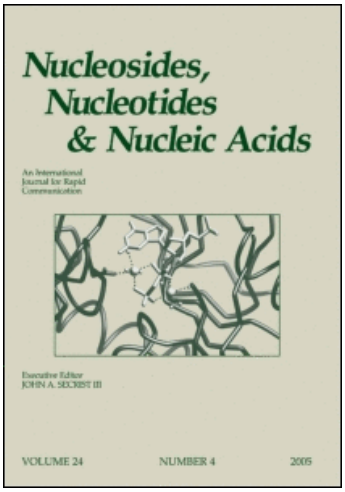

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Recognition of Point Mutations in Dna By Means of Time Resolved Fluorescence Methods

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RECOGNITION OF POINT MUTATIONS IN DNA BY MEANS OF TIME RESOLVED FLUORESCENCE METHODS

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ABSTRACT

A rapid recognition of aberrations in the base sequence of nucleic acids is an important step toward the diagnosis of genetic diseases. We have developed a hybridization method in which a fluorescently labeled oligonucleotide is used to detect point mutations in a target by a simple fluorescence lifetime analysis of the emission of the fluorescent label.

INTRODUCTION

Besides sequencing techniques, fluorescence *in situ* hybridization (FISH) became an established tool in genetic diagnosis because the location of a gene in a chromosome can be visualized[1]. In the microscope, the gene being recognized appears as a bright fluorescent spot on the chromosome. This indicates the hybridization of the labeled probe with the DNA and thus the existence and location of the complementary sequence in the target. However, from the fluorescence data it can not be derived whether or not the probe has really fully hybridized with all of its nucleotides to the corresponding target. Due to the length of the probe (about 1 kb) it might be possible that small aberrations (deletions, insertions or point mutations) in the target sequence are ignored by the hybridizing probe. This, in turn leads to a misinterpretation of the really existing sequence. Our method employes a hybridization technique in solution and allows the detection of a point mutation by measuring the fluorescence lifetime of a suitable label which is covalently bound to the hybridization probe.

PRINCIPLE OF THE METHOD

The fluorescence lifetime of a fluorophore is a good indicator for the electronic environment. It has been shown recently that different binding modes of dimeric oxazole dyes (YOYO and POPO) can be distinguished by fluorescence lifetime techniques even in the case of their simultaneous existence[2]. Thus, it should be possible to find a suitable fluorescent dye which detects a mismatched base pair if the dye is brought into close vicinity to the mismatch.

This can be obtained by using an oligonucleotide probe which contains the fluorescent label on its 5'-end and which is hybridized to the target in such a manner that the last nucleotide at the 5'-position is placed opposite to the point mutation to be analyzed. One can detect such a point mutation if the fluorophore changes significantly its fluorescence lifetime in comparison to those measured for a perfectly hybridized probe, i.e. hybridized to a target which does not contain the mismatch. The different kinds of nucleotides placed in the mismatch as well as their neighbouring bases should not influence the fluorescence properties of the fluorophore. FIG.1 shows the principle of the method as well as the nucleotide sequence of the oligonucleotides used as target and probe. The sequence was established in such a way to avoid selfassociation of the probe as well as of the targets.

MATERIALS AND METHODS

Dyes which are able to detect small aberrations in their environment usually have high dipole moments in their first excited singlet state[3]. Furthermore, they should contain linker groups in order to achieve covalent bonds to the 5'-end of the oligonucleotide. We have screened a variety of fluorescent dyes designed for DNA labeling[4] with respect to their sensitivity to changes in their molecular environment.

The dyes used are listed in TABLE 1. We measured their absorption and fluorescence spectra as well as the fluorescence lifetimes in different organic solvents and in mixtures of ethanol, acetone and dioxane with water. By stepwise increasing the amount of water a transfer of the fluorescent dye from a hydrophobic to a hydrophilic environment was simulated. Such a transfer is expected to occur in the environment of the fluorophore if the probe is linked to the target and forms hydrogen bonds.

All reagents used for the solid-phase oligodeoxyribonucleotide synthesis were purchased from Applied Biosystems (Foster City, CA, USA), MWG Biotech (Ebersberg,

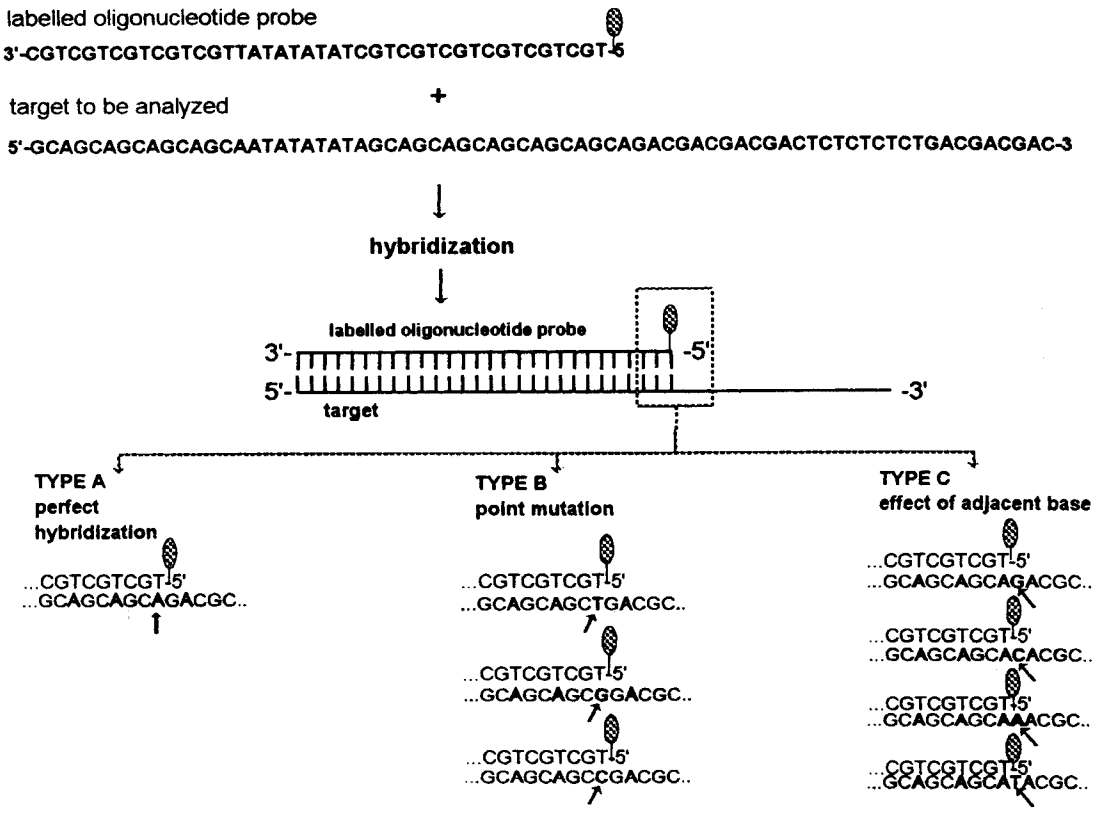

FIG 1: *Principle and oligonucleotides used for the recognition of point mutations by fluorescence lifetime analysis*

TABLE 1: *Fluorescent dyes used in the investigation*

chemical name	lab code	purchased from
5-(and 6)-carboxy-X-rhodamine,succinimidyl ester	Rh X	Molecular Probes*
5-carboxy-tetramethyl-rhodamine, succinimidyl ester	Rh Ch3	Molecular Probes *
4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester	BODIPY 2184	Molecular Probes*
4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester	BODIPY 2228	Molecular Probes*
4,4-difluoro-5-(2-pyrrolyl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester	BODIPY 2225	Molecular Probes*
4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester	BODIPY 2187	Molecular Probes*
Carboxy-methyl-sulfoindo-cyanine, succinimidyl ester	Cy3	Biological Detection Systems**

* Eugene, Oregon, USA ;** Pittsburgh,PA, USA

Germany) and Roth (Karlsruhe, Germany). The oligonucleotide synthesis was carried out on a Model 394 DNA synthesizer from Applied Biosystems according to the 0.2 μmol cycles. In the case of polysterene-grafted polytetrafluoroethylene support P29[7] coupling and washing steps for the initial five elongations were performed during double the time (60 s) as compared with the standard of 0.2-μmol cyanoethyl cycle for the ABI 394 sythesizer of Model 392/394T system software for a 65mer synthesis. 5'-Amino-Modifiers C6 (Glen Research, Sterling, VA, USA) were used to functionize the 5'-terminus of the oligonucleotide probes for dye labeling. The dye labeling and the purification of the labeled probes were carried out according to the product references. All purified oligonucleotide probes were lyophilized, resuspended in sterile distilled water and quantified spectrophotometrically.

For hybridization of probe and target we used a buffer containing 0.01 M TRIS-HCl pH=6.8, 0.15 M NACl, 0.01 M Na-Cacodylate. The water was purified by a Milli-Q Plus water system (Millipore, Bedford, MA, USA).

Instrumentation

The absorption spectra were recorded with a CARY 4E spectrophotometer (Varian Inc. Ltd., Mulgrave, Australia). The fluorescence lifetimes we measured by means of a SLM 48000 MHF Fourier transform spectrofluorimeter working on the phase-modulation principle (SLM Instruments, Urbana, Illinois , USA). The 568nm line of an argon-krypton laser Omnichrome 643-AP (Chino,CA,USA) was used for fluorescence excitation. The cw-laser light was modulated by a Pockel's cell driven by a multiharmonic high frequency generator. The desired frequencies, starting from 5 MHz, were superimposed with their harmonics up to 250 MHz and applied to the Pockel's cell. Thus, the sample was excited by a light pulse of definite shape. The fluorescence light emitted by the sample is intensity modulated too, but its Fourier spectrum differs from that of the exciting light. The fluorescent sample shows an impulse response containing a phase shift φ and a reduction of the modulation depth M for each frequency ω of the Fourier spectrum.

The quantities observed in the measurements, phase shift φ and modulation depth M, can be derived from the transform components as shown in detail elsewhere[6].

Finally one obtains the phase lifetime equation

$$\tau_{\varphi} = \frac{\tan \varphi}{\omega} \qquad (1)$$

and the modulation lifetime equation

$$\tau_{M} = \frac{1}{\omega} \sqrt{(1/M^2 - 1)} \qquad (2).$$

Phase and modulation data for all frequencies used in the Fourier analysis result in the phase-modulation curves which are fitted according to a single or multiexponential decay law by the Marquardt least square fitting algorithm.

Assuming a multiple lifetime population in the sample, the exponential decay law is defined by equation

$$I(t) = \sum_i A_i \exp\left(-\frac{t}{\tau_i}\right) \qquad (3)$$

where τ_i and A_i are lifetime and weighting factor for the component *i*, respectively.

RESULTS AND DISCUSSION

TABLE 2 shows the fluorescence lifetimes of selected dyes dissolved in different organic solvents and in water. The results outlined BODIPY 2225 to have the largest lifetime difference between ethanol and water (2.6 ns). Thus, we concluded that BODIPY 2225 is very sensitive to water molecules in its environment. This finding we proofed in further titration experiments. 10^{-5} M ethanolic solutions of four BODIPYs were stepwise titrated with a 10^{-5} M aquous solution of the respective BODIPY.

The fluorescence lifetimes of the four BODIPYs measured in the resulting water-ethanol mixtures are shown in FIG.2. It turned out that BODIPY 2225 retains its property to react very sensitive to changes in the solvent polarity by its fluorescence lifetime. Parallel measurements of the fluorescence intensities revealed decreasing intensities upon increasing water content. Thus, a dynamic quenching of the BODIPY 2225 fluorescence can be supposed.

For BODIPY 2225 dissolved in acetone or dioxane the absorption and the fluorescence emission spectra were recorded. The data were analyzed by application of the Lippert- Equation (Eq.4,5)[5].

TABLE 2: *The fluorescence lifetimes in ns of selected dyes dissolved in different organic solvents and in water.*

Solvent	BODIPY 2184	BODIPY 2228	BODIPY 2225	BODIPY 2187	Rh X*	Rh Ch3**	Cy3
Dioxane	6,1	4,4	4,9	5,7	3,7	-	-
Acetone	5,6	4,5	5,7	6,2	4,7	2,6	-
Ethanol	*6,2*	*4,0*	*5,2*	*6,0*	*4,7*	*2,9*	*0,67*
Propanol	5,5	4,4	5,7	5,3	4,6	2,7	-
Methanol	6,4	4,6	5,2	5,9	4,8	2,7	-
DMF	6,2	4,4	5,1	5,7	4,1	2,2	-
Acetonitrile	5,9	4,3	6,0	5,8	4,7	2,4	-
Water	*5,7*	*4,1*	*2,8*	*5,8*	*4,0*	*2,3*	*0,28*
lifetime-difference ethanol/water	*0,5*	*-0,1*	***2,6***	*0,2*	*0,7*	*0,6 n*	*0,39*

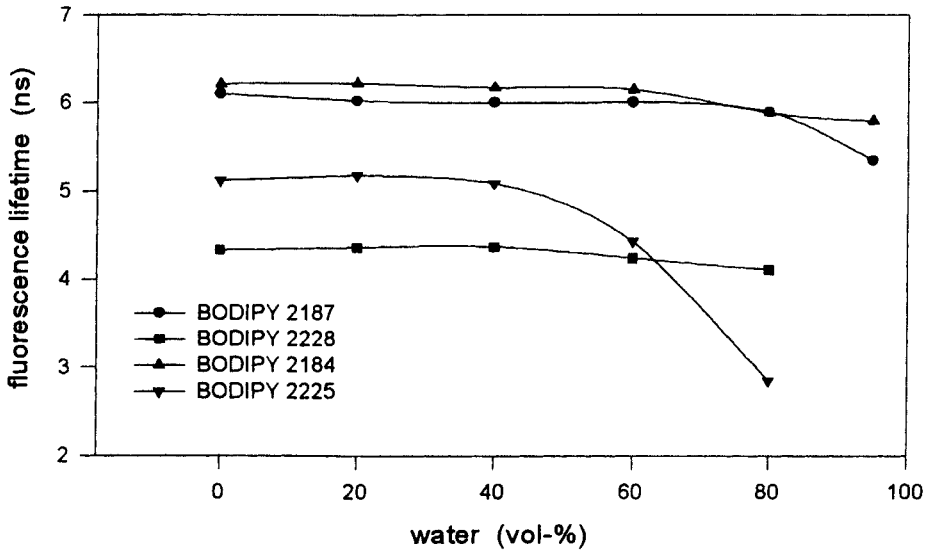

FIG.2: *Fluorescence lifetimes of four BODIPYs in ethanol with increasing amounts of water simulate the transfer of the fluorescent dye from a hydrophobic to a hydrophilic environment*

The equation takes into account the refraction index and the dielectric constant of the solvent mixtures. The parameter ΔF was correlated to the occurring shifts between the maximum of the absorption and the fluorescence spectra indicating the increased electric dipole moment of the dye in the excited state.

Lippert-Equation:

$$\Delta\nu=\nu_{max,abs} - \nu_{max,fluo} = 2\Delta F / hca^3 \ (\mu* - \mu)^2 \quad (4)$$

$$\Delta\nu=\nu_{max,abs} - \nu_{max,fluo} \propto \Delta F = (\varepsilon -1)/(2\varepsilon+1) - (n^2-1)/(2n^2+1) \quad (5)$$

($\Delta\nu$: wave number difference between the maximum of absorbance $\nu_{max,abs}$ and fluorescence $\nu_{max,fluo}$, a: Onsager radius, (μ*-μ): difference of the dipole moments in the first excited singlet state and ground state, n: refraction index of the solvent, ε: dielectric constant of the solvent)

According to Eq. 5 one expects a linear dependence between ΔF and $\Delta\nu$. Hence this could not be observed for BODIPY 2225 dissolved in dioxane and acetone upon stepwise increasing the water content in the solution. The data calculated by the application of the Lippert equation are shown in FIG.3.

We assume that the aberration from the linear dependence is due to the formation of a solvation shell around the fluorophore which consists of water molecules. Moreover, water is capable of forming hydrogen bonds whose interaction energies are not included in Lippert's approximation.

Mismatch detection by a labeled oligonucleotide probe

After having selected BODIPY 2225 as a suitable molecular sensor we synthesized a 72-mer target oligonucleotide and a 42-mer probe oligonucleotide which was BODIPY 2225 labeled at its 5'-end. The four target oligonucleotides synthesized contain a binding site of the probe into which three kinds of point mutations were introduced by exchanging the nucleotide opposite to the 5'-end position of the probe by C,T or G. Four types of hybridizations are expec.ed to occur:

type of hybridization	code	explanation
perfect hybridization	T1	5'-T of the probe is paired with target-A
point mutation	F1-G	5'-T of the probe is „paired" with target-G
point mutation	F1-C	5'-T of the probe is „paired" with target-C
point mutation	F1-T	5'-T of the probe is „paired" with target-T

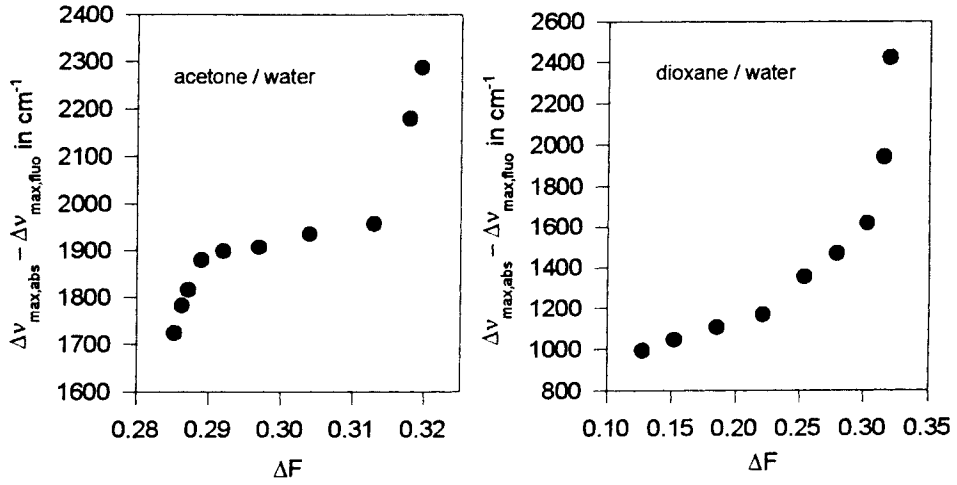

FIG.3. The application of the Lippert-Equation for BODIPY 2225. Water was stepwise added to BODIPY 2225 which was dissolved in dioxane or acetone. The paramter ΔF was calculated by usage of the approximations: $n = c_{water} \cdot n_{water} + c_{acetone} \cdot n_{acetone}$ *and* $\varepsilon = c_{water} \cdot \varepsilon_{water} + c_{acetone} \cdot \varepsilon_{acetone}$, *(c - relative concentration with* $c_{acetone} + c_{water} = 1$*).*

Furthermore, the effect of the neighbouring bases were tested in order to exclude their influence on the lifetime data measured for each point mutation. For that reason the neighbouring bases to the point mutation in the target were varied.

In principal a mismatch detection can be achieved if the fluorescence lifetime of the perfectly hybridized probe is significantly different from those measured for all of the mutated targets. Additionally, the fluorescence lifetime of the free, i.e. single stranded, probe must be different from all the other ones. In our experiments all the fluorescence decay curves showed a bi-exponential behaviour, i.e. they consist of the superposition of two decays with different fluorescence lifetimes reflecting different binding modes of the BODIPY 2225 label to double stranded DNA. We assume that those binding modes are possible due to the 9 carbon atoms containing linker between the 5'-end of the probe and the dye molecule. In the solution exists an equilibrium between dye molecules which are attached to the hydrophobic environment of the DNA and such which retain their higher flexibility. Dyes with higher flexibility usually show shorter fluorescence decay times. Our experiments showed that in the case of an optimal fit indicated by the lowest χ^2-parameter the shorter decay times can not be associated

with the occurrence of mismatches in the target. In contrast the longer living component which we assign to the attached molecules reflects mismatched bases in the DNA. In a bi-exponential fit with two fixed lifetimes (see FIG. 4) the contribution of the longer living component decreases if a mismatched base pair is present in the duplex. At room temperature (25°C) the averaged fluorescence lifetime (mono-exponential fit) of the free probe amounts to τ_{free} = 3.5±0.2 ns. After its perfect hybridization to the target the fluorescence lifetime of the probe increases up to $\tau_{perfect}$ = 5.1±0.1 ns. This is due to the transfer of the fluorophore into the hydrophobic environment of the double stranded DNA and the resulting restricted mobility of the label. If the target contains a point mutation at 5'-position of the probe the fluorescence lifetime is in the interval $\tau_{pointmu.}$=4.1±0.3 ns. FIG. 4 shows that independent of the model applied for the data analysis point mutations cause a decrease in the fluorescence lifetime of BODIPY 2225 when compared with the perfectly hybridized probe. However, it can not be distinguished between the different types of the mismatches, i.e. the new nucleotide in the target sequence remains unknown.

In order to avoid a misinterpretation of the fluorescence data we checked the influence of the ambient temperature on the fluorescence lifetimes. Data were collected for BODIPY 2225 labeled probes which are hybridized to targets which do not contain the point mutation but which have different adjacent bases („Type C" in FIG.1). Another data set was obtained for BODIPY 2225 labeled probes which are hybridized to targets containing mismatches („Type B" in FIG 1.). The longer living component of the bi-exponential fit of the decay data is plotted against the temperature in FIG. 5. For the perfectly hybridized probe a continuously decreasing fluorescence lifetime is observed with rising temperature. The adjacent bases do not considerably influence the fluorescence lifetime of the label. Thus, the perfectly hybridized probe can be taken as a reference in the mismatch detection. The aberration caused by the adjacent bases is indicated by the error bars in FIG.5.

BODIPY 2225 labeled probes hybridized to the targets which contain the point mutation in general show shorter fluorescence lifetimes. This finding allows the detection of the point mutation. The difference of the fluorescence lifetimes can be increased by performing the measurements at an appropriate temperature. In our system we obtained an optimal temperature region between 32°C and 38°C. We assume that in the temperature region mentioned a pre-melting of the double strand occurs which in

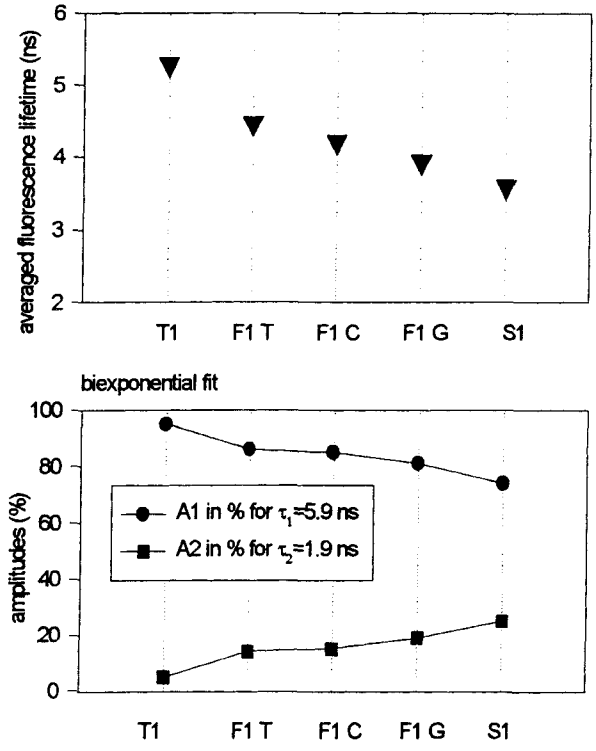

FIG. 4: *Results of the recognition of point mutations with the fluorescence label BODIPY 2225. In the upper panel the averaged fluorescence lifetime is given for the free probe (S1), the perfect hybridized probe (T1) and three mispaired bases (see text). In the lower panel the lifetimes* τ_1, τ_2 *are fixed in the bi-exponential fit and* A_1, A_2 *are calculated according to the bi-exponential model (see text). Independent of the model applied for the data analysis point mutations induce a significant decrease in the fluorescence lifetime of BODIPY 2225 in comparison to the perfectly hybridized probe.*

consequence elongates the C9-linker by the length of a single nucleotide due to the lack of hydrogen bonds in the mismatched bases. Thus, the fluorophors flexibility is further increased resulting in a further decreased fluorescence lifetime.

A BODIPY 2225 labeled oligonucleotide probe is an appropriate tool to detect whether or not a point mutation on a well defined site in the DNA is present. There is no need for sequencing the DNA fragment for each sample to be analyzed. It is sufficient to prepare a labeled oligonucleotide probe, to hybridize it to the target and to compare the fluorescence lifetime of the sample with those of the perfectly hybridized probe.

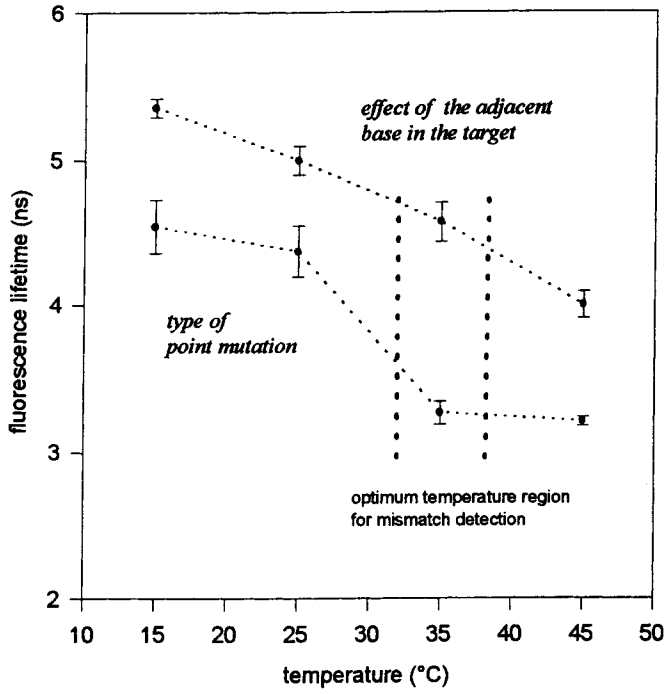

FIG.5: *Temperature dependence of the fluorescence lifetime of a BODIPY 2225 labeled probe hybridized to targets containing a point mutation in comparison to those which enable a perfect hybridization of the probe. In the optimal temperature region indicated by the dotted lines the difference between the fluorescence lifetimes reaches a maximum.*

Point mutations are responsible for a number of genetic diseases, for example cystic fibrosis. Experiments with PCR amplified wild-type DNA fragments, containing the gene for cystic fibrosis, are now under way.

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