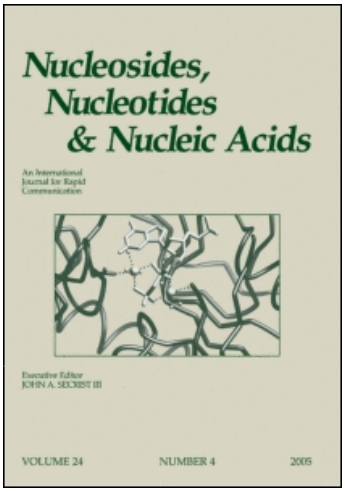

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Molecular Dynamics of Labeled Oligonucleotide Probes Used for the Detection of Point Mutations in DNA

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MOLECULAR DYNAMICS OF LABELED OLIGONUCLEOTIDE PROBES USED FOR THE DETECTION OF POINT MUTATIONS IN DNA

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ABSTRACT

The fluorescent label BODIPY 576/589 linked to the 5'-end of an oligonucleotide via alkyl chain linkers can be used as a probe to detect point mutations in DNA[1]. We have employed fluorescence anisotropy decay and dynamic fluorescence resonance energy transfer (FRET) in order to investigate the molecular origin of the fluorescence lifetime aberrations of BODIPY in the presence of a mismatched base. The results show that both, an increased flexibility of the alcyl chain linker to the BODIPY molecule, as well as relaxation of the secondary structure of the whole complex, contribute to the decreased fluorescence lifetimes reported previously[1].

INTRODUCTION

The occurrence of point mutations in a coding region of a chromosome can result in a variety of genetic diseases, such as, for example, cystic fibrosis. The location of the point mutation and the type of mutation can be detected by DNA sequencing techniques. It has been shown that a combination of particular point mutations are responsible for genetic diseases, for example approximately 600 in the case of cystic fibrosis. For clinical application of mutation diagnostics, a method is required which can detect the entire range of such mutations in a short time and which can be readily implemented. At present several hybridization techniques are used for DNA sequence determination in

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short DNA stretches[2,3]. Fluorescent labels are often used to report the hybridization state of DNA[4].

Recently, we reported a new method employing an isomer of BODIPY 576/589 (4,4-difluoro-5-(2-pyrrolyl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester; Molecular Probes, Eugene, Oregon, USA) to detect the presence of a mismatched base pair at the probe's 5'-end position. The mismatch is detected by the reduction of the fluorescence lifetime of BODIPY in comparison to the one measured for the perfect double-stranded duplex[1].

The present paper describes time-resolved fluorescence anisotropy decay studies and the determination of distance distributions between probe and target, both of which give information about the molecular origin of the fluorescence lifetime alterations of BODIPY upon introduction of a mismatched base pair.

MATERIALS AND METHODS

Fluorescence anisotropy decay studies

The fluorescent label BODIPY linked to the 5'-end of a 16mer oligonucleotide probe and hybridized to the target under investigation exhibits time-dependent fluorescence depolarization following excitation with vertically polarized light. The depolarization of the emission is due to segmental and overall motions of the BODIPY/DNA complex during the lifetime of the excited electronic state. We used the time resolved fluorescence anisotropy decay technique in order to distinguish between different motions of the BODIPY/DNA complex. Different modes of motion were expected to occur due to the rotations of the alkyl chain linker, having a length of 2 or 6 CH_2 groups (C_2 or C_6), between the dye and the 5'-end of the probe oligonucleotide, as well as the overall tumbling of the whole BODIPY/DNA complex (see **FIG. 1**). Time-resolved decays of fluorescence anisotropy were measured using pulsed excitation. The time resolved decays of the parallel polarization component of the emission, $I_{II}(t)$, and the corresponding perpendicular component, $I_{\perp}(t)$, were recorded using a time-correlated single photon counting setup. The emissions are described by the following equations taking into account the convolution with the impulse response function $F(t)$ of the detection system. Since a depolarizer was used in front of the monochromator there was no need to introduce a G-factor describing the different sensitivity of the detection

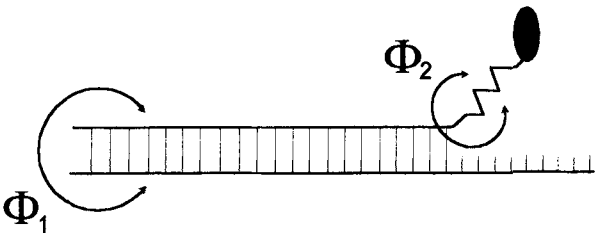

FIG.1: Segmential motions of the BODIPY dye linked to a double stranded DNA
(● - BODIPY molecule, Φ_1, Φ_2 denote the corresponding rotational correlation times)

channel for parallel and perpendicular polarization. Furthermore *F(t)* remains the same for both components.

$$I_{II}(t) = \{[1 + 2\, r(t)]\, K(t)\} * F(t) \qquad \text{Eq.1}$$

$$I_{\perp}(t) = \{[1 - r(t)]\, K(t)\} * F(t) \qquad \text{Eq.2}$$

In these equations *r(t)* and *K(t)* denote the time-dependent behaviour of the anisotropy and the population of the excited state, respectively. Both *r(t)* and *K(t)* are convolved with the instrument response function *F(t)* and must be described by a sum of exponential functions to account for the fluorescence properties of a dye linked to an oligonucleotide[5].

$$r(t) = \sum_{i=1}^{n} \beta_i e^{-t/\Phi_i} \qquad \text{Eq.3}$$

$$K(t) = \sum_{j=1}^{m} \alpha_j e^{-t/\tau_j} \qquad \text{Eq.4}$$

Φ_i and β_i are the rotation correlation time and amplitude of the i-th anisotropy decay component, respectively; and τ_j and α_j are the fluorescence lifetime and fractional contribution of the j-th intensity decay component, respectively.

The data measured for $I_{II}(t)$ and $I_{\perp}(t)$ were fitted according to Eqs. 1 and 2 using a least-squares fitting, allowing for deconvolution with the instrument response function *F(t)*.

The parameters Φ_i , β_i , τ_j and α_j were varied to obtain the optimum fit, which was indicated by a reduced χ^2 value close to unity.

The time-dependent fluorescence anisotropy was also calculated directly from the polarized intensity decays according to Eq. 5:

$$r(t) = [\, I_{II}(t) - I_{\perp}(t) \,] / [\, I_{II}(t) + 2\, I_{\perp}(t) \,] \qquad \text{Eq. 5}$$

Materials for anisotropy decay studies

In order to study the influence of a single mismatched base pair at the end of a double-stranded stretch of DNA on both, the fluorescence lifetime and the fluorescence anisotropy of the dye BODIPY, the following partially double-stranded DNA samples were investigated:

3'-ATATCGTCGTCGTCGT-C_n-**[Bo576]** 16mer
5'-GCAGCAGCAGCAATATATATAGCAGCAGCAGC**X**GACGACGAC-3' 42mer

X := A	no mismatch (reference)
X := T	T : T mismatch
X := G	T : G mismatch
X := C	T : C mismatch
[Bo576]	BODIPY 576
C_n	alkyl chain linker containing n CH_2-groups

Two different lengths between the BODIPY molecule and the probe oligonucleotide were used, containing either 2 or 6 CH_2-groups. In the following they are referred to as C_2 or C_6 linker, respectively. Oligonucleotide synthesis, labeling and purification were performed at TIB MolBiol, Berlin, Germany.

Each sample to be investigated was prepared by annealing 520 pmol (3.5 μM) of labeled oligonucleotide and 530 pmol (3.6μM) of single-stranded 42mer target oligonucleotide in 150 μl buffer containing 10 mM Tris-HCl, 150 mM NaCl and 10 mM Na-cacodylate at pH 6.8. The molar dye concentration amounted to 3.5μM.

Time resolved fluorescence data were recorded for each sample at 20°C and 37°C. Fluorescence was excited by 100 ps pulses from a mode-locked argon ion laser (Innova 100-12, Coherent, Palo Alto, USA) operating at 514 nm. Pulses were selected from the 78 MHz pulse train by a pulse picker to obtain a 6 MHz repetition frequency at the sample. Prior to reaching the detector, the fluorescence light passed through a polarizing filter which was switched from vertical to horizontal position every 60 seconds during data acquisition. After having passed the polarizer the fluorescence light was depolarized

before reaching the monochromator. Data were stored seperately for $I_{\parallel}(t)$ and $I_{\perp}(t)$ in the memory of multichannal analyzer (PCA3, Oxford Instruments, Oxford, Great Britain). The detector, a microchannel plate photomultiplier (R6809U-01, Hamamatsu, Japan), was operated in the single photon counting mode. The anode pulses from the photomultiplier were selected by a constant fraction discriminator (Tennelec TC 455, Oxford Instruments, Great Britain) and used to provide the START signal for a time-to-amplitude converter TAC (Tennelec TC864, Oxford Instruments, Great Britain). The STOP signal for the TAC was obtained from a fast photodiode illuminated by the 6 MHz pulse train.

Dynamic fluorescence energy transfer experiments

The energy transfer between a donor, D, and an acceptor, A, is a very sensitive method to measure distances in molecules, because the rate of energy transfer varies with the inverse sixth power of the D-A-distance according to Förster[6]. Usually the distance between D and A is not fixed during the lifetime of the excited state. As a result, one obtaines a distribution of distances which gives information about the relative probability to find a particular D-A-distance. Furthermore, time-resolved fluorescence measurements provide a means of distinguishing between several components of such a distribution, thus taking account of different binding modes of the donor which might exist in a complex macromolecule. We employed time-resolved fluorescence resonance energy transfer (FRET) to study the rigidity of the partially double-stranded DNA duplex which is obtained in our method of mismatch detection.

The energy transfer process used here is a nonradiative transfer which is mediated by a dipole-dipole interaction of donor and acceptor. The rate of energy transfer, K_t, depends on the spectral overlap of the emission spectrum of the donor and the absorption spectrum of the acceptor, the relative orientation of their transition dipoles and the D-A-distance. It is given by Eq. 6:

$$K_t = \frac{1}{\tau_d}\left(\frac{R_0}{R}\right)^6 \qquad \text{Eq. 6}$$

where τ_d is the lifetime of the donor in the absence of an acceptor, R is the D-A-distance and R_0 is the Förster distance which denotes the D-A-distance for 50% transfer efficiency.

The fluorescence decays were recorded for both the donor without the presence of an acceptor, $I_D(t)$, and the donor undergoing energy transfer to the acceptor $I_{DA}(t)$. The data measured for $I_{DA}(t)$ were fitted according to a model incorporating a continuous distribution of D-A-distances[7]:

$$I_{DA}(t) = \int \sum_{i=1}^{n} \alpha_i \exp\left\{\left(-\frac{t}{\tau_i}\right)\left[1+\left(\frac{R_0}{R}\right)^6\right]\right\} P(R)dR \qquad \text{Eq. 7}$$

where $P(R)$ is the probability distribution of the D-A-distances. This distribution is presented by a weighted Gaussian distribution of distances according to Eq. 8:

$$P(R) = c4\pi R^2 \exp\left\{-a\left(R-b\right)^2\right\} \qquad \text{Eq. 8}$$

where a and b are adjustable parameters that describe the shape of the distribution and c is a normalization constant. The intrinsic decay parameters α_i and τ_i obtained from the donor-only fluorescence $I_D(t)$. A non-linear least-squares method was used to adjust the values of a and b in order to obtain the best fit to the D-A-decay according to Eqs. 6 and 7.

$$I_D(t) = \sum_{i=1}^{n} \alpha_i e^{-\frac{t}{\tau_i}} \qquad \text{Eq. 9}$$

Materials for energy transfer experiments

For the intramolecular distance measurements we employed the well known fluorescein-rhodamine system[8] in which FAM (fluorescein) represents the donor and TAMRA (tetramethylrhodamine) acts as the acceptor. For this system we used the R_0-value known from the literature[5]. FAM was linked to the 5'-end of the 16mer probe oligonucleotide and TAMRA was attached to the 3'-end of the 26mer target, both via a C6 linker. The

target and the probe were annealed in such a way that the resulting duplex contained a mismatched base pair at the 5'-position of the probe. For comparison a duplex was produced which did not contain any mismatch. To study the effect of the nucleotide at the 5'-position, the probe oligonucleotide contained either a pyrimidine (T) or a purine (G) at their 5'-ends. Four different samples were obtained, as follows:

3'-ATATCGTCGTCGTCG**Y**-C_6-[FAM]	16mer
5'-TATAGCAGCAGCAGC**Z**GACGACGACT-C_6-[TAMRA]	26mer

	Y	Z	
1	T	A	perfect match
2	T	C	mismatch
3	G	C	perfect match
4	G	A	mismatch

The 26mer target was also synthesized without the C6 linker or TAMRA for measurement of the „donor-only" decay data.

Each sample consisted of 850 pmol (5.7μM) single-stranded FAM-labeled oligonucleotide annealed with 2550 pmol (17μM) single-stranded 26mer target oligonucleotide in 150μl buffer. In order to keep the amount of free labeled probe as small as possible, the target was added in 2 fold molar excess. The resulting excess of the acceptor is not critical at concentrations in the micromolar range because the unbound single-stranded TAMRA labeled oligonucleotides are too far away on average from the donors to have any effect.

The fluorescence decays of the FAM donor in each sample were recorded using the instrument described above with the emission polarizer set to the „magic angle", i.e. 54.7° to the polarization of the exciting light.

RESULTS AND DISCUSSION

Fluorescence Anisotropy Decay

We assumed that the sensitivity of BODIPY to a mismatched base in its vicinity is due to a dynamic quenching process which could be the result of the following alterations in the molecular structure in comparison to a perfectly matched probe:

1. Elongation of the carbon atom linker arm between the 5'-end of the probe oligonucleotide and the fluorescent dye due to fraying of the mismatched terminal base pair.

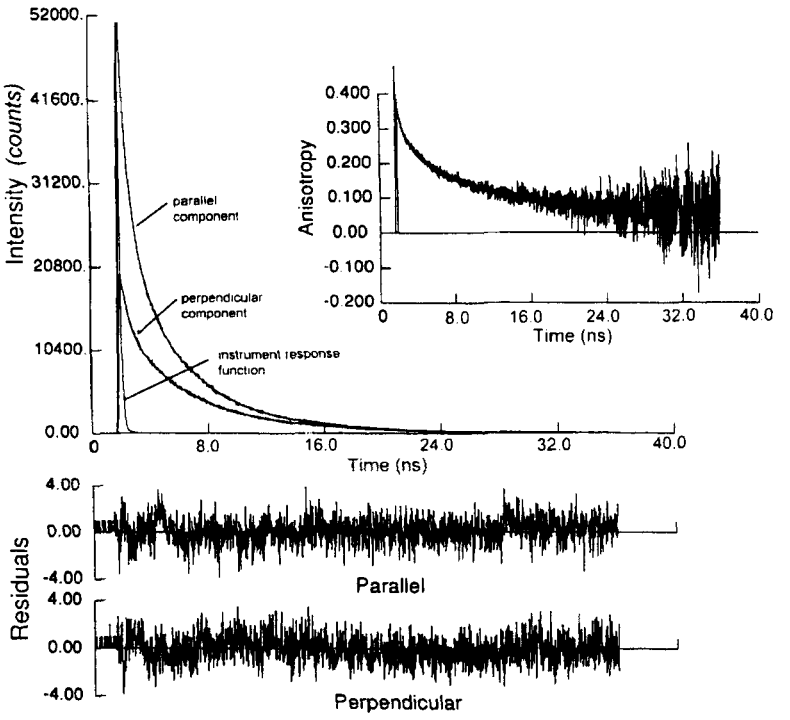

FIG. 2: Typical fluorescence decay curves for the parallel and the perpendicular components of the emission from which the anisotropy decay (insert) is calculated (sample T:T mismatch, C2 linker)

2. Global changes in the secondary structure of the double-stranded dye-DNA complex.

3. Interaction between the BODIPY molecule and the DNA bases and/or sugar phosphate backbone due to the increased flexibility of the mismatched dye-DNA complex.

FIG. 2 shows typical fluorescence anisotropy decay data, i.e., the decays of the parallel and perpendicular polarization component of the emitted light with respect to the polarization of the exciting light, as well as the time dependent anisotropy calculated using equation Eq.3 (insert). The data were fitted to a model in which the anisotropy, $r(t)$, and excited state population, $K(t)$, were both represented by sums of exponential decays as in Eqs. 3 and 4. The best fit was obtained using a four component decay law for $K(t)$ and two decay components for $r(t)$.

Due to the intramolecular flexibility of the double-stranded complex, two different rotation times of the dye molecule are expected to occur (see **FIG. 1**).

1. Overall tumbling of the entire double-stranded dye-DNA complex.
2. Local rotational diffusion of the dye about the alkyl linker arm.

These modes of motion are described mathematically by their amplitudes, β_1 and β_2, and their rotational correlation times, Φ_1 and Φ_2. The angle θ which denotes the space of the dyes rotational diffusion can be calculated from the amplitudes as follows:

$$\theta = \cos^{-1}\left\{\frac{1}{2}\left[\left(1+8S\right)^{1/2}-1\right]\right\} \qquad \text{Eq. 10}$$

with

$$S = \sqrt{\frac{\beta_2}{\beta_1+\beta_2}}$$

TABLE 1 shows the results of the anisotropy decay measurements performed at 20°C and 37°C for samples having either a C_6 or a C_2 linker to the dye molecule. Complexes having the C_6 linker show for the mismatched base pairs at 20°C a rotation time Φ_1 which is increased by approximately 2ns in comparison to the perfect match. Since Φ_1 denotes the overall tumbling of the whole double-stranded complex an increase in this correlation time indicates an enlargement of the overall volume of the complex due to the mismatch. The alterations of Φ_2 and θ are not significant. Thus, we conclude that both the flexibility and the length of the C_6 linker remain unchanged in the mismatched DNA substrates. These results indicate that at 20°C a changed secondary structure of the double-stranded complex is likely responsible for the alteration of the fluorescence lifetime of the reporter molecule BODIPY, which is the basic process of the mismatch detection described previously[1]. At a temperature of 37°C, the rotation times Φ_1 are generally shorter than at 20°C and their increase upon occurrence of a mismatch is less pronounced, because at higher temperatures the secondary structure of DNA becomes more and more relaxed. The cone semi-angles derived from the amplitudes β_1 and β_2 are decreasing by 2 degrees if a mismatch is present. It is likely that at 37°C, pre-melting of the duplex starts closed to the mismatched base, effectively elongating the C_6 linker arm by the dimension of the unpaired nucleotide.

At 20°C the anisotropy decay data measured for samples containing the C_2 linker revealed a more relaxed structure of the duplex only in the case of a T:C mismatch. The

TABLE 1: Molecular rotation times and cone semi-angles measured for BODIPY linked to the oligonucleotide by a linker containing either six or two carbon atoms at 20°C and 37°C (Φ_1-correlation time of the overall tumbling of the double stranded complex, Φ_2-rotation time of the alkyl chain linker, θ - cone semi-angle of the rotation of the linker arm, χ^2- goodness of the fit)

	Temp (°C)	no mismatch T : A	mismatched bases at the 5'-end of the probe T : T	T : C	T : G
dye linked to		*the oligonucleotide*	*by a* ***C6-linker***		
Φ_1(ns)	20	10.4	12.1	12.0	12.1
Φ_2(ns)	20	0.7	0.8	0.7	0.8
θ (deg)	20	41	40	38	39
χ^2	20	1.28	1.19	1.24	1.23
Φ_1(ns)	37	6.8	7.2	8.2	8.0
Φ_2(ns)	37	0.4	0.4	0.5	0.5
θ (deg)	37	48	46	46	46
χ^2	37	1.36	1.24	1.38	1.40
dye linked to		*the oligonucleotide*	*by a* ***C2-linker***		
Φ_1(ns)	20	14.7	15.4	20.9	14.8
Φ_2(ns)	20	1.0	1.2	0.5	1.0
θ (deg)	20	32	33	34	28
χ^2	20	1.25	1.19	1.35	1.25
Φ_1(ns)	37	8.6	8.7	8.8	9.1
Φ_2(ns)	37	0.6	0.7	0.7	0.8
θ (deg)	37	38	38	35	35
χ^2	37	1.37	1.20	1.37	1.32

mismatches T:T and T:G show anisotropy data very close to the perfect match T:A. At 37°C the aberrations in anisotropy between the perfect match and all the mismatches are too small to allow for a mismatch discrimination. Indeed, with a C_2 linker our method of mismatch detection did not work satisfactorily during our previous experiments.

Fluorescence Energy Transfer Experiments

The determination of a probability distribution of donor-acceptor distances is a suitable method to study the flexibility of the C_6 linker which carries the fluorescent reporter dye molecule at one of its ends. The fluorescence decays of the donor FAM in the presence of the acceptor TAMRA at the 3'-end of the target strand, as well as of the unquenched donor, were recorded with the emission polariser set to the magic angle. The temperature was fixed to 20°C throughout all the experiments. Typical decay curves are shown in

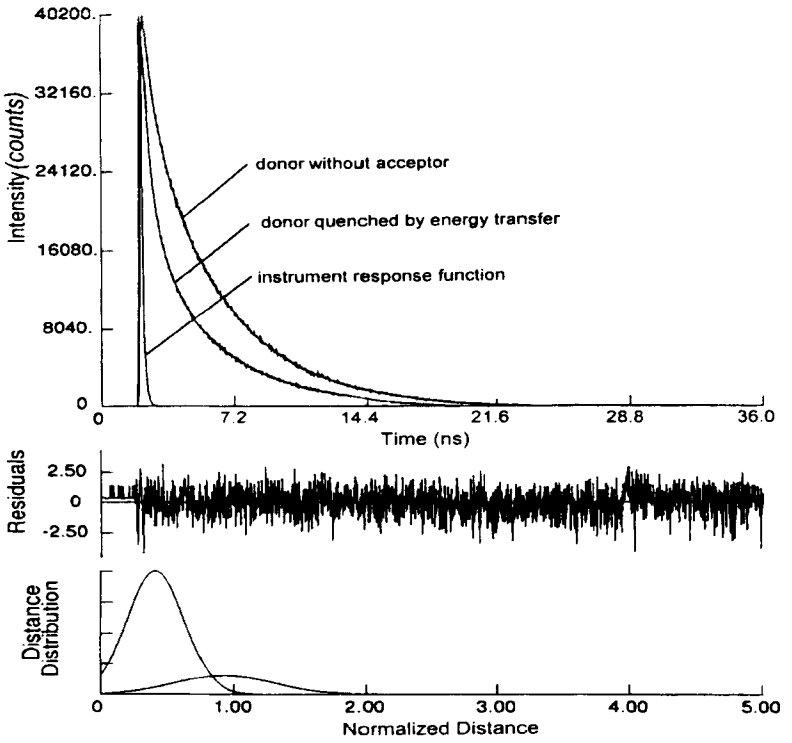

FIG. 3: Fluorescence decay curves of the donor quenched by an acceptor and without quenching (sample: A:G mismatch). The residuals indicate the goodness of the fit which is best in case of using a model which includes two distance distributions. The lower part shows the widths of the distributions and their maxima in arbitrary units (x-axis: D-A distance normalized to the Förster distance).

FIG. 3. The data were fitted according to equation Eqs.7 and 8. First we assumed that the donor is not restricted in its flexibility. In this case, calculations should reveal a single distribution of distances between the donor and the acceptor. However, the fits obtained using only a single distance distribution were unsatisfactory, as indicated by the reduced χ^2 values ($\chi^2 > 10$). After including a second distance distribution, the data could be fitted with reasonable χ^2. The results are shown in **TABLE 2**. For each distance distribution the mean distances, R_1 and R_2 , and the widths at half maximum, $FWHM_1$ and $FWHM_2$, are given. The parameters β_1 and β_2 account for the fraction of donors associated with the corresponding distribution. The existence of a second distance distribution indicates that the donor molecule at the 3'-end of the probe may exist in two

TABLE 2: Distributions of the donor-acceptor distances obtained from dynamic energy transfer experiments for dsDNA containing a single mismatched base in comparison to the perfect duplex. ($\mathbf{R_1}$-mean value of distance component 1, $\mathbf{FWHM_1}$- width of distance distribution of component 1, β_1-relative contribution of component 1; $\mathbf{R_2}$, $\mathbf{FWHM_2}$, β_2 denote the corresponding data of component 2, χ^2-goodness of fit)

	A : T no mismatch	**A : G** mismatch	**C : G** no mismatch	**C : T** mismatch
$\mathbf{R_1}$ (nm)	5.1	6.0	4.8	3.5
$\mathbf{FWHM_1}$ (nm)	4.8	3.4	5.8	5.5
β_1 (%)	13	6	22	21
$\mathbf{R_2}$ (nm)	2.3	2.3	2.5	2.0
$\mathbf{FWHM_2}$ (nm)	2.8	2.8	2.1	2.6
β_2 (%)	87	94	78	79
χ^2	1.14	1.12	1.43	1.10

different configurations, e.g., projecting into solution or interacting with the DNA structure. We assume that the component 1 showing a broader distribution, $FWHM_1$, is due to the donor molecules which remain flexible and do not interact appreciably with the DNA. Both the linkers to the donor and to the acceptor, as well as the 13mer single-stranded „overhang" of the target DNA, may contribute to the broadness of the distribution, $FWHM_1$, because these parts of the duplex are highly flexible. The second distance distribution, which is narrower, is assigned to those donor molecules which interact closely with the DNA target and are restricted in their flexibility. The mean D-A distance for this population amounts to 2.3 nm. Since the length of the 13mer „overhang" plus the C6 linker length are estimated to be approximately 6 nm in the case of a linear conformation, we conclude that the donor lies along the single-stranded „overhang" pointing towards the acceptor. A comparison of the amplitudes β_1 and β_2 shows that the majority of the donor molecules are interacting with the „overhang".

Neither the occurrence of a mismatch at the 5'-end of the probe, nor the identity of the nucleotide to which the donor is attached have any significant influence on the distance distributions measured. It is apparent that if the majority of the fluorescein dye is attached to the „overhang" the dye is not able to detect a mismatched base pair in these particular dye-DNA constructs.

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