J Fluoresc (2008) 18:413–421
DOI 10.1007/s10895-007-0281-2

ORIGINAL PAPER

Probing DNA Hybridization in Homogeneous Solution and at Interfaces via Measurement of the Intrinsic Fluorescence Decay Time of a Single Label

Bianca K. Hoefelschweiger · Otto S. Wolfbeis

Received: 30 September 2007 / Accepted: 31 October 2007 / Published online: 19 December 2007

Abstract The hybridization of DNA oligomers including molecular beacons can be detected by measurement of either the decay time or the intensity of a single fluorescent label attached to the end of the respective oligonucleotide. The method works both in solution and solid phase and can distinguish between fully complementary and mismatch sequences as demonstrated for a 15-mer oligonucleotide and a 25-mer molecular beacon. The fluorescence lifetime method is advantageous in (a) requiring a single label (and therefore a single labeling step) only; and (b), being based on measurement of a self-referenced magnitude that is hardly affected by parameters such as fluctuations in light intensity that make measurement of intensity more prone to interferences.

Keywords DNA assay · Fluorescence lifetime · Fluorescent label · Molecular beacon · Affinity assay

Fluorescent DNA probes are widely used in various kinds of hybridization assay including studies on gene expression, detection of gene mutations and translocations, and mapping and sequencing of genes. In order to qualitatively detect or to quantitatively determine a specific sequence, different methods are applied [1]. One large group of probes consists of fluorogenic intercalators, the other of fluorescent covalent labels. Intercalators and related probes such as groove binders associate (in the overwhelming majority) with double stranded DNA or RNA to undergo a significant change in their properties, in most cases an increase in fluorescence intensity or a spectral shift [2]. Intercalators interact non-convalently.

Fluorescent labels, in contrast, are covalently attached to oligonucleotides, often to the desoxyribose unit via phosphoramidite chemistry, or to an aminohexyl side group that has been introduced, mostly by chemical means, into a specific oligonucleotide. One or more labels may be used [3–5]. Two labels, one on each strand, are needed when detecting hybridization by fluorescence resonance energy transfer (FRET) between donor-labeled and acceptor-labeled DNA [6]. Alternatively, a fluorescent label along with a quencher may be applied. Increased FRET efficiency is found if matched pairs are brought into a spatial proximity of typically <7 nm as a result of hybridization [7, 8]. In the competitive hybridization approach, increasing fractions of labeled and non-labeled target DNA compete for the formation of duplexes [6, 7]. FRET is a self-referenced method in that the ratio of the intensities of two emission bands (at one excitation wavelength) can be related to concentration of the label, and thus the oligomer. On the other side, all FRET methods require both the probe and the target DNA to be labeled.

DNA assay may be performed in solution or on solid supports. The products of a PCR can by immobilized on a surface (e.g. of a microtiterplate), and the specific sequence can be detected with fluorescent primers. After removal of unhybridized primers, the remaining fluorescence is measured. DNA-"ELISAs" are performed based on the same scheme. These two methods are heterogeneous in that washing steps are required which impair sensitivity [1, 9]. Therefore, homogeneous assays using fluorescently labeled oligonucleotides are a quite desirable alternative.

It is well known that the hybridization of a fluorescently labeled single strand oligonucleotide with a complementary

B. K. Hoefelschweiger · O. S. Wolfbeis (✉)
Institute of Analytical Chemistry, Chemo- and Biosensors,
University of Regensburg,Germany,
93040 Regensburg, Germany
e-mail: otto.wolfbeis@chemie.uni-r.de
URL: www.wolfbeis.de

sequence can substantially modify the fluorescence properties of the fluorophore. New fluorescent dyes that intercalate or groove bind to DNA double helix and, consequently, undergo a significant change their fluorescence properties therefore are of considerable interest. Only one marker is needed in such methods [10]. However, the fluorescence properties also are dependent on how the marker is being linked to the oligonucleotide. Perylene, for example, displays a fluorescence that is different depending on whether it is directly linked to the DNA backbone or via a spacer group [11]. Both quenching and fluorescence enhancement can be observed.

Fluorescence intensity represents an analytical information that is influenced by several parameters. Parker's law ($F=I_0 \cdot \varepsilon \cdot l \cdot c \cdot QY$) relates the measured fluorescence intensity (F) to the intensity (I_0) of the exciting (laser) beam, the molar absorbance (ε) of the label, the penetration length of the exciting beam (l), the quantum yield of the label (QY), and instrumental geometries by introducing a geometrical factor k. Obviously, variations in l, c and k, not to talk about inner filter effects, may bias the accuracy and precision of a measurement.

Fluorescence lifetime (more precisely: decay time), in contrast, is a self-referenced parameter in that the decay profile (or phase shift) of the emission always is related to the respective excitation signal (pulse or phase) [7, 8]. Hence, fluorescence decay time represents an intrinsic molecular property that is independent of the setup or adjustment of the instrument and—in a first approximation—of any effects of photobleaching.

Fluorescence lifetime-based methods have been used in DNA technology in various ways, but not so far for the detection of hybridization. Acridone dyes were characterized [12] for use in 4-decay-DNA sequencing. Lasseter et al. [13] have applied near infrared dyes for lifetime identification of DNA in capillary electrophoresis, a scheme that is also applicable [14] to intercalating dyes and in combination with multiplex fluorescence lifetime detection. Waddell et al. [15] have deposited NIR dyes directly on substrates typically used for DNA microarrays, and it was shown that the lifetime of these dyes change if oligonucleotides are being deposited on such surface. Ying et al. [16] found that the efficiency of fluorescence resonance energy transfer depends on the heterogeneity of a model DNA system (a 12-mer), whilst the group of Clegg [17] exploited the fluorescent characteristics of a label that was linked to a oligonucleotide to detect multiple conformers of single-stranded and double-stranded DNA complexes. The luminescence lifetime of labelled DNA fragments in the vicinity to the surface of (nano) particles from either silver [18] or gold [19] has been investigated by fluorescence lifetime spectroscopy. The same group has shown that fluorescence lifetime imaging of DNA can be accomplished via changes in the efficiency of fluorescence resonance energy transfer between two intercalating dyes [20]. Finally, He et al. [21] have separated mixtures of labeled DNA primers by capillary gel electrophoresis (CGE). Fluorescence lifetime measurements allowed for multiplex DNA sequencing.

Recent advantages in lifetime based sensing [22–24] have led to fairly small-sized and laser-based instrumentation for measurements of lifetime in the ns time domain. This development is also driven by the fact that most of the organic labels used so far (and unlike labels based on metal ligand complexes) have decay times in the order of 0.5 to 5 ns. When using such labels, the excitation light source can be pulsed at high rates, thus enabling a large number of measurements to be performed in short time. Lifetimes therefore can be determined with high precision and a high signal-to-noise ratio. The major advantages of recently available microplate readers [23–25] are speed and ease of detection and analysis. Microplates also are compatible with the FLAA scheme which has bee shown to function excellently in high-throughput screening for proteins [26].

We have now extended FLAA to lifetime-based homogeneous hybridization assays, again using a single label only. It is shown here that the measurement of fluorescence decay times is a powerful means to detect hybridization, and that lifetime data represent referenced data that are clearly superior to intensity-based data.

Results and discussion

Label Py-1 Labels whose fluorescence is affected by their environment may act as molecular probes for detecting almost any kind of biomolecular interaction. The sensitivity of a label to its micro-environment can be caused by changes in the polarity of the solvent, by shielding the label from quenching by water molecules or protons, to mention the two most frequent reasons. In addition to changes in fluorescence intensity and emission maxima, there are few labels that undergo a change in their decay time.

Numerous labels are known that can be used for fluorescently labeling oligonucleotides. Good labels are insensitive to their micro-environment and display strong absorption and quantum yield, the product of the two parameters often being referred to as brightness (Bs). However, whilst almost any pair of labels may be used for FRET if they match the fundamental conditions of the Förster or Dexter relationships [8], the choice of label is much more critical in case of a fluorescence lifetime affinity assay (FLAA). Crockett & Witter [27] have exploited the inherent quenching capacity of desoxyguanosine nucleotides on the intensity of the fluorescence of

fluorescein for the design of real-time PCR assay using a single fluorescein-labeled probe. Turconi et al. [25] have performed a binding study using Cy5 as a single fluorescent label for lifetime detection of proteins with a confocal microplate reader. The fluorescence lifetime of the label increased upon affinity binding of the labeled biotin to streptavidin. It was shown that labels undergo changes in their lifetime not only on conjugation but also on affinity binding. Hennig et al. [28] have used an azabicyclooctenone as a fluorescent probe having decay times as long as 1 μs in a time resolved protease assay which exploits the quenching capabilities of a tryptophane moiety contained in a protease substrate. More recently [29], metalloporphyrins with RT phosphorescence have been used as labels with comparably long luminescence decay times. These allow very low limits of detection of nucleic acids, even though the probes are susceptible to strong quenching by molecular oxygen. The labels used in our work have much shorter decay times and are not at all quenched by oxygen.

We have screeened several labels out of a series of pyrylium and cyanine dyes for changes in decay time, but only Py-1 showed a significant $\Delta\tau$ when linked to an ss-oligonucleotide and hybridized with a complementary strand. An important criterion set by ourself is that the fluorescence lifetime, expected to be >90% mono-exponential, should be retained in the FLAA. The label Py-1 (see Fig. 1) in aqueous solution is blue and virtually nonfluorescent before reaction with a primary amino group [30]. It undergoes a color change from blue to red (which is equivalent to a shift in the peaks of the absorption spectra from 611 nm to 503 nm) upon conjugation to the terminal amino group of a modified oligonucleotide, and the fluorescence quantum yield increases from <0.01 to >0.4.

The reaction conditions outlined in the Experimental Part need to be followed strictly. As was shown before [31], the reaction of pyrylium salts with amines can go into various directions and may not only lead to the (red) pyridinium conjugates, but also to various other products if the reaction conditions are varied.

We now have reasons to assume that certain acridones and naphthalimides (with their decay times between 10 and 20 ns) will represent attractive alternatives to the Py-1 labels since they may undergo even larger changes in their decay time upon hybridization. Unfortunately, such fluorophores have not yet been converted into amino-reactive forms.

Selection of oligomers The oligomers listed in Table 1 represent a series of 15-mers with either complete match of with various degrees of mismatches. They have been used in the experiments described in the following. Complementary sequences are framed.

Hybridization assay on solid phase

In a typical experiment, labeled oligo-2 was attached, via its biotin residue, to the surface of a streptavidin-coated well of a 96-well microplate. Each well contains enough streptavidin to bind 5 pmol of biotin (Bt). The quantity of oligos immobilized may be smaller, though, since not each binding site at the streptavidin will be accessible to Bt because of steric hindrance. Different quantities of the complementary oligonucleotide (oligo-3) were then added and lifetimes were measured after a washing step. Figure 2 shows a plot of the fluorescence lifetime of oligo-2* versus the ratio of oligo-3 to oligo-2*. An asterisk indicates a labeled species in this article. The first data point (x=0) in Fig. 2 is the fluorescence lifetime of single-strand (ss) oligo-2* bound to the surface. If this first point is omitted, the residual titration curve can be described by a single exponential decay curve. The change in lifetime due to complete hybridization is from 1.40 to 0.84 ns. The signal change $\Delta\tau$ is 0.56 ns and approximately 10 times larger than the resolution of the instrument used. The titration with oligo-1, a non-complementary strand, showed no characteristic change in lifetime (plot not shown). As little as 0.5 pmol of the complementary oligonucleotide can be determined by this method, which in our experiment corresponds to a concentration of 11.5 ng/mL.

Hybridization assay in homogeneous solution

The decay times of labeled ss-oligonucleotides are different in solutions and when bound to a solid phase. Py-1 labeled ss-oligo-1 has a lifetime of 1.54 ns free in solution. Py-1 labeled ss-oligo-2 has the same sequence, but is bound to

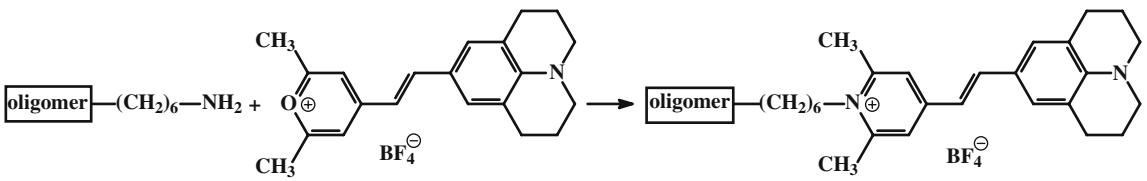

Fig. 1 Labeling chemistry: reaction of the amino group with Py-1 causes a substantial change in the electronic structure of the chromophore (fluorophore) which leads to a color change from blue to red

Table 1 Oligonucleotides used, their sequences, and modifications

oligomer	*Sequence*	*complementary oligo*	*lifetime of labeled form*
oligo-1	amino-C6-5'-CCG GCA GCA AAA TGT-3'	oligo-3	1.5 ns [(a)]
oligo-2	amino-C6-5'-CCG GCA GCA AAA TGT-3'-biotin	oligo-3	1.4 ns [(b)]
oligo-3	5'-ACA TTT TGC TGC CGG-3'	oligo-1; oligo-2	-
oligo-4	5'-CCG GCA GCA AAA TGT-3'	equiv. to oligo-1	-
oligo-5	5'-ACA TTT TGC TGC CAA-3'	mismatch to oligo-1	-
oligo-6	5'-ACA TTT TGC TGC TGC-3'	mismatch to oligo-1	-
oligo-7	amino-C6-5'-CCC ACC CGG CAG CAA	oligo-8 [(c)]	2.3 ns [(a)]
(beacon)	AAT GTG GGG G-3'		
oligo-8	5'-GTT ACA TTT TGC TGC CGG GTA TT-3'	oligo-7 (beacon) [(c)]	-

(a) In solution; (b) bound to the surface of a microtiter plate; (c) see Fig. 5

the surface of a microplate via a Bt-streptavidin interaction. Its lifetime is 1.40 ns. Obviously, the decrease in decay time is larger if hybridization is performed in solution. Figure 3 shows results of the titration of oligo-3 with its labeled counter strand (oligo-1*) which is present in constant concentration of $3.3 \cdot 10^{-8}$ mol/L. One has to differentiate between two effects that may cause changes in lifetime. The first is caused by immobilization of an oligo* onto a solid support, the second by hybridization.

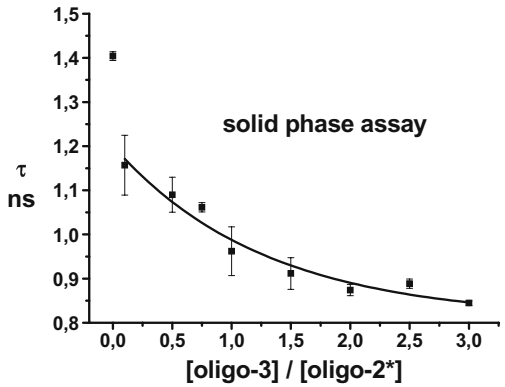

Fig. 2 Plot of the lifetime (τ) vs the ratio of complementary oligo-3 to oligo-2* which is immobilized (via biotin-streptavidin binding) to the surface of the well of a microtiter plate

Increasing fractions of the complementary oligonucleotide (oligo-3) cause both the fluorescence intensity (left panel) and the fluorescence decay time (right panel) to decrease. Both plots display a distinct endpoint at a molar ratio of 1:1. The detection limit (LOD) of this FLAA was graphically determined to be 12 nmol/L for the fluorescence intensity plot, which is equivalent to 1.9 pmol per well, and 8 nmol/L for the lifetime plot, which is equivalent to 1.2 pmol or 36 ng/mL per well. These LODs and the plots in Fig. 3 show that the lifetime method is more sensitive.

In order to improve the LODs, which is needed for example in competitive inhibition assays, the concentration of the label may be reduced. Moreover the error bars for the decay time-based assay are much smaller than for intensity-based assays. As can be expected from Parker's equation, fluorescence intensity is more strongly affected by external parameters, e.g. by inner filter effects and geometrical factors, and thus is a parameter that is clearly inferior to lifetime. It should be noted that in the lifetime

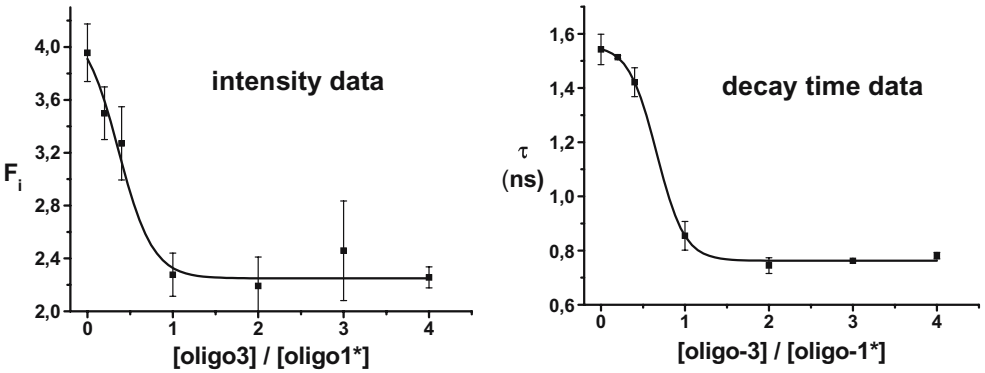

Fig. 3 Left: Plot of fluorescence intensities at 630 nm versus the ratio of the concentrations of oligo-3 and its counterstrand oligo-1 labeled with Py-1 (oligo-1*) Right: Same plot for the respective fluorescence decay times Data are in the two graphs from the same experiment (all in triplicate)

assays the *relative* change in lifetime represents adequate information and that precise data on lifetimes do not need to be known.

Detection of mismatches in homogeneous hybridization assay

The effect of two mismatches in the sequence of the complementary oligonucleotide was examined by titrating oligo-1*, at a constant concentration of 2.5 10^{-7} mol/L, with oligo-3 (no mismatch), oligo-5 (two mismatches), oligo-6 (two mismatches but one matching base in between) and oligo-4 (non-complementary), respectively. The resulting plots are shown in Fig. 4. Oligo-3, which is exactly complementary to oligo-1*, causes the most distinct effect on fluorescence lifetime which drops from 2.2 ns to 1.4 ns. Two mismatches were placed at the end of the sequence of oligo-5, and a small decrease in lifetime from 2.2 ns to 1.86 ns is observed, not the least because the mismatch is close to the site of labeling.

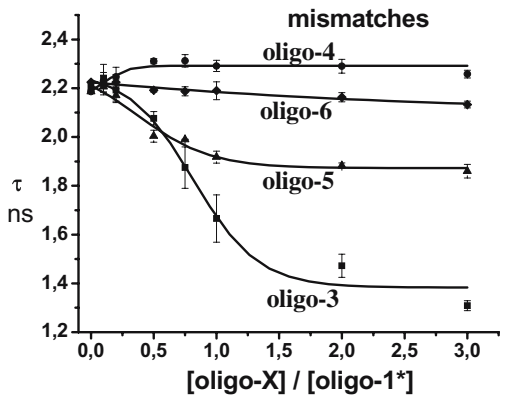

Fig. 4 Plot of changes in the lifetime τ of oligo-1* on titration with complementary and non-complementary oligonucleotides Oligo-1* (in a constant concentration of 250 nmol/L) was mixed with oligo-3 (no mismatch) oligo-5 (double mismatch) oligo-6 (two mismatches with one matching base between) and oligo-4 (non-complementary oligo)

If a matching base is placed between the two mismatches (oligo-6), the fluorophore at the complementary oligonucleotide is even less influenced by hybridization, and the drop in decay time from 2.2 ns to 2.13 ns is almost within the experimental error of +/− 50 ps in this time domain. As expected, oligo-4 has virtually no effect on the decay time of the label. Emission intensity plots of the same experiments resulted in very similar shapes (data not shown here), but display more scattered data and, therefore, larger error bars.

Molecular beacon with a single label

Molecular beacons (MBs) are single stranded DNA molecules that possess a stem-and loop structure. The loop portion of the MB can form a double-stranded DNA in the presence of a complementary strand of nucleic acid. MBs can recognize and report the presence of specific nucleic acids in homogeneous solution with high sensitivity. Tyagi and Kramer [32] have utilized a novel design of fluorescence resonance energy transfer (FRET) inside an MB that was labeled with a fluorophore and quencher at the two ends of the stem. Five to eight bases at each side of the two ends of the MB are complementary to each other. The stem keeps these two moieties in close proximity to each other, causing the fluorescence of the fluorophore to be quenched or the fluorescence of the acceptor to be increased by energy transfer. These probes undergo a spontaneous fluorogenic conformational change when they hybridize to their targets.

MBs are useful in situations where it is either not possible or not desirable to isolate the probe-target hybrids from an excess of the hybridization probes, such as real-time monitoring of DNA/RNA amplification reactions and the detection of RNA within living cells. Molecular beacons have been used for protein-DNA interaction studies, enzymatic cleavage measurements, and for real-time monitoring of polymerase chain reactions, and in for DNA biosensors with even micrometer to submicrometer dimensions [33]. On the other hand, self-quenching or fluores-

cence energy transfer in molecular beacons suffer from their complexity of synthesis which makes costs prohibitive for many routine applications so that other approaches may be more elegant [34]. Unfortunately, specific labeling of both termini of molecular beacons needs two different reaction steps using two different reactive dyes, and usually gives small yields only. Another limitation of using doubly labeled MBs can result from photo-destruction of the acceptor chromophore and the increase of donor fluorescence intensity which renders an unequivocal identification of hybridization events more difficult.

In addition to previous experiments, we have designed an MB consisting of 25 bases and a C_6-amino residue at the 5′ end. The MB possesses a stem-loop structure with (a) a loop consisting of a 13-nucleotide sequence that is complementary to that in the target oligo, and (b) a stem that is formed by annealing of two complementary arm sequences, one a 5-nucleotide and the other a 7-nucleotide arm, which are unrelated to the target sequence. Its sequence is shown in Fig. 5. The dye was conjugated to the MB via the protocol described above. The label is located in close proximity to a run of desoxyguanosines presented in the complementary stem part.

It is known that the fluorescence intensity of fluorophores such as coumarins, rhodamines and oxazins are affected by DNA bases. In particular, guanosine can act as a more or less efficient quencher of certain dyes. Covalent linkage of such dyes to oligonucleotides containing desoxyguanosine (dG) results in a reduced fluorescence quantum yield and decay time depending on the distance between the dG moiety and the fluorophore. This has been observed by several groups [1, 27, 35, 36] and was used to study conformational fluctuations in DNA oligonucleotides at the single-molecule level by FRET and time-resolved fluorescence. The difference in behavior of neighboring dG compared to dA, dT, or dC bases can be attributed to the lower oxidation potential of dG [37].

As long as label Py-1 is in close contact with the 3′ end of the MB which is rich in dG, its fluorescence intensity will be strongly quenched. When the hairpin probe encounters its target (oligo-8), it will form a longer and more stable hybrid (Fig. 5). Consequently, the dye will be more distant from the terminal guanosine, thereby causing an increase of the fluorescence intensity and a decrease of the decay time of Py-1. The fluorescence decay is monoexponential throughout.

The findings have been used to establish the response curves shown in Fig. 6. As can be seen, the spontaneous formation of the more stable probe target duplex from the MB can be used to detect, via measurement of either τ or fluorescence intensity, the presence of the specific target sequence. The limit of detection of this FLAA was graphically determined from to be $1.1 \cdot 10^{-7}$ mol/L of oligo-8.

Unlike in the case of *non-beacon* single strand probes, the fluorescence lifetime of the label attached to the MB *decreases* upon opening of the stem-loop structure by the complementary target. The emission intensity is 4-fold enhanced upon the hybridization experiment, while the fluorescence lifetime undergoes a rather small decrease from 2.32 to 2.06 ns. We assume that this is the result of the combined but counterdirected actions of static and dynamic quenching. In the intact (labeled) MB, the fluorescence of the label is statically quenched by close-lying dGs. Static quenching does not affect lifetime, but fluorescence intensity only which in this case remains comparatively small. On opening the MB, the quenching process is no more operative and fluorescence intensity increases strongly. Lifetime decreases by about 11%, this probably being the result of dynamic quenching by water molecule now surrounding the probe.

Conclusion

The label Py-1 is a viable probe for detecting and quantifying complementary target DNA via measurement of fluorescence decay time. Unlike in certain other

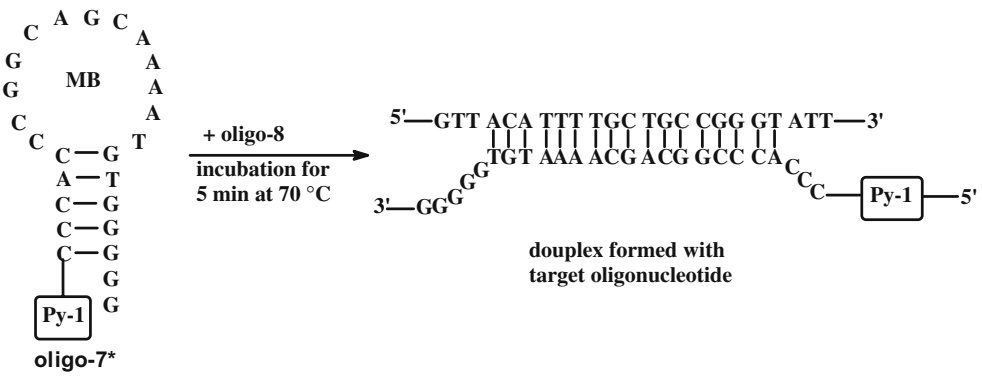

Fig. 5 Sequence of the MB used in this fluorescence lifetime affinity assay and of the product of hybridization with a complementary target oligonucleotide

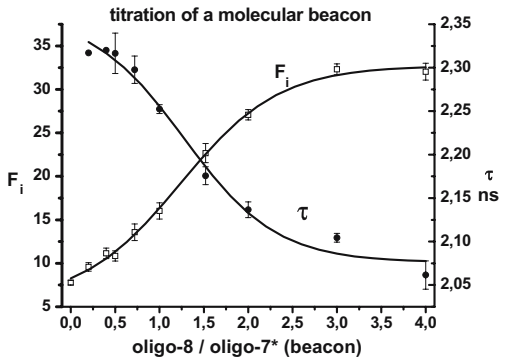

Fig. 6 Relative fluorescence intensity plot (F_i; open squares) and fluorescence decay time plot (τ solid circles) for the titration of a 33 × 10^{-7} molar pH 75 solution of a MB (oligo-7*) with its complementary target (oligo-8)

methods, single-labeling is adequate. The methods can be performed both in homogeneous solution and on solid phase. The lifetime of labeled oligonucleotides also is very sensitive to mismatches, and the approach also works for molecular beacons. The method is simple, and unlike in FRET requires the DNA to be mono-labeled only. Thus, efforts and costs for labels, labeling and purification are strongly reduced.

Experimental section

Acquisition of Spectra and Lifetimes Absorption spectra and absorbances that were used to calculate or adjust the respective concentration of labeled oligonucleotides were acquired on a Cary 50 Bio UV-visible spectrophotometer (Varian, Australia, http://www.varian.com). All experiments were performed at 25 °C unless otherwise stated. Fluorescence spectra were acquired in 1×1×3 cm quartz cuvettes on an Aminco Bowman AB2 luminescence spectrometer (http://www.thermo.com) equipped with a 150-W continuous wave xenon lamp as the excitation source. Relative fluorescence intensities and quantum yields were determined from the areas under the corrected fluorescence spectra divided by their absorbance at λ_{exc}.

Ensemble decay times of the labeled nucleotides were determined in either black 96-well microplates (black plates 96F; from Nunc; http://www.nunc.de) or in black streptavidin-coated plates (Pierce, product no. 15119; http://www.perbio.com) under standard conditions using an LF 401 NanoScan HT microplate reader (from IOM, Berlin; see http://www.iom-berlin.de). In this reader, the excitation beam is pulsed at a repetition rate of 50 Hz (a single excitation pulse). Excitation and emission wavelengths were set to 505 and 630 nm, respectively. The signal for single data points were averaged over 16, 32, or 64 laser pulses. The resolution of the reader is ± 80 ps at a decay time of 0.5 ns, 50 ps at a lifetime of 1.5 ns, and of 40 ps at 2.5 ns. A 96-well plate can be scanned within 110 s when averaged over 16 pulses per well, and a 384-well plate within 300 s. A mono-exponential fit was adequate in all cases to describe the decay curves.

Chemicals, label, buffer and solvents Chemicals and solvents were purchased from either Sigma or Fluka (http://www.sigmaaldrich.com), or from Merck (http://www.merckeurolab.com). They were of analytical grade and used as received. The label Py-1 was synthesized according to a procedure described previously [30] but may be obtained commercially now (Chromeo P-502; see http://www.activemotif.com). Py-1 is remarkable in that it is blue with a λ_{max} of 611 nm and virtually nonfluorescent, but on conjugation to amino groups turns red with a λ_{max} of 503 nm and becomes fluorescent with a λ_{max} of 602 nm. In contrast to any other covalent label for amino groups, it does not change the charge of the species labeled (see Fig. 1).

The 22 mM phosphate buffer (PB) of pH 7.2 was prepared by dissolving 5.67 g of $Na_2HPO_4 \cdot 12\ H_2O$ and 0.96 g of $NaH_2PO_4 \cdot 2\ H_2O$ in 1 L of doubly distilled water. A 50 mM bicarbonate buffer (BCB) pH 9.0 was prepared by dissolving 2.1 g of $NaHCO_3$ in 500 mL doubly distilled water. The 5x saline sodium citrate (SSC) buffer of pH 7.5 was prepared by dissolving 22 g (75 mmol) of trisodium citrate dihydrate and 43.8 g (750 mmol) of sodium chloride in 1 L of doubly distilled water. The 1x SSC buffer of pH 7.5 was prepared by dilution of the 5x SSC buffer by 1:5 (v/v). The 10 mM Tris/HCl buffer of pH 7.5 (used in the beacon experiments) was prepared by dissolving 0.788 g of tris(hydroxymethyl)aminomethane·HCl, 1.86 g KCl and 0.119 g $MgCl_2$ in doubly distilled water. All buffers were adjusted to the corresponding pH value with 1 N HCl and 1 N NaOH, respectively. pH measurements were performed with a Schott pH meter with temperature compensation.

Oligonucleotides and labeling procedure The oligonucleotides listed in Table 1 were custom-synthesized by Metabion GmbH (http://www. metabion.de). They were provided with an amino-modified C_6 chain at their 5′-termini. The coupling reactions were carried out as follows: Twenty nanomoles of the respective amino-modified oligonucleotide (see Table 1) were dissolved in 10 μL of sterile water, and diluted with (200 μL carbonate buffer of pH 9. Label Py-1 (1 mg) was pre-dissolved in 100 μL of dry dimethylformamide (DMF), and 10 μL of the blue solution was added to the solution of the oligonucleotide. After incubating for 4 h at room temperature, the red labeled oligonucleotide was purified by reversed-phase

(RP18 column) HPLC (from Knaur, http: www.knaur.com) using a gradient from 10 to 65% acetonitrile in 0.1 M aqueous triethylammonium acetate. Reaction yields were around 50 %. The oligonucleotides were precipitated with diethyl ether. Purified oligonucleotides were redissolved in 100 mM Tris/HCl buffer of pH 7.5 for storage and diluted with the corresponding SSC buffer for further experiments. Labeled oligonucleotides are marked with an asterisk throughout this article.

The concentrations of labeled oligonucleotides were adjusted via absorbance measurements at 260 and 503 nm, after correction for the intrinsic absorbance of the dye at 260 nm which is 9,000 L cm^{-1} mol^{-1}. The molar absorbances of the unlabeled oligonucleotides at 260 nm were calculated using standard software [http://www.gensetoligos.com/calculation/ calculation.html].

The fluorescence quantum yield of oligo-1* was determined to be 42% using cresyl violet as a standard with a reported quantum yield of 0.56 in methanol for dyes absorbing at 550 nm [7, 8, 38]. The molar absorbance of the bound label is 24 000 L/(cm mol) in water as concluded from the value of a model compound that was obtained by labeling n-propylamine with Py-1.

Hybridization Assay on Lifetime Plates (LPs) Streptavidin-coated microplates blocked with non-proteomic blocking buffer were obtained from Pierce (product no. 15119; http://www.perbio.com). Their binding capacity as specified by the manufacturer is ~5 pmol of biotin per well. Each well was washed three times with PB of pH 7.2, and 50 μL of biotinylated labeled oligo-2 (= oligo-2*, concentration 1 μmol/L in PB) were added. After incubation at room temperature for 1 h under slow shaking, excess oligo-2* was washed off with 1x SSC buffer to give wells where the streptavidin on the bottom of each well is covered with biotinylated oligo-2*. Oligo-3 is complementary to oligo-2*. It was diluted to a concentration of 0.1 μmol/L in 1x SSC buffer. Different quantities of this solution, typically 5 to 150 μL, were placed in the coated wells with their total volume of 200 μL. After an incubation time of 30 min, the plate was washed with 1x SSC buffer and filled with 100 μL of this buffer. Fluorescence lifetimes were determined thereafter of these solutions.

Homogeneous Hybridization Assays Oligo-1 was labeled with Py-1 to give oligo-1*, and then was diluted to a concentration of 0.1 μmol/L in 1x SSC buffer. Varying quantities, typically 5-100 μL, of oligo-3 solution (0.2 μmol/L) in 1x SSC buffer were diluted in the wells of a black microplate to a final volume of 100 μL. Then, 50 μL of a solution of oligo-1* were added. After an incubation for 30 min at 25 °C, both fluorescence intensity and fluorescence lifetime were determined at excitation/emission wavelengths of 505/630 nm, respectively.

Homogeneous Hybridization Assays; Detection of Mismatches First, oligo-1 was labeled with Py-1 to give oligo-1*. Oligo-1*, oligo-3, oligo-4, oligo-5, and oligo-6 were diluted to a concentration of 1 μmol/L in 5x SSC. Quantities between 5 and 150 μL of these solutions were diluted with 5x SSC buffer to a final volume of 150 μL. Then, 50 μL of oligo-1* solution were added to give a final volume of (200 μL. After incubation for 30 min at 25 °C, fluorescence lifetimes were determined.

Hybridization Assay Using a Mono-Labeled Molecular Beacon (MB) Oligo-7 was labeled with Py-1 to give oligo-7*, and then was diluted with Tris/HCl buffer of pH 7.5 to a concentration of 1 μmol/L. Quantities of 2.5 to 100 μL of oligo-8 in a concentration of 2 μmol/L were diluted to a final volume of 100 μL in cups with safe locks. Thereafter, 50 μL of oligo-7* solution were added. The final volume in the assay was 150 μL. The samples were incubated for 5 min at 70 °C, slowly cooled to room temperature and then transferred to the wells of a microplate. Fluorescence lifetimes were then determined at 23±1 °C.

Acknowledgment We thank IOM (Innovative Optische Messtechnik GmbH), Berlin, for providing the LF40 lifetime reader and technical support.

References

1. Knemeyer JP, Marmé N, Sauer M (2000) Probes for Detection of Specific DNA Sequences at the Single-Molecule Level. Anal Chem 72:3717–3724
2. Rye HS, Dabora JM, Quesada MA, Mathies RA (1993) Fluorometric assay using dimeric dyes for double- and single-stranded DNA and RNA with picogram sensitivity. Anal Biochem 208:144–150
3. Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol 18:233–234
4. Goodchild J (1990) Conjugates of oligonucleotides and modified oligonucleotides: a review of their synthesis and properties. Bioconjug Chem 1:165–187
5. Nagl S, Schaeferling M, Wolfbeis OS (2005) Fluorescence Analysis in Microarray Technology. Microchim Acta 151:1–21
6. Wolfbeis OS, Boehmer M, Duerkop A, Enderlein J, Gruber M, Klimant I, Krause C, Kuerner J, Liebsch G, Lin Z, Oswald B, Wu M (2002) In: R Kraayenhof (ed.) Springer Series in Fluorescence, vol. 2. Springer, Berlin, Heidelberg
7. Lakowicz JR (1999) Principles of fluorescence spectroscopy, 2nd edn. Kluwer/Plenum, New York
8. Valeur B (2002) Molecular Fluorescence: Principles and Applications Wiley-VCH Weinheim
9. Jeltsch A, Fritz A, Alves J, Wolfes H, Pingoud A (1993) A fast and accurate enzyme-linked immunosorbent assay for the deter-

mination of the DNA cleavage activity of restriction endonuclease. Anal Biochem 213:234–240
10. Lee SP, Porter D, Chirikjian JG, Knutson JR, Han MK (1994) A fluorometric Assay for DNA Cleavage Reactions characterized with BamHJ Restriction Endonuclease. Anal Biochem 220:377–383
11. Skorobogatyi MV, Malakhov AD, Pchelintseva AA, Turban AA, Bondarev SL, Koreshun VA (2006) Fluorescent 5-alkynyl-2'-deoxyuridines: high emission efficiency of a conjugated perylene nucleoside in a DNA duplex. ChemBioChem 2:810–816
12. Mihindukulasuriya SH, Morcone TK, McGown LB (2003) Characterization of acridone dyes for use in four-decay detection in DNA sequencing. Electrophoresis 24:20–25
13. Lassiter SJ, Stryjewski W, Owens CV, Flanagan JH, Hammer RP, Khan S, Soper SA (2002) Optimization of sequencing conditions using near-infrared lifetime identification methods in capillary gel electrophoresis. Electrophoresis 23:1480–1489
14. McIntosh SL, Deligeorgiev TG, Gadjev NI, McGown LB (2002) Mono- and bis-intercalating dyes for multiplex fluorescence lifetime detection of DNA restriction fragments in capillary electrophoresis. Electrophoresis 23:1473–1479
15. Waddell E, Wang Y, Stryjewski W, McWhorter S, Henry AC, Evans D, McCarley RL, Soper SA (2000) High-Resolution Near-Infrared Imaging of DNA Microarrays with Time-Resolved Acquisition of Fluorescence Lifetimes. Anal Chem 72:5907–5917
16. Ying L, Wallace MI, Balasubramanian S, Klenerman D (2000) FRET Fluctuation Spectroscopy: Exploring the Conformational Dynamics of a DNA Hairpin Loop. J Phys Chem B104:5171–5178
17. Vamosi G, Gohike C, Clegg RM (1996) Fluorescence characteristics of 5-carboxytetramethylrhodamine linked covalently to the 5' end of oligonucleotides: multiple conformers of single-stranded and double-stranded dye-DNA complexes. Biophys J 71:972–994
18. Malicka J, Gryczynski I, Maliwal BP, Fang J, Lakowicz JR (2003) Fluorescence spectral properties of cyanine dye labeled DNA near metallic silver particles. Biopolymers 72:96–104
19. Jennings TL, Singh MP, Strouse GF (2006) Fluorescent Lifetime Quenching near d=1.5 nm Gold Nanoparticles: Probing NSET Validity. J Am Chem Soc 128:5462–5467
20. Murata S, Herman P, Lin H, Lakowicz J (2001) Texture analysis of fluorescence lifetime images of AT- and GC-rich regions in nuclei. J Histochem Cytochem 49:1443–1452
21. He H, Nunnally BK, Li L, McGown LB (1998) On-the-fly fluorescence lifetime detection of dye-labeled DNA primers for multiplex analysis. Anal Chem 41:178–185
22. Jaeger S, Brand L, Eggeling C (2003) New Fluorescence Techniques for High-Throughput Drug Discovery. Curr Pharm Biotechnol 4:463–476
23. Eggeling C, Brand L, Ullmann D, Jaeger S (2003) Drug Discovery Today (DDT). Drug Discov Today (DDT) 8:632–641
24. French TE, Bailey B, Stumbo DP, Modlin DN (1999) Time-resolved fluorometer for high-throughput screening. Proc Soc Photo-opt Instrum Eng (SPIE) 3603:272–280
25. Turconi S, Bingham RP, Haupts U, Pope AJ (2001) Developments in fluorescence lifetime-based analysis for ultra-HTS. Drug Discov Today (DDT) 6:S27–S39
26. Wetzl BK, Pfeifer L, Wolfbeis OS (2005) Screening Scheme Based on Measurement of Fluorescence Lifetime in the Nanosecond Domain. J Biomol Screen 10:685–694
27. Crockett AO, Wittwer CT (2001) Fluorescein-Labeled Oligonucleotides for Real-Time PCR: Using the Inherent Quenching of Deoxyguanosine Nucleotides. Anal Biochem 290:89–97
28. Hennig A, Roth D, Enderle T, Nau WM (2006) Nanosecond time-resolved fluorescence protease assays. ChemBiochem 7:733–737
29. O'Shea DJ, O'Sullivan PJ, Ponomarev GV, Papkovsky DB (2005) Post-PCR detection of nucleic acids using metalloporphyrin labels and time-resolved fluorescence. Anal Chim Acta 537:111–117
30. Wetzl BK, Yarmoluk SM, Craig DB, Wolfbeis OS (2004) Chameleon labels for staining and quantifying of proteins. Angew Chem Int Ed 43:5400–5402
31. Katritzky AR (1980) Conversions of primary amino groups into other functionality mediated by pyrylium cations. Tetrahedron 36:679–699
32. Tyagi S, Kramer FR (1996) Molecular beacons: probes that fluoresce upon hybridisation. Nat Biotechnol 14:303–308
33. Liu X, Farmerie W, Schuster S, Tan W (2000) Molecular beacons for DNA biosensors with micrometer to submicrometer dimensions. Anal Biochem 283:56–63
34. Kourentzi KD, Fox GE, Willson RC (2003) Hybridization-responsive fluorescent DNA probes containing the adenine analog 2-aminopurine. Anal Biochem 322:124–126
35. Marras SAE, Kramer FR, Tyagi S (2002) Efficiency of Fluorescence resonance energy transfer and contact-mediate quenching in oligonucleotide probes. Nucleic Acid Res 30:e122
36. Kim J, Doose S, Neuweiler H, Sauer M (2006) The initial step of DNA hairpin folding: a kinetic analysis using fluorescence correlation spectroscopy. Nucleic Acid Res 34:2516–2527
37. Lewis FD, Wu T, Zhang Y, Letsinger RL, Greenfield SR, Wasielewski MR (1997) Distance-dependent electron transfer in DNA hairpins. Science 277:673–676
38. Demas JN, Crosby GA (1971) The Measurement of Photoluminescence Quantum Yield. J Phys Chem 75:991–1024