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# A New Fluorescence Resonance Energy Transfer Pair and Its Application to Oligonucleotide Labeling and Fluorescence Resonance Energy Transfer Hybridization Studies

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We describe two new fluorescence resonance energy transfer (FRET) compatible labels, their covalent linkage to oligonucleotides, and their use as donor and acceptor, respectively, in FRET hybridization studies. The dyes belong to the cyanine dyes, and water solubility is imparted by a phosphonate which represents a new solubilizing group in DNA labels. They were linked to amino-modified synthetic oligonucleotides *via* oxysuccinimide (OSI) esters. The studies performed include binding assays, determinations of molecular distances, homogeneous competitive assays, and limits of detection, which are in the order of 5 pmol/L for a 15-mer.

**KEY WORDS:** Fluorescence hybridization assay; fluorescence resonance energy transfer; long-wavelength fluorescent labels.

## NOMENCLATURE

*Chromeon 546*: 2-[3[1-(7-Carboxyheptyl)-1,3-dihydro-3,3-dimethyl-2H-indol-2-ylidene] trienyl]-1-[2-(ethoxyhydroxyphosphoryl)ethyl]-3,3-dimethyl-indole OSI ester.

*Chromeon 642:* 2-[5[1-(5-Carboxypentyl)-1,3-dihydro-3,3-dimethyl-2H-indol-2-ylidene]-1,3-pentadienyl]-1-[2-(ethoxyhydroxyphosphoryl)ethyl]-3,3-dimethyl-indole OSI ester.

## INTRODUCTION

There is growing interest in the use of fluorescent labels for the detection and quantitation of DNA. Long wavelength labels are particularly attractive because of the decreased autoabsorption and autofluorescence of biological material in the range between 600 and 700 nm, and the fact that such fluorophores can be excited by rather inexpensive diode lasers or LEDs [1].

Cyanine dyes are widely used for making labels. They display high molar absorbances, high quantum yields (QYs) if bound to a target, and often lower QYs if present as free dyes, or labels. (*Note:* We refer to *dyes* as the chromophoric/fluorophoric systems, and to *labels* if the dyes are activated so to react with an amino group or hydroxy group.) The photostability of cyanines is high [1]. Cyanines form a group of dyes that combine relatively long-wavelength absorption with comparatively small molecular size, a feature that is desirable for labels in order not to disturb the system to be probed. In addition, the color of a cyanine dye is fairly predictable from its molecular structure, and therefore it is

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*Abbreviations:* BCBS, bicarbonate buffer solution, 50 mM, pH 9; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; FRET, fluorescence resonance energy transfer; NHS, *N*-hydroxysuccinimide; OSI, oxysuccinimidyl; PBS, phosphate-buffered saline, 22 mM, pH 7.2; trisacetate buffer, 50 mM, pH 7.2 with 25 mM NaCl.

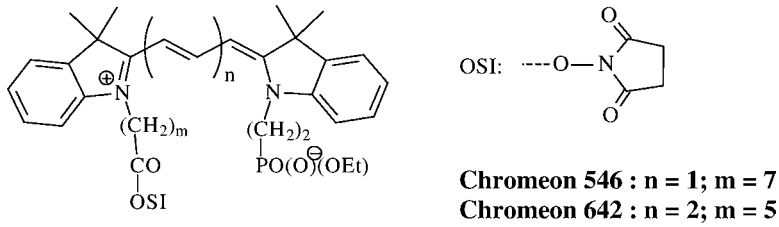


**Fig. 1.** Chemical structures of the new cyanine labels.

possible to design dyes with the desired spectral properties [1,2].

The new labels presented in this work (see Fig. 1) perfectly match the requirements for fluorescent biolabels such as high molar absorbance, high QYs, one reactive group for covalent coupling to oligonucleotides, and high photostability. The fact that the emission spectrum of the donor Chromeon 546 overlaps the absorption spectrum of the acceptor Chromeon 642 makes them ideal candidates for FRET studies. The donor Chromeon 546 is compatible to the 535 nm diode laser. A major advantage of using both fluorescent donors and acceptors is the possibility of ratiometric determination of the concentration of DNA [2,3].

Detection of DNA hybridization is often required in molecular biology, genetics, and forensics. A variety of methods has been used to detect DNA hybridization by fluorescence. Many of those rely on energy transfer between donor- and acceptor-labeled DNA [2,3]. The presence of complementary DNA sequences can be detected by the increased energy transfer efficiency if matched pairs are brought into spatial proximity by hybridization (see Figs. 2, $A_1$ and $A_2$). Competitive hybridization (Fig. 2B) may also be performed in which increased fractions of non-labeled target DNA compete with the formation of donor–acceptor pairs [2].

## MATERIALS AND METHODS

### Chemicals, Oligonucleotides and Buffers

All chemicals and solvents used were purchased from Acros (Geel, Belgium), Sigma-Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland), or Merck (Darmstadt, Germany) and were of analytical grade. Oligonucleotide syntheses were carried out by Metabion (Martinsried, Germany). The following sequences were used: *oligo-1*: amino-5′-CCG GCA GCA AAA TGT-3′, *oligo-2*: amino-5′-ACA TTT TGC TGC CGG-3′, *oligo-3*: 5′-ACA TTT TGC TGC CGG-3′-amino, *oligo-3* (with one mismatch): 5′-ACA TTT TCC TGC CGG-3′, *oligo-3* (with two mismatches): 5′-ACA TTT ACC TGC CGG-3′. The following buffers were used: PBS, 22 mM, pH 7.2; trisacetate buffer, 50 mM, pH 7.2 with 25 mM NaCl.

### Spectroscopy

Absorption spectra were acquired on a Cary 50 Bio UV-Visible Spectrophotometer from Varian. Emission spectra were recorded with a luminescence spectrometer (Aminco Bowman, Series 2; from SLM) with a standard 150-W xenon lamp as the excitation source.

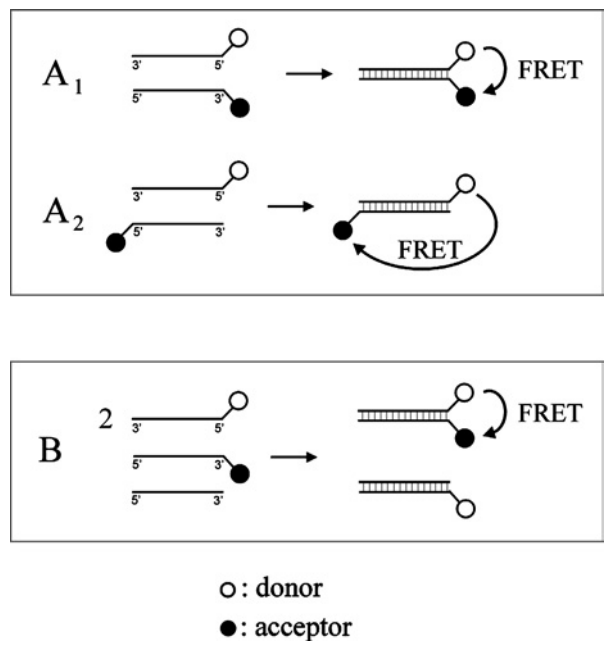


**Fig. 2.** Typical methods for studying the hybridization of oligonucleotides *via* fluorescence resonance energy transfer (FRET): binding studies ($A_1$ and $A_2$), competitive hybridization (B). The ratio of the product formed in a type B hybridization depends on the ratio between labeled and non labeled oligonucleotides.

### Syntheses

The synthetic routes for the dyes and labels shall be outlined here briefly. Dyes Chromeon 546 and Chromeon 642 were synthesized by analogy to [4,5]. The dyes were purified by column chromatography using silica gel 60 (40–63 $\mu$m) as the stationary phase and chloroform/methanol mixtures as the mobile phase, or silica gel 60 RP-18 (40–63 $\mu$m) as the stationary phase and methanol/water mixtures as the mobile phase (all from Merck). The dyes were activated to the corresponding OSI ester *via* the established NHS/DCC method [6]. The formation of OSI ester was observed by TLC using RP-18 $F_{254s}$ aluminum sheets (from Merck, Darmstadt, Germany) as the stationary phase and a methanol/water mixture as the eluent. In all cases the OSI esters showed lower $R_f$ values than the free acid. Chemical structures were proven by $^1$H-NMR and mass spectroscopy.

### Physical Properties of Labels

*Chromeon 546-OSI-Ester:* purple powder, $C_{37}H_{46}N_3O_{10}PS$ (755.82 g/mol). $R_f$ value (on silica gel RP-18, methanol:water 70:30, v/v): 0.11.

*Chromeon 642-OSI Ester:* blue powder, $C_{39}H_{48}N_3O_7P$ (701.80 g/mol). $R_f$ value (on silica gel RP-18, methanol:water 70:30, v/v): 0.08.

### General Oligonucleotide Labeling Procedure

First, 0.30 $\mu$mol of the respective amino-modified 15-mer oligonucleotide were dissolved in 500 $\mu$L of BCBS. The respective OSI ester (2 mg, dissolved in 50 $\mu$L of dry DMF) was added and the solution was allowed to stand for 15 hr in the dark. Then, 1.5 mL of ice-cold ethanol were added and the solution mixed well, placed at $-18^\circ$C for 1–2 hr, and centrifuged at 15,000 rpm for 10 min. The supernatant containing unreacted dye was removed carefully and the pellet containing labeled and unlabeled oligonucleotide was rinsed twice with cold ethanol.

Labeled oligonucleotides were purified by HPLC on a Knauer HPLC 64 apparatus. A Hibar pre-packed column RT (250 mm $\times$ 4 mm) packed with LiChrosorb RP 18 (10 $\mu$m) was used as the stationary phase.

The pellet was dissolved in 0.1 M triethylammonium acetate (TEAA) of pH 7.1 and loaded onto the column. A linear 10–65% acetonitrile gradient was run over 30 min. In all cases the unlabeled oligonucleotide migrated fastest, followed first by the labeled oligonucleotide, and then the free dye and the OSI ester [7]. The solvent of the HPLC fraction was removed on a rotary evaporator. The residue was dissolved in 100 $\mu$L of water, 1 mL of ice-cold ethanol was added, and the solution mixed well, placed at $-18^\circ$C for 1–2 hr, and centrifuged at 15,000 rpm for 10 min. The supernatant was removed carefully, and the pellet containing the labeled oligonucleotide either stored at 4°C or dissolved in 50 mM trisacetate buffer, pH 7.2 for further experiments.

### Determination of Molar Absorbances

Approximately 1 mg of dye was dissolved in PBS. This stock solution was diluted such that the absorbance was about 0.1, which then was measured exactly. The molar absorbances of the free dyes were calculated according to Beer's law.

It is often tacitly assumed that the molar absorbance of a label and its conjugate are the same, but this is often not the case. We therefore have determined the molar absorbances of the labeled oligonucleotides by recording the absorption spectra of both the free dye and the dye-oligonucleotide conjugate, both in PBS solution. The molar absorbance of the conjugate at its maximum was determined *via* the following formula:

$$\varepsilon_{\text{conj}}(\max) = \frac{A_{\text{conj}}(\max)\varepsilon_{\text{oligo}}(260)}{A_{\text{conj}}(260) - A_{\text{conj}}(\max)A_{\text{dye}}(260)/A_{\text{dye}}(\max)} \quad (1)$$

where $\varepsilon_{\text{conj}}(\max)$ is the molar absorbance of the conjugate at its maximum, $\varepsilon_{\text{oligo}}(260)$ the molar absorbance of the oligonucleotide at 260 nm, $A_{\text{dye}}(260)$ and $A_{\text{conj}}(260)$ the absorptions of the dye and its conjugate, respectively, at 260 nm, and $A_{\text{dye}}(\max)$ and $A_{\text{conj}}(\max)$ the absorptions of the dye and its conjugate, respectively, at the absorption maximum.

### Determination of Fluorescence Quantum Yields

The quantum yields (QYs) of the dyes and the dye conjugates, respectively, were determined in phosphate buffer (22 mM, pH 7.2) relative to established reference fluorophores of known QY. We used (a) the cyanine dye Cy5 (from Amersham, Pittsburgh, PA) with a reported QY of 0.28 in phosphate buffered saline (PBS) [8], useful for Chromeon 642, and (b) rhodamine 6G (from Aldrich) with a reported QY of 0.95 in ethanol [9], useful for Chromeon 546.

### Determination of the Förster Distance and Donor–Acceptor Distances

The Förster radius $R_0$ was calculated by the standard formula

$$R_0^6 = \frac{9\kappa^2}{8\pi(2\pi)^4 n^4}\phi_d \frac{\int d\lambda F_d(\lambda)\sigma(\lambda)\lambda^4}{\int d\lambda F_d(\lambda)} \quad (2)$$

where $\kappa^2$ is a factor accounting for the relative orientation of the emission and absorption dipoles of donor and acceptor, respectively; $n$ the refractive index of the solvent (for water $n = 1.33$); $F_d(\lambda)$ the emission intensity of the donor (in arbitrary units) at wavelength $\lambda$; and $\sigma(\lambda)$ the absorption cross section of the acceptor, which can be calculated from its molar absorbance $\varepsilon(\lambda)$ by

$$\sigma(\lambda) = \frac{10^3 \cdot \ln 10\ \varepsilon(\lambda)}{N_A} \approx \frac{2303}{N_A}\varepsilon(\lambda) \quad (3)$$

where $N_A$ is Avogadro's number. In the calculations, freely rotating donors and acceptors were assumed, so that for $\kappa^2$ the standard value of 2/3 can be adopted [3].

The donor–acceptor distance $r$ was calculated *via* the following two formulae:

$$E = \frac{R_0^6}{R_0^6 + r^6} \quad (4)$$

$$E = 1 - \frac{F_{DA}}{F_D} \quad (5)$$

where $E$ is the efficiency of energy transfer, $R_0$ the Förster distance, and $F_D$ and $F_{DA}$ the relative fluorescence intensities of the donor in absence and in presence of the acceptor, respectively [3].

### Hybridization Studies Using FRET

Varying quantities (typically 12.5–200 $\mu$L) of the acceptor solution ($c = 0.1$ $\mu$mol/L) were diluted with 50 mM trisacetate buffer of pH 7.2 to a final volume of 200 $\mu$L. Fifty microliters of donor solution ($c = 0.1$ $\mu$mol/L) were added. Fluorescence was measured at 20°C after an incubation time of 20 min at room temperature. All experiments were repeated five times.

### Competitive Hybridization Assays Using FRET

Quantities of typically 12.5–200 $\mu$L of non-labeled oligonucleotide solution ($c = 0.1$ $\mu$mol/L) were diluted with 50 mM trisacetate buffer of pH 7.2 to a final volume of 200 $\mu$L. Then, 50 $\mu$L of donor labeled oligonucleotide solution ($c = 0.1$ $\mu$mol/L) and 50 $\mu$L of acceptor labeled complementary oligonucleotide solution ($c = 0.1$ $\mu$mol/L) were added in this order. Fluorescence was measured at 20°C after an incubation time of 20 min at room temperature. All experiments were repeated five times.

## RESULTS AND DISCUSSION

### Dyes and Conjugates

We have chosen the two new cyanines as long-wave absorbing labels because of their high photostability and their relatively small molecular size (see Fig. 1). Both dyes contain only one reactive group. While this is more challenging from a synthetic point of view, a single reactive group is highly preferential in order to avoid crosslinking. In order to label both the 3′- and the 5′-end of oligonucleotides, we have synthesized oxysuccinimidyl (OSI) esters which bind to amino-modified synthetic oligonucleotides.

OSI esters are employed in aqueous solutions. Hence, they have to be water-soluble. The new labels presented here contain phosphonate groups (a new solubilizing substituent in fluorochrome chemistry), so to impart adequate water solubility. For the chemical structures of the dyes and labels see Fig. 1.

The new dyes and labels display high molar absorbances (85,000–250,000 L/mol cm) and high QYs (up to 31%) when covalently bound to oligonucleotides (see Table I). The QY increases in the case of Chromeon 546 seven-fold on binding to an oligonucleotide. The increase in QY may be attributed to (a) the rigidization of the molecular dynamics upon attachment to the oligonucleotide (this effect is known to increase QYs), and (b) to partial shielding of the fluorophore from water molecules (known to quench fluorescence). The high molar absorbances and QYs result in a rather low detection limit of the dye conjugates. Closely related protein labels have been shown to be detectable on a single molecule level [1]. The absorption and emission maxima of the dyes undergo a 5 nm long wave shift upon labeling.

Since the emission spectra of some of the donor dye efficiently overlaps the absorption spectra of the acceptor

**Table I.** Absorption and Fluorescence Maxima (in nm), Molar Absorbances (L/(mol cm)), and Quantum Yields (QYs; %) of the Dyes and Their Conjugates in PBS (22 mM, pH 7.2)

| Name | $\lambda_{abs}$ | $\lambda_{em}$ | $\varepsilon$ | QY |
|---|---|---|---|---|
| Chromeon 546 | 545 | 562 | 86,000 | 4 |
| Chromeon 546/oligo-1 | 550 | 565 | 142,000 | 31 |
| Chromeon 642 | 642 | 663 | 170,000 | 17 |
| Chromeon 642/oligo-2 | 647 | 666 | 217,000 | 22 |
| Chromeon 642/oligo-3 | 647 | 667 | 246,000 | nd |

*Note*. nd: not determined.

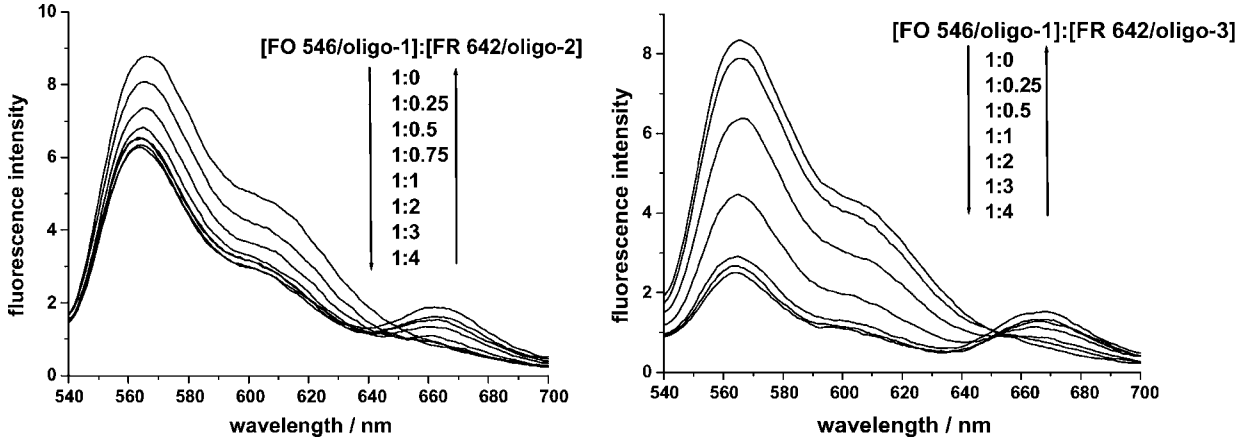


**Fig. 3.** Energy transfer study in which Chromeon 546/oligo-1 (5′-labeled) of constant concentration was titrated with Chromeon 642/oligo-2 (5′-labeled, left side) and Chromeon 642/oligo-3 (3′-labeled, right side), respectively. $\lambda_{exc} = 500$ nm. [Chromeon 546/oligo-1] = 0.02 $\mu$mol/L, molar ratios are 1:0, 1:0.25, 1:0.5, 1:0.75, 1:1, 1:2, 1:3, 1:4.

dye, this pair of dyes can be used in FRET studies. The use of fluorescent acceptors (instead of quenchers [10–13]) has the additional advantage of enabling ratiometric data evaluation. Ratiometric methods can help to eliminate bias that may be caused by instrumental artifacts such as fluctuations of the light source or drifts in the stability of photodetector.

## Hybridization Studies

Various hybridization studies were performed. Two representative experimental results are shown in Fig. 3. In both experiments the donor (the Chromeon 546/oligo-1 conjugate), being present in constant concentration, is titrated with increasing quantities of the acceptor. In the first experiment we tested the Chromeon 642/oligo-2 conjugate, in the second the Chromeon 642/oligo-3 conjugate (Figs. 3A and B). In both cases the donor dye Chromeon 546 was labeled to the 5′-end of oligo-1. In the first experiment (Fig. 3, left) the acceptor was attached to the 5′-end, in the second experiment (Fig. 3, right) to the 3′-end of the complementary oligonucleotide. The fluorescence intensity of the donor generally decreases with increasing quantities of acceptor, while the fluorescence intensity of the acceptor increases due to FRET. However, the FRET efficiency is much weaker in first experiment (Fig. 3, left) compared to the second experiment (Fig. 3, right), because the donor–acceptor distance is much larger in first experiment (5′, 5′) than in the second (5′, 3′).

Figure 4 shows the so-called binding curves, i.e. a plot of the ratio of the two peak intensities versus the ratio of concentrations of the labeled oligonucleotides. Curve A displays the binding curve of the first experiment (5′, 5′-labeling), curve B displays the binding curve of the second experiment (5′, 3′-labeling). The slope of plot B is bigger than that of plot A due to the fact that the energy transfer rate is higher in case of the second experiment B.

The Förster radii ($R_0$) of the donor–acceptor pairs were determined in order to estimate the proximity of the two labels in the hybrid. The Förster radii of the two systems described were 5.8 and 5.9 nm, respectively (see Table II). Compared to known systems (2.2 nm in [14]), the $R_0$ values of the dye pairs chosen are higher which enable energy transfer measurements over much larger distances. This is of particular significance in case of 5′–5′ labeling: if $R_0$ was smaller, the energy transfer efficiency certainly would be too low to be detectable.

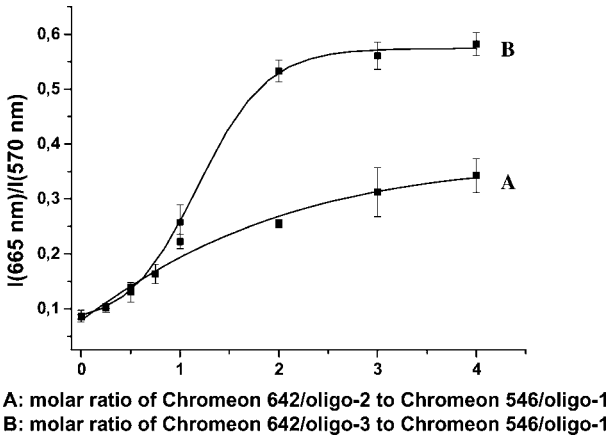


**Fig. 4.** Plot of the ratio of the intensities at 665 and 570 nm versus the ratio of the concentrations of Chromeon 546/oligo-1 (labeled at 5′) and its counterstrand, depending on the site of labeling. Graph A gives the plot for a counter oligonucleotide labeled in 5′-position (Chromeon 642/oligo-2), while B gives data for a counterstrand labeled in 3′-position (Chromeon 642/oligo-3).

**Table II.** $R_0$ Values and Average Distances (in nm) Between Donor and Acceptor Molecules as Calculated *via* Equitastions (3–6) as a Function of the Site of Labeling

| Donor | Acceptor | $R_0$ | Distance | Labeling positions (donor–acceptor) |
|---|---|---|---|---|
| Chromeon 546/oligo-1 | Chromeon 642/oligo-2 | 5.8 | $7.2 \pm 0.3$ | 5′–5′ |
| Chromeon 546/oligo-1 | Chromeon 642/oligo-3 | 5.9 | $5.2 \pm 0{,}07$ | 5′–3′ |

The average distances between donor and acceptor were computed using these $R_0$ values. If the position of the labels is 5′ (donor) and 3′ (acceptor), the average donor–acceptor distance was found to be 5.2 nm (Table II). The spacer groups between the fluorophores and the oligonucleotides are in the range between 2 and 2.5 nm considering the $C_6$-spacer of the oligonucleotide, and the $C_6$- or $C_8$-spacer of the respective labels. Therefore the value of the distance between the fluorophores would be expected to be in the range between 0 and 5 nm. We find a value of 5.2 nm which is indication that the oligonucleotides do not perfectly hybridize at the ends of the chain where the labels are located. As a result, distance id found to be somewhat higher than expected.

If both donor and acceptor are attached to the 5′-ends of the complementary oligonucleotides, the average donor–acceptor distance was found to be around 7.2 nm (Table II). Again in this case, the spacer groups between the fluorophores and the oligonucleotides are in the range between 2 and 2.5 nm. In addition, the length of the 15 mer oligonucleotide of 5.1 nm has to be taken into account. Therefore the value of the distance between the fluorophores would be expected to be in the range between 5 and 9 nm. The found value of 7.2 nm is therefore in the expected range.

In both cases the calculated values need to be considered as average distances because the spacer groups between oligonucleotide and dye are spatially flexible and result in a variable distance of the fluorophores to each other.

## Competitive Hybridization Assays

Competitive hybridization studies were performed in order to evaluate the potential of FRET in qualitative and quantitative hybridization assays. Figure 5 shows the results of a competitive hybridization assay. Chromeon 642/oligo-3 (3′-labeled, the *donor*) of constant

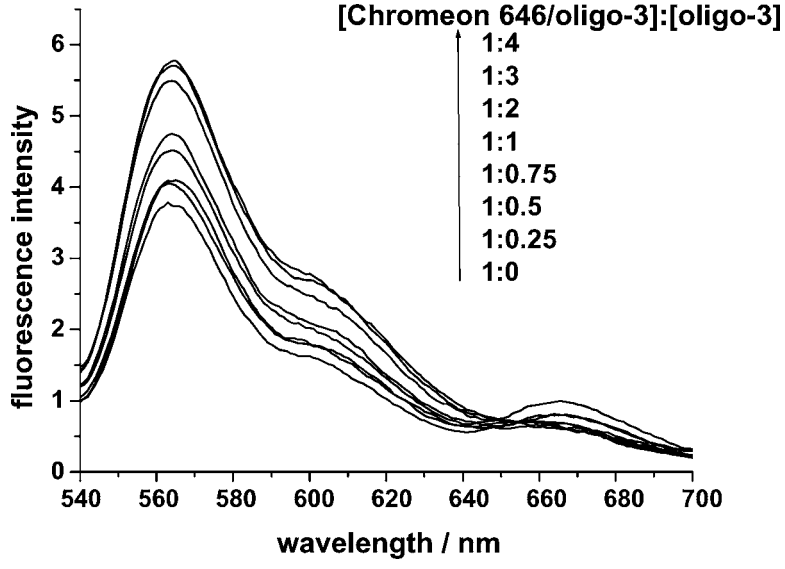


**Fig. 5.** Competitive hybridization assay in which Chromeon 642/oligo-3 (3′ labeled, donor) of constant concentration and various fractions of non-labeled oligo-3 were mixed with constant fractions of Chromeon 546/oligo-1 (5′ labeled, acceptor). $\lambda_{exc} = 500$ nm. [Chromeon 546/oligo-1] = [Chromeon 642/oligo-3] = 0.017 $\mu$mol/L, molar ratios are 1:0, 1:0.25, 1:0.5, 1:0.75, 1:1, 1:2, 1:4.

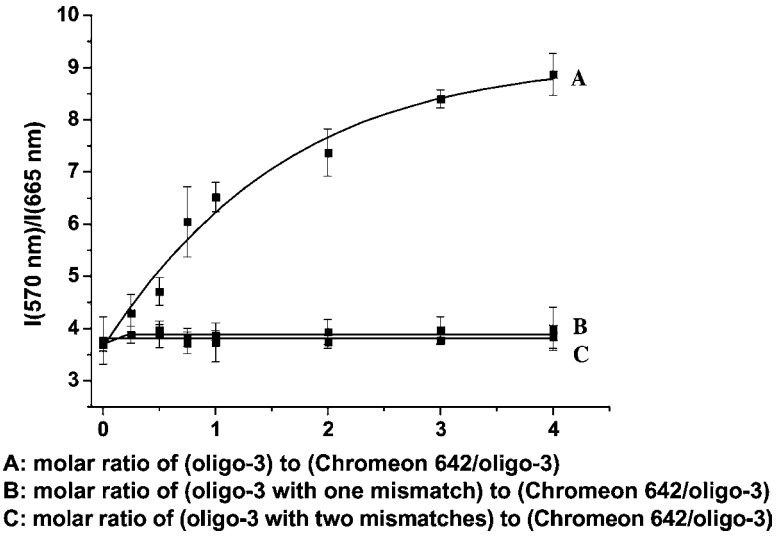


**Fig. 6.** Ratiometric data evaluation of the competitive hybridization assay shown in Fig. 5. The ratio of the intensities at 570 and 665 nm were plotted versus the concentration ratio of non-labeled oligonucleotide to the acceptor.

concentration and various fractions of non-labeled oligo-3 were mixed with constant fractions of Chromeon 546/oligo-1 (5′-labeled, the *acceptor*). The fluorescence of the donor increases with increasing quantities of oligo-3, because the oligo-3 binds to the donor, the acceptor is freely moving in solution, and energy transfer efficiency is reduced. However, a decrease in the intensity of the acceptor is not detectable, most likely because of the low fluorescence QY of the acceptor.

A plot of the ratio of the two peak intensities versus the ratio of the concentrations of the non-labeled oligonucleotides to the acceptor results in the binding plot shown in Fig. 6 (plot A). Saturation is reached at an about three-fold excess of oligo-3. From this and other plots we calculate the range over which the concentration of a 15-mer oligonucleotide can be quantified is in the range between $6 \times 10^{-9}$ to $8 \times 10^{-8}$ mol/L. The limit of detection is at 5 pmol $L^{-1}$.

The effect of single mismatches in an oligonucleotide on the energy transfer rate was examined *via* a competitive hybridization study. Constant concentrations of Chromeon 546/oligo-1 (donor) were mixed with the following non-labeled oligonucleotides: oligo-3 (no mismatch), oligo-3 (single mismatch), or oligo-3 (double mismatch). Constant fractions of complementary Chromeon 642/oligo-3 (acceptor) were added. The binding curves thus obtained are shown in Figs. 6, plots A–C). While the fully complementary oligonucleotide gives a distinct response (Fig. 6, plot A), both the single mismatch oligomer and the 2-mismatch oligomer cause no effect on the efficiency of FRET (Figs. 6, plots B and C).

## CONCLUSION

We introduce two new long-wavelength fluorescent labels for DNA oligonucleotides that are well suited for FRET studies. The spectral properties of the labels can be fine-tuned so to match the desired wavelengths of commonly available light sources. The dyes containing reactive OSI esters were used for labeling amino modified DNA oligonucleotides, which then were submitted to hybridization studies using FRET. These enabled the determination of donor-to-acceptor distances. The competitive assays enable the determination of non-labeled oligonucleotides in the range from $6 \times 10^{-9}$ to $8 \times 10^{-8}$ mol/L. In addition, FRET can be used to distinguish between complementary oligonucleotides without, and with a single mismatch in the sequence. The scheme is therefore well suited for detecting hybridization reactions, not only in solution, but conceivably also in optical DNA biosensor arrays [15].

## ACKNOWLEDGMENT

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