measuring the fluorescence increase of 2-AP during the unwinding process.¹⁰⁹

2AP can also be used to detect DNA-binding enzymes, such as junction revolving enzyme CCE1 and thrombin. CCE1 causes a major local structure distortion of central base pairs upon binding and a 39-fold fluorescence enhancement was observed when 2AP was placed at the center of the junction.¹¹⁰ Thrombin binding produces a lesser geometric distortion, and a 10-fold fluorescence enhancement was observed.⁶³

2AP has also proven useful in monitoring RNA-related enzymes. For example, the catalytic process of RNA polymerases involves recognizing upstream DNA sequence, binding to a promoter, unwinding the promoter DNA duplex, and then synthesizing a RNA chain. DNA duplex promoters with 2AP incorporated into different positions were used as probes to study the binding and initiation process of RNA polymerases, such as enzyme binding site and preference,¹¹¹ binding dynamics and kinetics of template and nontemplate promoters,^{112–114} mechanism of transcription initiation and open complex formation.^{115–117}

2AP was also incorporated directly into a RNA sequence to study the adenosine deamination reaction catalyzed by the RNA-editing enzyme adenosine deaminase (ADAR). A 3.3-fold fluorescence enhancement and 14 nm blue shift were observed when 2AP was placed at the

editing site.¹¹⁸ Some details of the catalytic process were revealed by using a 2AP labeled RNA duplex.¹¹⁹

Other fluorescent purine analogs are also very useful for different enzymatic studies, and show unique advantages in some cases. 3-Methyl isoxanthopterin (3-MI, a guanine analog, Fig. 7A) was used in the assay of HIV-1 integrase,¹²⁰ O⁶-alkylguanine-DNA alkyltransferase (AGT),¹²¹ and DNA-binding RNA polymerase.¹²² 6-Methyl isoxanthopterin (6-MI, a guanine analog, Fig. 7A) was used in DNA-RNA polymerase binding studies.¹²² A mutated thrombin-binding aptamer with 4-amino-6-methylpteridone (6-MAP, an adenine analog, Fig. 7A) showed 30-fold fluorescence enhancement,⁶³ which is greater than the enhancement for aptamers with 2-AP (10-fold), fluorophore-quencher (around 2-fold) and FRET-fluorophore pairs (10–15 fold).

4.2 Fluorescent expanded natural base analogs

Although fluorescent structural natural base analogs have intriguing chemical and fluorescent properties, and have been widely used in basic biological studies, they still have some drawbacks. One major problem encountered is short excitation and emission wavelengths. The fluorescent purine analogs discussed above have an absorption in the UV range and a blue fluorescence emission lower than 450 nm, which is problematic for *in vivo* studies. Moreover, it has been difficult to design a fluorescent analog for pyrimidines due to their small size. Expanded fluorescent analogs have thus been designed to address these issues (Fig. 7B). These larger-sized fluorescent natural base analogs have one or more aromatic rings added to or inserted into the natural base, leading to a red shift in both absorption and fluorescence emission. These analogs also retain part or all of the standard hydrogen bonding patterns, thus allowing them to pair with natural bases. Their environmental sensitivity varies from case to case. For example, cytosine analogs tC and tC^O (Fig. 7B) are not sensitive to the local environment, while pyrrolo-cytosine (pC) is environmentally sensitive much like 2AP. The choice of which to use will depend strongly on the enzyme sensing application.

Many fluorescent expanded natural base analogs have been developed, and some of them have been used in enzymatic studies. Because these analogs are larger in size and different in shape compared to natural bases, their activity and specificity to certain enzymes (for direct reactivity) may be different than those of the natural bases. However, these analogs can be used to test the tolerance and preference of certain enzymes to their substrates, and to reveal the details of the catalytic process. For example, the 5'-triphosphate forms of deoxycytidine analogs tC and tC^O were efficiently incorporated into DNA sequences, which indicated that the Klenow fragment (KF) polymerase is flexible enough to tolerate size-expanded base pairs in the major groove.¹²³ By using a tC-labeled DNA primer and TEMPO-labeled KF polymerase, nucleotide-induced conformational changes of the KF polymerase bound to duplex DNA were characterized.¹²⁴ Another cytidine analog, pC, was used to monitor the binding process of DNA-reverse transcriptase,¹²⁵ and to characterize the promoter structural rearrangement generated by T7 RNA polymerase during the transition from initiation to elongation steps.^{126,127}

A different class of expanded DNA base analogs involves insertion of a benzene ring into the middle of the nucleobase structure. The earliest of these, lin-benzo-A, was reported by Leonard, and full sets of expanded nucleobases (xDNA and yDNA) were described by Kool (Fig. 7B). As a group, such compounds can form stable duplexes with their counter-complementary natural bases, yielding base pairs with sizes expanded by ca. 2.4 Å.¹²⁸ All the nucleobases of the xDNA and yDNA sets are fluorescent.^{129,130}

Leonard's early work evaluated the expanded adenosine ribose analog rxA with several DNA/RNA related enzymes to check the enzyme tolerance and specificity. Xanthine oxidase and adenosine deaminase showed a reduced activity and poor selectivity, while nucleoside phosphorylase and adenine phosphoribosyltransferase did not accept rxA as a substrate.¹³¹ Later replication experiments with DNA repair polymerases showed higher efficiency.¹³² A template-independent polymerase, terminal deoxynucleotidyl transferase (TdT), was shown to accept the xDNA nucleotides efficiently, resulting in fluorescent polymers that could be analyzed in solution or by microscopy (Fig. 8).¹³³

4.3 Nonpolar and other fluorescent analogs

Some fluorescent nucleoside analogs have been designed to act simply as fluorophores without mimicking a specific natural base structure. Examples include simple aromatic hydrocarbons^{134,135} and known fluorescent dyes (Fig. 7C).⁷⁹ Despite their lack of close resemblance to natural nucleobases, they have proven quite useful in a number of enzymatic studies.

The pyrene nucleobase replacement (Fig. 7C)¹³⁵ is the most widely used nonpolar fluorescent analog due to its chemical stability, its ability to form a long-wavelength excimer state with a neighboring pyrene, and its sensitivity to the local environment. Pyrene is larger in size than natural bases (indeed, it has been used as a surrogate for a base pair), and it does not have functional groups for hydrogen bonding. Despite this, the 5'-triphosphate derivative of pyrene analog dPTP can be selectively and efficiently inserted into DNA opposite an abasic site by KF polymerase I and other polymerases, which confirmed that steric complementarity is important in DNA synthesis.¹³⁶ Later, pyrene nucleotide dPMP was used as a probe for the mechanism study of the formation of a transient abasic site-like intermediate in the bypass of dipyrimidine photoproducts by T7 DNA polymerase.¹³⁷ Terminal deoxynucleotidyl transferase (TdT) can also accept the triphosphate form of pyrene nucleoside (dPTP) as a substrate and insert three or four pyrenes at the end of a DNA primer without a template strand.¹³⁸ Jiang *et al.* reported a "chemical rescue" approach to completely or partially restore the weak binding affinity and low catalytic activity of mutated uracil DNA glycosylases (UDG) by substituting a pyrene analog opposite to the uracil base, and showed that pyrene can push the uracil base from the base stack and hinder its reinsertion after base flipping.¹³⁹

A coumarin 102-containing nucleoside analog (Fig. 7C) was also reported. It also can pair with an abasic site in the DNA duplex without significant structural distortions, and is sensitive to the local environment. This analog was used to study the binding dynamics and mechanism of the DNA-APE1 endonuclease complex.¹⁴⁰

4.4 DNA polyfluorophore sensors

In some recent DNA-based enzyme sensors, the DNA backbone has served simply as a scaffold to arrange multiple fluorophores in a stack. This leads to multiple favorable photophysical properties that do not exist in single-fluorophore compounds. In this light, Kool and co-workers have developed an oligodeoxyfluoroside (ODF) system, which has several fluorophores replacing the DNA bases, directly attached to the backbone in a close proximity. This ODF system enables sets of cooperative multicolor fluorophores that can be excited at single wavelength, and fluorophores with Stokes shifts of as much as 250 nm.^{134,141,142} The delocalized excited states that occur in these systems can result in the useful phenomenon of “superquenching”,^{143–145} allowing very dark initial states for turn-on sensors.

ODF-based sensors for esterases, lipases and proteases have been synthesized and tested recently.¹⁴⁵ In the sensor design for esterases and lipases, an ODF tetramer was conjugated to a dabcyl quencher *via* different ester linkages (Fig. 9A). Experiments showed that different ODF sequences could be quenched with a common dabcyl quencher with high efficiency (20 to 60-fold), and the substrate selectivity was not affected by the structure and sequence of ODFs (Fig. 9B).¹⁴⁵ Interestingly, the ODF sensors were shown to be cell permeable and yielded signals for esterases in human cell culture; this may be the only example to date of the application of synthetic DNA-based enzyme sensors *in vivo* (Fig. 9C).

This approach has considerable promise for future applications with a broader array of enzymes than has yet been examined with DNA-based sensors. In principle, a sensor can be designed for any bond-cleaving enzyme with any emission color by simply changing the substrate (*i.e.* cleavable bond) structure and ODF sequence. The properties of single wavelength excitation, favorable quenching and varied fluorescence color choice make this ODF system promising for real-time multi-color, multi-enzyme assays, especially in live systems.

5. Perspectives

Fluorescence techniques have been extensively explored and widely utilized in basic biological, biomedical and clinical studies. The field is still in rapid development on topics such as new fluorophores with better photophysical properties and biological compatibility, new probe designs with higher specificity and signal-to-noise ratio, and new techniques for more rapid and accurate analysis. As a result of the rapid progress, there is an ever-increasing expectation and demand. How can we monitor an enzymatic event in greater detail, or follow a process on a molecular level in a living system? Can we distinguish and track each enzyme involved in a continuous catalytic process? Can we identify and characterize a large number of enzymes simultaneously in a complex system? To address these issues, promising technological developments are underway and may make a significant impact on enzymatic studies in the near future. Two examples are single molecule spectroscopy and DNA microarrays.

5.1 Single molecule technology

Single molecule techniques comprise one of the most rapidly developing areas of science in the last decade. Due to their super-high resolution and sensitivity, single molecule spectroscopy and microscopy can provide information on individual target distributions and time trajectories, which cannot be obtained by conventional ensemble-averaged methods. All kinds of fluorescence information, such as FRET, polarization, lifetime, localization and intensity, can be utilized for the real-time detection and monitoring of biochemical events. Single molecule technology shows particular advantages in the studies of non-equilibrated time-dependent systems, such as reaction intermediates and pathways.

DNA-based fluorescent probes have been combined with single molecule technology to reveal new details of certain enzymatic catalytic processes. Fluorescent probes for single molecule spectroscopy are usually designed by immobilizing traditional fluorescent substrates on a solid surface. It can be a single fluorophore system with monitoring of fluorescence decay or intensity change, or a multi-fluorophore system, monitoring real-time FRET signals. In the past few years, fluorescent DNA-based FRET probes have been used in the studies of RNA polymerase translocation, pausing and folding,^{146–148} three-state folding mechanism of RNase H,¹⁴⁹ the DNA/RNA helicase initiated unwinding and catalytic mechanism,^{40–42} staphylococcal nuclease conformational dynamics and cleavage mechanisms,¹⁵⁰ primase and helicase function in a T7 replication complex,¹⁵¹ the DNA polymerase synthesis rate,¹⁵² binding orientation effects on HIV reverse transcriptase activity,¹⁵³ and many more.

In vitro enzymatic studies using single molecule technology are providing detailed information on the enzymatic mechanism, kinetics and dynamics, which is not available with traditional fluorescence methods. However, there remain scientific and technological challenges to track an enzymatic process at a single molecule level *in vivo* due the high background and complexity in a living system. Recently, single molecule techniques have been used for *in vivo* imaging,^{154,155} and tracking single enzyme activities in cells may become a reality in the near future. This will advance enzymatic studies to a more biologically relevant context.

5.2 DNA microarrays

Single molecule technology is trying to solve sensing problems in a “micro” way by providing higher detection sensitivity and resolution. Microarray technology, on the other hand, aims to address the issues in a “macro” way, by characterizing a large number of biomolecules simultaneously. Microarrays and the concept of high-throughput screening are not new, but with the development of new technologies especially in bioengineering and manufacturing, microarray technologies (biochips) for enzyme sensing are in rapid development.

DNA microarrays have been developed and applied in nearly every area of nucleic acid research, such as large-scale low-cost genome sequencing, DNA/RNA sequence identification and profiling, DNA-protein interaction, and in *situ* synthesis of oligonucleotides. In the sensing of enzyme activities, polymerase-catalyzed “on-chip” primer

extension¹⁵⁶ and activity-based phosphatase identification¹⁵⁷ have been reported. DNA microarrays can also hybridize with complementary peptide nucleic acids (PNA), and have been employed to pull down PNA-encoded peptide libraries for protease and kinase screening.^{158–160} With the improvement in sensitivity and specificity of fluorescent enzyme probes, and development of substrate trapping and printing methods, DNA microarrays are likely to make significant new contributions in practical enzyme-related analysis, including enzyme/protein identification, drug and drug metabolite screening, and disease diagnosis.

5.3 Other topics for future development

In addition to the rapid developments in single molecule technologies and microarrays, DNA-based sensors are likely to find applications in new directions that have only begun to be explored to date. One limitation of DNA-based enzymes has been their primary use in nucleic acid-based enzyme monitoring. Since DNA-based sensors have some real advantages over classical small molecule sensors—water solubility and ease of synthesis being among them—it seems attractive to consider whether one might apply some of these advantages to a broader array of enzymes beyond DNA- and RNA-processing enzymes. The recent work involving aptamers for enzymes that do not typically bind DNA (described above) shows one path to making this a reality; but it seems likely that additional creative approaches may be developed for other enzymes as well.

It is clear that one strong theme in the recent development of sensors in general is the move from simple *in vitro* solution-based sensing to intracellular sensing and to imaging in multicellular organisms. This presents a challenge to future developers of nucleic acid-based sensors: can new approaches be found that can be applied in living systems? Considering the known properties of nucleic acids, researchers will need to address issues of biostability and intracellular delivery. However, these areas are advancing rapidly in nucleic acid sciences, so it is likely that an area for future growth of DNA-based enzyme sensing lies in this direction.

Acknowledgments

We thank the U.S. National Institutes of Health (GM067201 and GM068122) for support of research on fluorescent DNA sensors.

Biographies

Nan Dai: Nan Dai was born in Zhengzhou, P. R. China in 1979. He obtained his BS degree in Chemistry in 2001 from Peking University, China. After that, he moved to Virginia Tech and got his PhD degree in Organic Chemistry under the supervision of Prof. Felicia A. Etzkorn in 2008. Then he joined Prof. Eric T. Kool's group as a postdoctoral fellow at Stanford University. His research there focused on the design and synthesis of enzyme sensors based on DNA-scaffolded oligodeoxyriboside fluorophores (ODFs).

Eric T. Kool: Eric Kool is the George and Hilda Daubert Professor of Chemistry at Stanford University. He obtained his undergraduate degree from Miami University (Ohio) and his PhD in Organic Chemistry from Columbia University in the lab of Ronald Breslow. His postdoctoral training was carried out at Caltech with Peter Dervan. After beginning his independent academic career at the University of Rochester, he joined the faculty at Stanford in 1999. His research focuses on the design, synthesis and biological applications of molecules inspired by DNA.

Notes and references

1. Lavis LD, Chao TY, Raines RT. ACS Chem Biol. 2006; 1:252–260. [PubMed: 17163679]
2. Giepmans BN, Adams SR, Ellisman MH, Tsien RY. Science. 2006; 312:217–224. [PubMed: 16614209]
3. Johnsson N, Johnsson K. ACS Chem Biol. 2007; 2:31–38. [PubMed: 17243781]
4. Rao J, Dragulescu-Andrasi A, Yao H. Curr Opin Biotechnol. 2007; 18:17–25. [PubMed: 17234399]
5. Sadaghiani AM, Verhelst SH, Bogoy M. Curr Opin Chem Biol. 2007; 11:20–28. [PubMed: 17174138]
6. Juliano R, Alam MR, Dixit V, Kang H. Nucleic Acids Res. 2008; 36:4158–4171. [PubMed: 18558618]
7. Luo D, Saltzman WM. Nat Biotechnol. 2000; 18:33–37. [PubMed: 10625387]
8. Chen Y, Yang CJ, Wu Y, Conlon P, Kim Y, Lin H, Tan W. ChemBioChem. 2008; 9:355–359. [PubMed: 18181133]
9. Potenza N, Salvatore V, Migliozi A, Martone V, Nobile V, Russo A. Nucleic Acids Res. 2006; 34:2906–2913. [PubMed: 16738129]
10. Erskine SG, Halford SE. J Mol Biol. 1998; 275:759–772. [PubMed: 9480767]
11. Rizzo J, Gifford LK, Zhang X, Gewirtz AM, Lu P. Mol Cell Probes. 2002; 16:277–283. [PubMed: 12270269]
12. James DA, Woolley GA. Anal Biochem. 1998; 264:26–33. [PubMed: 9784184]
13. Roberts JA, White MF. Nucleic Acids Res. 2005; 33:6662–6670. [PubMed: 16314325]
14. Lowman HB, Draper DE. J Biol Chem. 1986; 261:5396–5403. [PubMed: 2420800]
15. Harvey JJ, Lee SP, Chan EK, Kim JH, Hwang ES, Cha CY, Knutson JR, Han MK. Anal Biochem. 2004; 333:246–255. [PubMed: 15450799]

16. Koval VV, Kuznetsov NA, Ishchenko AA, Saparbaev MK, Fedorova OS. *Mutat Res.* 2010; 685:3–10. [PubMed: 19751748]
17. Maksimenko A, Ishchenko AA, Sanz G, Laval J, Elder RH, Saparbaev MK. *Biochem Biophys Res Commun.* 2004; 319:240–246. [PubMed: 15158468]
18. Heid CA, Stevens J, Livak KJ, Williams PM. *Genome Res.* 1996; 6:986–994. [PubMed: 8908518]
19. Tan W, Wang K, Drake TJ. *Curr Opin Chem Biol.* 2004; 8:547–553. [PubMed: 15450499]
20. Li Y, Zhou X, Ye D. *Biochem Biophys Res Commun.* 2008; 373:457–461. [PubMed: 18489905]
21. Wang K, Tang Z, Yang CJ, Kim Y, Fang X, Li W, Wu Y, Medley CD, Cao Z, Li J, Colon P, Lin H, Tan W. *Angew Chem, Int Ed.* 2009; 48:856–870.
22. Huang Y, Kong D, Yang Y, Niu R, Shen H, Mi H. *Biotechnol Lett.* 2004; 26:891–895. [PubMed: 15269536]
23. Solinas A, Brown LJ, McKeen C, Mellor JM, Nicol J, Thelwell N, Brown T. *Nucleic Acids Res.* 2001; 29:E96. [PubMed: 11600715]
24. Li Q, Luan G, Guo Q, Liang J. *Nucleic Acids Res.* 2002; 30:E5. [PubMed: 11788731]
25. Santangelo PJ, Nix B, Tsourkas A, Bao G. *Nucleic Acids Res.* 2004; 32:e57. [PubMed: 15084672]
26. Marras SA, Tyagi S, Kramer FR. *Clin Chim Acta.* 2006; 363:48–60. [PubMed: 16111667]
27. VanGuilder HD, Vrana KE, Freeman WM. *BioTechniques.* 2008; 44:619–626. [PubMed: 18474036]
28. Conze T, Shetye A, Tanaka Y, Gu J, Larsson C, Goransson J, Tavoosidana G, Soderberg O, Nilsson M, Landegren U. *Annu Rev Anal Chem.* 2009; 2:215–239.
29. Yuan C, Lou XW, Rhoades E, Chen H, Archer LA. *Nucleic Acids Res.* 2007; 35:5294–5302. [PubMed: 17686784]
30. Brennan CA, Gumpert RI. *Nucleic Acids Res.* 1985; 13:8665–8684. [PubMed: 4080551]
31. Kinoshita Y, Nishigaki K, Husimi Y. *Nucleic Acids Res.* 1997; 25:3747–3748. [PubMed: 9278501]
32. Tang H, Yang X, Wang K, Tan W, Li H, He L, Liu B. *Talanta.* 2008; 75:1388–1393. [PubMed: 18585229]
33. Nilsson M, Dahl F, Larsson C, Gullberg M, Stenberg J. *Trends Biotechnol.* 2006; 24:83–88. [PubMed: 16378651]
34. Mardis ER. *Annu Rev Genomics Hum Genet.* 2008; 9:387–402. [PubMed: 18576944]
35. Dillingham MS, Wigley DB, Webb MR. *Biochemistry.* 2002; 41:643–651. [PubMed: 11781105]
36. Bjornson KP, Amaratunga M, Moore KJ, Lohman TM. *Biochemistry.* 1994; 33:14306–14316. [PubMed: 7947840]
37. Boguszewska-Chachulska AM, Krawczyk M, Stankiewicz A, Gozdek A, Haenni AL, Stokovskaya L. *FEBS Lett.* 2004; 567:253–258. [PubMed: 15178332]
38. Tani H, Fujita O, Furuta A, Matsuda Y, Miyata R, Akimitsu N, Tanaka J, Tsuneda S, Sekiguchi Y, Noda N. *Biochem Biophys Res Commun.* 2010; 393:131–136. [PubMed: 20117090]
39. Tani H, Akimitsu N, Fujita O, Matsuda Y, Miyata R, Tsuneda S, Igarashi M, Sekiguchi Y, Noda N. *Biochem Biophys Res Commun.* 2009; 379:1054–1059. [PubMed: 19150342]
40. Theissen B, Karow AR, Kohler J, Gubaev A, Klostermeier D. *Proc Natl Acad Sci U S A.* 2008; 105:548–553. [PubMed: 18184816]
41. Myong S, Bruno MM, Pyle AM, Ha T. *Science.* 2007; 317:513–516. [PubMed: 17656723]
42. Ha T, Rasnik I, Cheng W, Babcock HP, Gauss GH, Lohman TM, Chu S. *Nature.* 2002; 419:638–641. [PubMed: 12374984]
43. Belon CA, Frick DN. *BioTechniques.* 2008; 45:433–440. 442. [PubMed: 18855770]
44. Bock LC, Griffin LC, Latham JA, Vermaas EH, Toole JJ. *Nature.* 1992; 355:564–566. [PubMed: 1741036]
45. Macaya RF, Schultze P, Smith FW, Roe JA, Feigon J. *Proc Natl Acad Sci U S A.* 1993; 90:3745–3749. [PubMed: 8475124]
46. Hamaguchi N, Ellington A, Stanton M. *Anal Biochem.* 2001; 294:126–131. [PubMed: 11444807]
47. Tang Z, Mallikaratchy P, Yang R, Kim Y, Zhu Z, Wang H, Tan W. *J Am Chem Soc.* 2008; 130:11268–11269. [PubMed: 18680291]

48. Padmanabhan K, Padmanabhan KP, Ferrara JD, Sadler JE, Tulinsky A. *J Biol Chem.* 1993; 268:17651–17654. [PubMed: 8102368]
49. Liu J, Cao Z, Lu Y. *Chem Rev.* 2009; 109:1948–1998. [PubMed: 19301873]
50. Tasset DM, Kubik MF, Steiner W. *J Mol Biol.* 1997; 272:688–698. [PubMed: 9368651]
51. Fang X, Li JJ, Tan W. *Anal Chem.* 2000; 72:3280–3285. [PubMed: 10939400]
52. Tok J, Lai J, Leung T, Li SF. *Talanta.* 2010; 81:732–736. [PubMed: 20188990]
53. Li JJ, Fang X, Tan W. *Biochem Biophys Res Commun.* 2002; 292:31–40. [PubMed: 11890667]
54. Heyduk E, Heyduk T. *Anal Chem.* 2005; 77:1147–1156. [PubMed: 15858998]
55. Li N, Ho CM. *J Am Chem Soc.* 2008; 130:2380–2381. [PubMed: 18247609]
56. Li B, Wei H, Dong S. *Chem Commun.* 2007:73–75.
57. Nutiu R, Li Y. *J Am Chem Soc.* 2003; 125:4771–4778. [PubMed: 12696895]
58. Elowe NH, Nutiu R, Allali-Hassani A, Cechetto JD, Hughes DW, Li Y, Brown ED. *Angew Chem, Int Ed.* 2006; 45:5648–5652.
59. Rupcich N, Nutiu R, Li Y, Brennan JD. *Angew Chem, Int Ed.* 2006; 45:3295–3299.
60. Song C, Zhao M. *Anal Chem.* 2009; 81:1383–1388. [PubMed: 19170527]
61. Yang CJ, Jockusch S, Vicens M, Turro NJ, Tan W. *Proc Natl Acad Sci U S A.* 2005; 102:17278–17283. [PubMed: 16301535]
62. Green LS, Jellinek D, Jenison R, Ostman A, Heldin CH, Janjic N. *Biochemistry.* 1996; 35:14413–14424. [PubMed: 8916928]
63. Katilius E, Katiliene Z, Woodbury NW. *Anal Chem.* 2006; 78:6484–6489. [PubMed: 16970324]
64. Charter NW, Kauffman L, Singh R, Eglen RM. *J Biomol Screening.* 2006; 11:390–399.
65. Nutiu R, Yu JM, Li Y. *ChemBioChem.* 2004; 5:1139–1144. [PubMed: 15300841]
66. Nutiu R, Li Y. *Angew Chem, Int Ed.* 2005; 44:5464–5467.
67. Kubik MF, Stephens AW, Schneider D, Marlar RA, Tasset D. *Nucleic Acids Res.* 1994; 22:2619–2626. [PubMed: 7518917]
68. Jiang Y, Fang X, Bai C. *Anal Chem.* 2004; 76:5230–5235. [PubMed: 15373466]
69. Di Giusto DA, Wlasoff WA, Gooding JJ, Messerle BA, King GC. *Nucleic Acids Res.* 2005; 33:e64. [PubMed: 15817563]
70. Di Giusto DA, Knox SM, Lai Y, Tyrelle GD, Aung MT, King GC. *ChemBioChem.* 2006; 7:535–544. [PubMed: 16482500]
71. Wang XL, Li F, Su YH, Sun X, Li XB, Schluesener HJ, Tang F, Xu SQ. *Anal Chem.* 2004; 76:5605–5610. [PubMed: 15456277]
72. Srinivasan J, Cload ST, Hamaguchi N, Kurz J, Keene S, Kurz M, Boomer RM, Blanchard J, Epstein D, Wilson C, Diener JL. *Chem Biol.* 2004; 11:499–508. [PubMed: 15123244]
73. Levy M, Ellington AD. *Chem Biol.* 2002; 9:417–426. [PubMed: 11983331]
74. Mei SH, Liu Z, Brennan JD, Li Y. *J Am Chem Soc.* 2003; 125:412–420. [PubMed: 12517153]
75. Li D, Shlyahovsky B, Elbaz J, Willner I. *J Am Chem Soc.* 2007; 129:5804–5805. [PubMed: 17432859]
76. Jayasena SD. *Clin Chem (Washington, D C).* 1999; 45:1628–1650.
77. Potyrailo RA, Conrad RC, Ellington AD, Hieftje GM. *Anal Chem.* 1998; 70:3419–3425. [PubMed: 9726167]
78. McCauley TG, Hamaguchi N, Stanton M. *Anal Biochem.* 2003; 319:244–250. [PubMed: 12871718]
79. Sinkeldam RW, Greco NJ, Tor Y. *Chem Rev.* 2010; 110:2579–2619. [PubMed: 20205430]
80. Ward DC, Reich E, Stryer L. *J Biol Chem.* 1969; 244:1228–1237. [PubMed: 5767305]
81. Nordlund TM, Andersson S, Nilsson L, Rigler R, Graslund A, McLaughlin LW. *Biochemistry.* 1989; 28:9095–9103. [PubMed: 2605243]
82. Gowher H, Jeltsch A. *J Mol Biol.* 2000; 303:93–110. [PubMed: 11021972]
83. Vilkaitis G, Dong A, Weinhold E, Cheng X, Klimasauskas S. *J Biol Chem.* 2000; 275:38722–38730. [PubMed: 11102456]

84. Pues H, Bleimling N, Holz B, Wolcke J, Weinhold E. *Biochemistry*. 1999; 38:1426–1434. [PubMed: 9931007]
85. Reddy YV, Rao DN. *J Mol Biol*. 2000; 298:597–610. [PubMed: 10788323]
86. Allan BW, Reich NO. *Biochemistry*. 1996; 35:14757–14762. [PubMed: 8942637]
87. Allan BW, Beechem JM, Lindstrom WM, Reich NO. *J Biol Chem*. 1998; 273:2368–2373. [PubMed: 9442083]
88. Holz B, Klimasauskas S, Serva S, Weinhold E. *Nucleic Acids Res*. 1998; 26:1076–1083. [PubMed: 9461471]
89. Allan BW, Garcia R, Maegley K, Mort J, Wong D, Lindstrom W, Beechem JM, Reich NO. *J Biol Chem*. 1999; 274:19269–19275. [PubMed: 10383435]
90. Neely RK, Daujotyte D, Grazulis S, Magennis SW, Dryden DT, Klimasauskas S, Jones AC. *Nucleic Acids Res*. 2005; 33:6953–6960. [PubMed: 16340006]
91. Su TJ, Tock MR, Egelhaaf SU, Poon WC, Dryden DT. *Nucleic Acids Res*. 2005; 33:3235–3244. [PubMed: 15942026]
92. Stivers JT, Pankiewicz KW, Watanabe KA. *Biochemistry*. 1999; 38:952–963. [PubMed: 9893991]
93. Stivers JT, Drohat AC. *Arch Biochem Biophys*. 2001; 396:1–9. [PubMed: 11716455]
94. McCullough AK, Dodson ML, Scharer OD, Lloyd RS. *J Biol Chem*. 1997; 272:27210–27217. [PubMed: 9341165]
95. Malta E, Verhagen CP, Moolenaar GF, Filippov DV, van der Marel GA, Goosen N. *DNA Repair*. 2008; 7:1647–1658. [PubMed: 18638572]
96. Arndt JW, Gong W, Zhong X, Showalter AK, Liu J, Dunlap CA, Lin Z, Paxson C, Tsai MD, Chan MK. *Biochemistry*. 2001; 40:5368–5375. [PubMed: 11330999]
97. Shah AM, Li SX, Anderson KS, Sweasy JB. *J Biol Chem*. 2001; 276:10824–10831. [PubMed: 11154692]
98. Fidalgo da Silva E, Mandal SS, Reha-Krantz LJ. *J Biol Chem*. 2002; 277:40640–40649. [PubMed: 12189135]
99. Purohit V, Grindley ND, Joyce CM. *Biochemistry*. 2003; 42:10200–10211. [PubMed: 12939148]
100. Zhang H, Cao W, Zakharova E, Konigsberg W, De La Cruz EM. *Nucleic Acids Res*. 2007; 35:6052–6062. [PubMed: 17766250]
101. Johnson SJ, Taylor JS, Beese LS. *Proc Natl Acad Sci U S A*. 2003; 100:3895–3900. [PubMed: 12649320]
102. Tippin B, Kobayashi S, Bertram JG, Goodman MF. *J Biol Chem*. 2004; 279:45360–45368. [PubMed: 15339923]
103. DeLucia AM, Grindley ND, Joyce CM. *Biochemistry*. 2007; 46:10790–10803. [PubMed: 17725324]
104. Beechem JM, Otto MR, Bloom LB, Eritja R, Reha-Krantz LJ, Goodman MF. *Biochemistry*. 1998; 37:10144–10155. [PubMed: 9665720]
105. Fidalgo da Silva E, Reha-Krantz LJ. *Nucleic Acids Res*. 2007; 35:5452–5463. [PubMed: 17702757]
106. Baker RP, Reha-Krantz LJ. *Proc Natl Acad Sci U S A*. 1998; 95:3507–3512. [PubMed: 9520396]
107. Bloom LB, Otto MR, Eritja R, Reha-Krantz LJ, Goodman MF, Beechem JM. *Biochemistry*. 1994; 33:7576–7586. [PubMed: 8011623]
108. Otto MR, Bloom LB, Goodman MF, Beechem JM. *Biochemistry*. 1998; 37:10156–10163. [PubMed: 9665721]
109. Raney KD, Sowers LC, Millar DP, Benkovic SJ. *Proc Natl Acad Sci U S A*. 1994; 91:6644–6648. [PubMed: 8022830]
110. Declais AC, Lilley DM. *J Mol Biol*. 2000; 296:421–433. [PubMed: 10669598]
111. Schroeder LA, Gries TJ, Saecker RM, Record MT Jr, Harris ME, DeHaseth PL. *J Mol Biol*. 2009; 385:339–349. [PubMed: 18976666]
112. Ujvari A, Martin CT. *Biochemistry*. 1996; 35:14574–14582. [PubMed: 8931555]
113. Fedoriw AM, Liu H, Anderson VE, deHaseth PL. *Biochemistry*. 1998; 37:11971–11979. [PubMed: 9718322]

114. Jia Y, Kumar A, Patel SS. *J Biol Chem*. 1996; 271:30451–30458. [PubMed: 8940010]
115. Bandwar RP, Patel SS. *J Biol Chem*. 2001; 276:14075–14082. [PubMed: 11278877]
116. Sastry SS, Ross BM. *Biochemistry*. 1996; 35:15715–15725. [PubMed: 8961934]
117. Tang GQ, Paratkar S, Patel SS. *J Biol Chem*. 2009; 284:5514–5522. [PubMed: 19116203]
118. Stephens OM, Yi-Brunozzi HY, Beal PA. *Biochemistry*. 2000; 39:12243–12251. [PubMed: 11015203]
119. Yi-Brunozzi HY, Stephens OM, Beal PA. *J Biol Chem*. 2001; 276:37827–37833. [PubMed: 11479320]
120. Hawkins ME, Pfeleiderer W, Mazumder A, Pommier YG, Balis FM. *Nucleic Acids Res*. 1995; 23:2872–2880. [PubMed: 7659509]
121. Moser AM, Patel M, Yoo H, Balis FM, Hawkins ME. *Anal Biochem*. 2000; 281:216–222. [PubMed: 10870838]
122. Hawkins ME. *Cell Biochem Biophys*. 2001; 34:257–281. [PubMed: 11898867]
123. Sandin P, Stengel G, Ljungdahl T, Borjesson K, Macao B, Wilhelmsson LM. *Nucleic Acids Res*. 2009; 37:3924–3933. [PubMed: 19401439]
124. Tahmassebi DC, Millar DP. *Biochem Biophys Res Commun*. 2009; 380:277–280. [PubMed: 19167347]
125. Dash C, Rausch JW, Le Grice SF. *Nucleic Acids Res*. 2004; 32:1539–1547. [PubMed: 15004241]
126. Liu C, Martin CT. *J Biol Chem*. 2002; 277:2725–2731. [PubMed: 11694519]
127. Liu C, Martin CT. *J Mol Biol*. 2001; 308:465–475. [PubMed: 11327781]
128. Liu H, Gao J, Lynch SR, Saito YD, Maynard L, Kool ET. *Science*. 2003; 302:868–871. [PubMed: 14593180]
129. Krueger AT, Kool ET. *J Am Chem Soc*. 2008; 130:3989–3999. [PubMed: 18311973]
130. Lee AH, Kool ET. *J Am Chem Soc*. 2005; 127:3332–3338. [PubMed: 15755149]
131. Leonard NJ, Sprecker MA, Morrice AG. *J Am Chem Soc*. 1976; 98:3987–3994. [PubMed: 819479]
132. Lu H, Krueger AT, Gao J, Liu H, Kool ET. *Org Biomol Chem*. 2010; 8:2704–2710. [PubMed: 20407680]
133. Jarchow-Choy SK, Krueger AT, Liu H, Gao J, Kool ET. *Nucleic Acids Res*. 2010 Oct 14. [Epub ahead of print].
134. Gao J, Strassler C, Tahmassebi D, Kool ET. *J Am Chem Soc*. 2002; 124:11590–11591. [PubMed: 12296712]
135. Ren RX-F, Chaudhuri NC, Paris PL, Rumney S IV, Kool ET. *J Am Chem Soc*. 1996; 118:7671–7678. [PubMed: 20865136]
136. Matray TJ, Kool ET. *Nature*. 1999; 399:704–708. [PubMed: 10385125]
137. Sun L, Wang M, Kool ET, Taylor JS. *Biochemistry*. 2000; 39:14603–14610. [PubMed: 11087416]
138. Cho Y, Kool ET. *ChemBioChem*. 2006; 7:669–672. [PubMed: 16502476]
139. Jiang YL, Kwon K, Stivers JT. *J Biol Chem*. 2001; 276:42347–42354. [PubMed: 11551943]
140. Sen S, Paraggio NA, Gearheart LA, Connor EE, Issa A, Coleman RS, Wilson DM 3rd, Wyatt MD, Berg MA. *Biophys J*. 2005; 89:4129–4138. [PubMed: 16199493]
141. Gao J, Watanabe S, Kool ET. *J Am Chem Soc*. 2004; 126:12748–12749. [PubMed: 15469249]
142. Teo YN, Wilson JN, Kool ET. *J Am Chem Soc*. 2009; 131:3923–3933. [PubMed: 19254023]
143. Wilson JN, Teo YN, Kool ET. *J Am Chem Soc*. 2007; 129:15426–15427. [PubMed: 18027944]
144. Teo YN, Wilson JN, Kool ET. *Chem–Eur J*. 2009; 15:11551–11558. [PubMed: 19780115]
145. Dai N, Teo YN, Kool ET. *Chem Commun*. 2010; 46:1221–1223.
146. Solomatin SV, Greenfield M, Chu S, Herschlag D. *Nature*. 2010; 463:681–684. [PubMed: 20130651]
147. Herbert KM, La Porta A, Wong BJ, Mooney RA, Neuman KC, Landick R, Block SM. *Cell* (Cambridge, Mass). 2006; 125:1083–1094.
148. Abbondanzieri EA, Greenleaf WJ, Shaevitz JW, Landick R, Block SM. *Nature*. 2005; 438:460–465. [PubMed: 16284617]

149. Cecconi C, Shank EA, Bustamante C, Marqusee S. *Science*. 2005; 309:2057–2060. [PubMed: 16179479]
150. Ha T, Ting AY, Liang J, Caldwell WB, Deniz AA, Chemla DS, Schultz PG, Weiss S. *Proc Natl Acad Sci U S A*. 1999; 96:893–898. [PubMed: 9927664]
151. Pandey M, Syed S, Donmez I, Patel G, Ha T, Patel SS. *Nature*. 2009; 462:940–943. [PubMed: 19924126]
152. Schwartz JJ, Quake SR. *Proc Natl Acad Sci U S A*. 2009; 106:20294–20299. [PubMed: 19906998]
153. Abbondanzieri EA, Bokinsky G, Rausch JW, Zhang JX, Le Grice SF, Zhuang X. *Nature*. 2008; 453:184–189. [PubMed: 18464735]
154. Biteen JS, Thompson MA, Tselentis NK, Bowman GR, Shapiro L, Moerner WE. *Nat Methods*. 2008; 5:947–949. [PubMed: 18794860]
155. Shroff H, Galbraith CG, Galbraith JA, Betzig E. *Nat Methods*. 2008; 5:417–423. [PubMed: 18408726]
156. Tonisson N, Zernant J, Kurg A, Pavel H, Slavin G, Roomere H, Meiel A, Hainaut P, Metspalu A. *Proc Natl Acad Sci U S A*. 2002; 99:5503–5508. [PubMed: 11960007]
157. Hu Y, Chen GY, Yao SQ. *Angew Chem, Int Ed*. 2005; 44:1048–1053.
158. Diaz-Mochon JJ, Bialy L, Keinicke L, Bradley M. *Chem Commun*. 2005:1384–1386.
159. Diaz-Mochon JJ, Bialy L, Bradley M. *Chem Commun*. 2006:3984–3986.
160. Winssinger N, Damoiseaux R, Tully DC, Geierstanger BH, Burdick K, Harris JL. *Chem Biol*. 2004; 11:1351–1360. [PubMed: 15489162]

Fig. 1. Designs of fluorescent probes for enzymes that act on the DNA backbone as substrates. (A) Probes for enzymes with cleavage activities. The probe contains a DNA-based molecular beacon (blue), and a complementary target RNA strand (red) which can be degraded by ribonuclease H. (B–D) Probes for enzymes with elongation activities. (B) TaqMan probe; (C) molecular beacon; (D) labeled DNA duplex. (E) Probes for enzymes with ligation activities. The probe yields a fluorescence signal change by opening the stem–loop structure of a molecular beacon after ligation. (F) Probes for enzymes with conformation-changing activities. The representative is a single-fluorophore system with an environmentally sensitive fluorophore.

Fig. 2.

DNA-thrombin binding strategies. (A) X-Ray structure of 15-mer aptamer G15D binding to human α-thrombin.⁴⁸ Figure was remade with PDB file 1HUT. (B) The 15-mer aptamer G15D binds to the fibrinogen-recognition exosite;^{44,49} (C) aptamer 60-18[29] targets the heparin-binding exosite.^{49,50} Reprinted in part with permission from ref. ⁴⁹. Copyright 2009 American Chemical Society.

Fig. 3. Schematic representations of fluorescent DNA–thrombin binding affinity probes. (A) A molecular beacon-like thrombin sensor with extended 5′-end.⁴⁶ Reprinted in part with permission from ref. ⁴⁹. Copyright 2009 American Chemical Society. (B) A turn-off sensor without extended sequence;⁵³ (C) a probe with two labeled aptamers binding to different non-catalytic exosites of thrombin;⁵⁴ Reprinted with permission from ref. ⁵⁴. Copyright 2005 American Chemical Society. (D) The three-strand FDNA–QDNA system (structure-switching signaling aptamer).⁵⁷ Reprinted with permission from ref. ⁵⁷. Copyright 2003 American Chemical Society.

Fig. 4. RNA-based affinity probes for thrombin. (A) DNA interacting luminescence dye $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$; (B) proposed secondary structure of RNA-based anti-thrombin probe.⁶⁸ Reprinted in part with permission from ref. ⁶⁸. Copyright 2004 American Chemical Society.

Fig. 5.

Examples of amplification affinity probes. (A) Anti-thrombin aptamer probe involving rolling circle amplification (RCA).⁶⁹ This probe contains a circular dumbbell CT-6 aptamer (**1**) which binds to thrombin exosite II, and a linear LT-6 aptamer (**2**) which binds to thrombin exosite I. The binding of two aptamers simultaneously to thrombin can initiate DNA polymerase-mediated RCA.⁶⁹ Reprinted from ref. ⁶⁹ by permission of Oxford University Press, Copyright 2005. (B) Scheme for thrombin detection involving exonuclease protection.⁷¹ (1) Anti-thrombin aptamers bound to thrombin. (2) Exonuclease I degraded unbound aptamers. (3) Two additional connector strands hybridized with anti-thrombin aptamers. (4) T4 DNA ligase joins the hybridization products. (5) The ligation products are quantified by fluorescence quantitative PCR.⁷¹ Reprinted with permission from ref. ⁷¹. Copyright 2004 American Chemical Society.

Fig. 6. ADP-specific ribozyme-based affinity sensors. (A) Proposed secondary structure of an ADP-specific RiboReporter sensor.⁷² (B) Signal-generating scheme of the ADP RiboReporter sensor. A short fluorescein-labeled oligonucleotide is released by self-cleavage upon binding to ADP.⁷² Reprinted from ref. ⁷², Copyright 2004, with permission from Elsevier.

Fig. 7. Structures of fluorescent nucleobase analogs. (A) Fluorescent structural nature base analogs; (B) fluorescent expanded natural base analogs; (C) nonpolar base analogs.

Fig. 8. DNA-elongating activity of TdT monitored by incorporation of fluorescent expanded DNA (xDNA) nucleotides.¹³³ (A, B) Spectra of xDNTP monomers (purple, red) and of TdT reaction products (green, blue). (A) Incorporation of dxATP; (B) incorporation of dxCTP. (C–F) Fluorescence microscopy images of TdT-mediated xDNA reaction products on glass with (dT)25 primer attached. (C) dxATP without TdT; (D) dxATP with TdT; (E) dxCTP without TdT; (F) dxCTP with TdT.¹³³ Reprinted from ref. ¹³³ by permission of Oxford University Press, Copyright 2010.

Fig. 9. DNA polyfluorophore sensor for esterases. (A) A tetra-pyrene esterase sensor shows good quenching efficiency by dabcyI quencher and can be efficiently turned on by esterases both (B) *in vitro* and (C) in live HeLa cells.¹⁴⁵