Published in final edited form as:
Anal Chem. 2003 December 1; 75(23): 6629–6633. doi:10.1021/ac034881e.

DNA Hybridization Using Surface Plasmon-Coupled Emission

Joanna Malicka, **Ignacy Gryczynski**, **Zygmunt Gryczynski**, and **Joseph R. Lakowicz**
Center for Fluorescence Spectroscopy, Department of Biochemistry and Molecular Biology, University of Maryland at Baltimore, 725 West Lombard Street, Baltimore, Maryland 21201

Abstract

We describe a new approach to measuring DNA hybridization using surface plasmon-coupled emission (SPCE). Excited fluorophores are known to couple with surface oscillations of electrons in thin metal films, typically 50 nm thick silver on a glass prism. These surface plasmons then radiate into the glass at a sharply defined angle determined by the emission wavelength and the optical properties of the glass and metal. This radiation has the same spectral profile as the emission spectrum of the fluorophores. We studied the emission due to Cy3-labeled DNA oligomers bound to complementary unlabeled oligomers which were themselves bound to the metal surface. Hybridization resulted in SPCE due to Cy3–DNA into the prism. Directional SPCE was observed whether the sample was illuminated from the sample side or through the glass substrate at the surface plasmon angle for the excitation wavelength. A large fraction of the total potential emission is coupled to the surface plasmons resulting in improved sensitivity. When illuminated through the prism at the surface plasmon angle, the sensitivity is increased due to the enhanced intensity of the resonance evanescent field. It is known that SPCE depends on proximity to the silver surface. As a result, changes in emission intensity are observed due to fluorophore localization even if hybridization does not affect the quantum yield of the fluorophore. The use of SPCE resulted in suppression of interfering emission from a noncomplementary Cy5–DNA oligomers due to weaker coupling of the more distant fluorophores with the surface plasmons. We expect SPCE to have numerous applications to nucleic acid analysis and for the measurement of bioaffinity reactions.

Measurement of DNA hybridization is now a central component of biotechnology and medical diagnostics. A variety of approaches are available to detect DNA hybridization including the use of intercalating fluorophores,[1,2] dyes which displayed increased quantum yields upon binding to double stranded (ds) DNA,[3,4] fluorescence resonance energy transfer,[5,6] and excimer formation.[7] In all these methods hybridization is detected by a change in the emission spectral properties of the probe which occurs upon formation of double-stranded DNA, typically an increased quantum yield of the fluorophore or an increase in resonance energy transfer.

We now describe a new approach to measurement of nucleic acid hybridization which does not depend on a spectral change in the fluorophores. In our approach the intensity change is due to localization of the fluorophore near a thin metal surface by the binding reaction. In several recent reports we described a phenomenon called surface plasmon-coupled emission (SPCE).[8–10] From theoretical studies it is known that this phenomenon occurs for excited fluorophores within about 200 nm of a thin continuous metallic surface,[11,12] in our case 50 nm thick silver on a glass substrate. A small number of experimental reports of SPCE have appeared,[13–16] but this phenomenon has not yet been applied to biological assays. The dipoles of the excited fluorophores coupled with surface plasmons, which are electron oscillations on

Correspondence to: Joseph R. Lakowicz.

the metal surface. The surface plasmons then radiate into the glass substrate at a sharply defined angle which satisfied the resonance between the fluorophores and the plasmons. Coupling to the surface plasmons is highly efficient and can collect 50% or more of the total possible emission.[11] The directional property allows selective observation of the desired emission. Emission from fluorophores more distant from the metal will radiate mostly isotropically, and most of this emission will reflect off the metal surface. SPCE is in a sense the reverse process of surface plasmon resonance (SPR) where light at a specific angle of incidence, through a prism, is strongly absorbed by a metal film on the surface.[17–19] While the present report uses silver films, we recently observed SPCE using gold films.[20] The use of gold is advantageous because of its chemical stability and well-known surface chemistry. It is known that the evanescence field present in the sample above the gold–glass substrate is enhanced due to the resonance interactions.[21,22] As a result, the intensity of the SPCE is expected to show this same resonance enhancement.

In this report we examined SPCE for fluorophore-labeled DNA oligomers, which were complementary (ssDNA–Cy3, Chart 1) or not complementary (ssDNA–Cy5) to a surface-bound capture oligomer. We found a dramatic increase in the directional SPCE emission of Cy3 upon hybridization. In contrast, the noncomplementary Cy5-labeled DNA did not show changes in the SPCE. The use of SPCE results in high sensitivity due to efficient coupling to the metal. We also show background rejection of the Cy5 emission due to the distance-dependent coupling. SPCE is a simple technology requiring only a thin nobel metal film and surface-localized fluorophores, which can be easily implemented in sensing applications. We expect SPCE to have numerous applications to DNA analysis and diagnostics.

THEORY

Since the phenomenon of SPCE is new to analytical chemistry, it is valuable to review the principles. The principles of SPCE can be understood from the physics of surface plasmons[23,24] and the theory of surface plasmon resonance (SPR).[25,26] SPR is observed as a decrease in reflectivity of a thin gold film at a specific angle of incidence (θ_{SP}) through a glass prism. This occurs when the wavevector of the incident light matches the wavevector of the surface plasmons (θ_{SP}). The wavevector of the incident light is given by

$$k=\frac{2\pi}{\lambda}=\frac{n_p\omega}{c}n_p k_0 \tag{1}$$

where n_p is the refractive index of the prism, λ is the wavelength in the prism, ω is the frequency in radians/s, and k_0 is the wavevector in free space. Calculation of the wavevector for the surface plasmon is more complex. The dielectric constant (ε) of a metal (m) is a complex quantity given by

$$\varepsilon_m=\varepsilon_r+i\varepsilon_{im} \tag{2}$$

where $i=\sqrt{-1}$ and the subscripts indicate the real (r) and imaginary (im) components. For a metal the wavevector for the surface plasmon at the metal–sample interface can be approximated by

$$k_{SP}=k_0\left(\frac{\varepsilon_r\varepsilon_s}{\varepsilon_r+\varepsilon_s}\right)^{1/2} \tag{3}$$

where ε_r and ε_s are the real parts of the dielectric constants of the metal (m) and sample (s), respectively.

The incident light interacts with the surface plasmon when its *x*-axis component equals the wavevector for the surface plasmons. The wavevector for the incident light in the prism is given by

$$k_p = k_0 n_p \tag{4}$$

and the component along the *x*-axis is equal by

$$k_x = k_0 n_p \sin \theta_I \tag{5}$$

where θ_I is the incidence angle in the prism. The conditions for SPR absorption are satisfied when

$$k_{SP} = k_x = k_0 n_p \sin \theta_{SP} \tag{6}$$

SPR only occurs for p-polarized incident light.[17,18] In reality SPR does not occur only at a single angle but over a relatively narrow range of angles determined by the optical constants and resonance response of the metal.

SPCE displays similar properties of SPR[17,18] except that the surface plasmons are created by interactions with the excited fluorophores. These plasmons then emit into the glass prism, which is possible because the wavevector of the plasmons can be matched with far-field radiation according to eq 6. For SPCE the wavelength is now the emission wavelength which radiates at an angle θ_F.

MATERIALS AND METHODS

Sample Preparation

All samples were prepared on quartz slides, which were the smooth, ungrooved parts of 1 mm demountable cuvettes (12.5 mm × 45 mm; Starna Cell, Inc., Atascadero, CA). Complementary oligonucleotides labeled with biotin or Cy3 (*N*,*N*′-dipropyltetramethylindocarbocyanine) and noncomplementary oligo labeled with Cy5 (*N*,*N*′-dipropyltetramethylindodicarbocyanine) (Chart 1) were obtained from the Biopolymer Core Facility at the University of Maryland, School of Medicine. Nanopure H_2O (>18.0 MΩ), purified a using Millipore Milli-Q gradient system, was used for all experiments. Buffer components were purchased from Sigma-Aldrich (St. Louis, MO).

Each quartz slide was half covered with continuous 50 nm thick silver film, which was vapor deposited by EMF Corp. (Ithaca, NY). The entire surface was then covered with 500 μL of an aqueous solution of 10 μM BSA–biotin (Sigma, St. Louis, MO) and placed in humid chamber for 20 h (5 °C, cold room). After being washed 3 times with water, the slides were placed again in humid chamber and 500 μL of 5 μM streptavidin (Molecular Probes, Eugene, OR) in 0.1× PBS buffer was deposited on each BSA–biotin-coated surface for 40 min (room temperature). These slides were than washed 3 times with 0.1× PBS buffer. Then 500 μL of biotinylated DNA samples in 5 mM HEPES (pH 7.5), 0.1 M KCl, and 0.25 mM EDTA was deposited for 1 h at room temperature. After being washed with the same buffer and covered with the second

part of the demountable cuvette, the samples that consisted of an ssDNA–streptavidin–BSA layer on the silver film were ready to use.

To determine the conditions needed to observe SPCE we used the double-stranded Cy3–DNA–biotin deposited on protein monolayer by incubation with 500 μL of 2 μM Cy3–DNA–biotin previously hybridized by mixing complementary oligos in 5 mM HEPES (pH 7.5), 0.1 M KCl, and 0.25 mM EDTA.

For hybridization experiments, the protein layer was covered with 500 μL of 2 μM single-stranded biotinylated oligo (ssDNA–biotin) in buffer solution. The 1 mm cuvette was then filled with hybridization buffer containing 5.4 nM complementary ss Cy3–DNA and 150 nM noncomplementary ss Cy5–DNA.

Fluorescence Measurements

The quartz slide with sample was attached with index-matching fluid to a hemicylindrical prism made of BK7 glass and positioned on a precise rotary stage.[9] The stage was equipped with an arm about 15 cm long for fiber optic detection. The fiber bundle was 3 mm in diameter. The input of the fiber optic bundle could be rotated around the prism, which allowed observation at any angle relative to the incident angle. The incident light was either normal to the glass water interface from the water side (reverse Kretschmann, RK) or incident at the SPR angle for the incident wavelength through the prism (Kretschmann configuration, KR). These optical configurations will be described in more detail in the Results and are shown schematically in Figure 4 (see below). For collection of the angle-dependent emission intensity a 200 μm air slit was placed on the fiber input. The output of the fiber was directed to the SLM 8000 single photon counting spectrofluorometer. For measurement of the emission spectra the 200 μm slit was removed from the fiber and the fiber input was positioned as close as possible to the sample. The 514 nm excitation was from a pulsed mode-locked argon ion laser (76 MHz repetition rate, 120 ps half-width). Scattered incident light at 514 nm was suppressed on observation by using a holographic supernotch-plus filter (Kaiser Optical System, Inc., Ann Arbor, MI). Unless otherwise indicated, the incident light was polarized horizontally in the laboratory axis, which is p-polarized relative to the plane of incidence.

RESULTS

Overview of Experiments

The DNA oligomers are shown in Chart 1. The surface-bound capture oligomer was labeled with biotin (ssDNA–biotin). The oligomer complementary to the capture oligomer was labeled with Cy3 (ssCy3–DNA). A shorter oligomer which was not complementary to the capture oligomer was labeled with Cy5 (ssCy5–DNA). The concept of the experiment is shown in Scheme 1. The ssDNA–biotin is bound to the silver surface by a layer of biotinylated BSA covered with streptavidin. ssDNA–biotin binds to this surface. The bathing solution can contain ssDNA–Cy3 and/or ssDNA–Cy5. We expect some of the ssDNA–Cy3 to bind to the surface and any excess to remain unbound. ssDNA–Cy5 will be unbound and more distant from the silver.

When using silver surfaces, there are two different modes of excitation. The sample can be excited through the aqueous phase, in our case with normal incidence to the interface. This is called the reverse Kretschmann (RK) configuration, which does not result in excitation of surface plasmons in the metal. A second mode of excitation is through the glass substrate with the incident angle equal to the SPR angle for the excitation wavelength. This is called the Kretschmann configuration (KR) as shown in Scheme 1. The excitation source is the evanescent field from the plasmon resonance. This field penetrates about a wavelength into

the aqueous phase. Because of the resonance interaction, the evanescent intensity is enhanced about 20-fold relative to the incident intensity.[13,14] This evanescent field is different from that found for total internal reflection (TIR) because it is the result of surface plasmons in the metal film. However, the origin is similar because the incident light cannot propagate into the aqueous phase.

Detection of SPCE

First we examined the sample with surface plasmon (KR) excitation. Because we wished to measure the angular distribution of the SPCE, this sample contained only surface-bound Cy3–DNA. The emission intensity was measured at all angles ±90° from the normal axis (Figure 1). The emission was found to be strongly directional at ±75°. Even though the emission is the result of surface plasmons, the emission spectrum is characteristic of Cy3. We compared this angle with the expected angle-dependent reflectivity of the silver film.[17,18] For this calculation we made the assumption that the protein–DNA layer was 15 nm thick and had a slightly larger dielectric constant ($\varepsilon_1 = 2.07$) than the aqueous phase ($\varepsilon_0 = 1.79$). This calculation (Figure 2) showed a reflectivity minimum near 74° for the emission wavelength of 565 nm, in good agreement with the observed value of 75°. The similarity of the observed and calculated angles, and the highly directional nature of the emission, support our claim that the emission at 75° is in fact surface plasmon-coupled emission. Additionally, the emission was p-polarized as expected for SPCE.[8] We also calculated the reflectivity for the excitation wavelength of 514 nm. The calculated reflectivity minimum agrees with the experimental value of 78°, which we found yielded the most intense emission.

DNA Hybridization

We tested the possibility of using SPCE to measure DNA hybridization. The excitation and emission angles were chosen from the results in Figure 1, 78 and 75°, respectively. The sample consisted of unlabeled ssDNA–biotin, which was bound to the protein layer. Upon injection of ssCy3–DNA there was a time-dependent increase in the emission of Cy3 (Figure 3). Upon injection of ssCy3–DNA to a sample which contained protein (BSA and streptavidin) but no capture oligomers, there was no increase in Cy3 emission. The latter result shows that there was no significant nonspecific binding of ssCy3–DNA to the protein surface which lacked the complementary oligomer. This result also shows there is little observable emission from ssCy3–DNA which was in the sample but not bound near the silver surface. In total, the data in Figure 3 demonstrated that DNA hybridization can be detected from the SPCE. Furthermore, detection of hybridization depends on proximity to the silver surface and does not require a change in quantum yield of the fluorophore.

Background Suppression Using SPCE

The dependence of SPCE on proximity to the silver surface suggested the possibility of suppressing the background from regions of the sample more distal from the metal. We tested this possibility by examining a sample containing both surface-bound Cy3–DNA and noncomplementary ssCy5–DNA. We measured both the SPCE and the free-space emission (Figure 4, top). By free space emission we mean the nondirectional emission which propagates away from the sample. For this measurement the probe was excited through the sample, and not through the prism, at normal incidence. In this configuration it is not possible to excite surface plasmons, so that the free-space emission is similar to that which would be observed in a standard cuvette without a metal film.

At the excitation wavelength of 514 nm Cy5 absorbs light more weakly than Cy3. To obtain comparable intensities in the free-space emission of Cy3 and Cy5 we used an approximate 30-fold higher concentration of ssCy5–DNA than Cy3–DNA, resulting in the free-space emission spectrum shown in Figure 4. We considered this comparable intensity due to Cy5 to be the

unwanted background signal. We then changed the optical configuration to use surface plasmon (KR) excitation and to observe the SPCE. Using these conditions the emission was almost completely due to Cy3 (Figure 4). The emission from Cy5 was suppressed 20-fold or more. Hence SPCE can be used with samples containing multiple fluorophores or autofluorescence, and only fluorophores close to the metal will result in SPCE.

DISCUSSION

SPCE appears to offer several advantages for measurement of DNA hybridization and other binding interactions. When using the KR configuration, excitation occurs selectively near the metal film due to the enhanced evanescent field. Irrespective of the use of the KR or RK configuration the increased intensity seen at the surface plasmon angle is due to fluorophore localization near the metal surface. Hence, binding can be detected without a change in probe intensity due to the binding event. Since the intensity change is due to surface localization, an intensity change can be observed for any association reaction and is not limited to fluorophores which display changes in quantum yield. Additionally, SPCE occurs over moderately large distances, typically up to several hundred nanometers from the metal surface.[8] Since the biomolecules are typically much smaller, several layers of the capture molecules can be placed on the metal surface for increased sensitivity.

Another advantage of SPCE is effective rejection of the emission from fluorophores more distant from the metal. This suppression occurs by two mechanisms. One mechanism is decreased efficiency of coupling at larger distances from the metal. When using the Kretschmann configuration, excitation occurs preferentially near the metal surface.

Another important characteristic of SPCE is high sensitivity because plasmon coupling can result in light collection efficiency near 50%,[11] much higher than efficiencies of a few percent with more typical optics. The use of SPCE is technically simple, requiring only a easily prepared thin silver film. And finally, we note that SPCE can be observed with thin gold films[20] which are chemically stable and for which the surface modification chemistry is well developed. These attributes suggest SPCE will find numerous applications for nucleic acid and protein binding reactions.

ACKNOWLEDGMENT

This work was supported by the NIH National Center for Research Resources, Grants RR-08119, HG-002655, EB-000682, and EB-00981.

References

1. Le Pecq J-B, Le Bret M, Barbet J, Roques B. Proc. Natl. Acad. Sci. U.S.A 1975;72(8):2915–2919. [PubMed: 127172]
2. Markovits J, Roques BP, Le Pecq JB. Anal. Biochem 1979;94:259–264. [PubMed: 464292]
3. Glazer AN, Peck K, Mathies RA. Proc. Natl. Acad. Sci. U.S.A 1990;87:3851–3855. [PubMed: 2339125]
4. Rye HS, Yue S, Wemmer DE, Quesada MA, Haugland RP, Mathies RA, Glazer AN. Nucleic Acids Res 1992;20(11):2803–2812. [PubMed: 1614866]
5. Parkhurst KM, Parkhurst LJ. J. Biomed. Opt 1996;1(4):435–441.
6. Morrison LE, Stols LM. Biochemistry 1993;32:3095–3104. [PubMed: 8457571]
7. Ebata K, Masuko M, Ohtani H, Kashiwasake-Jibu M. Photochem. Photobiol 1995;62(5):836–839. [PubMed: 8570721]
8. Lakowicz JR. Anal. Biochem. in press.
9. Gryczynski I, Malicka J, Gryczynski Z, Lakowicz JR. Anal. Biochem. in press.

10. Lakowicz JR, Malicka J, Gryczynski I, Gryczynski Z. Biochem. Biophys. Res. Commun 2003;307:435–439. [PubMed: 12893239]
11. Ford, GW.; Weber, WB. Electromagnetic Interactions of Molecules with Metal Surfaces. Vol. Vol. 113. Amsterdam: North-Holland Physics Publishing; 1984. p. 195-2987.
12. Weber WH, Eagen CF. Opt. Lett. 1979;4(8):236–238.
13. Gerbshtein, M Yu; Merkulov, IA.; Mirlin, DN. JETP Lett 1976;22:35–36.
14. Holland WR, Hall DG. Opt. Lett 1985;10(8):414–416. [PubMed: 19724466]
15. Shu QQ, Hansma PK. Thin Solid Films 2001;384:76–84.
16. Benner RE, Dornhaus R, Chang RK. Opt. Commun 1979;30(2):145–149.
17. Frutos AG, Corn RM. Anal. Chem :449A–455A.199
18. Kolomenskii AA, Gershon PD, Schuessler HA. Appl. Opt 1997;36(25):6539–6547. [PubMed: 18259515]
19. Hanken, DG.; Jordan, CE.; Frey, BL.; Corn, RM. Electroanalytical Chemistry: A Series of Advances. Vol. Vol. 20. New York: Marcel Dekker; 1998. Surface plasmon resonance measurements of ultrathin organic films at electrode surfaces; p. 141-225.
20. Gryczynski I, Malicka J, Gryczynski Z, Lakowicz JR. J. Phys. Chem. B. submitted for publication.
21. Neumann T, Johansson ML, Kambhampati D, Knoll W. Adv. Funct. Mater 2002;12:575–586.
22. Liebermann T, Knoll W. Colloids Surf 2000;171:115–130.
23. Raether, H. Surface Plasmons on Smooth and Rough Surfaces and on Gratings. New York: Springer-Verlag; 1988. p. 136
24. Forstmann, F.; R Gerhardts, R. Metal Optics Near the Plasma Frequency. Vol. Vol. 109. New York: Springer-Verlag; 1986. p. 132
25. Kurihara K, Suzuki K. Anal. Chem :696–701.74
26. Salamon Z, Macleod HA, Tollin G. Biochim. Biophys. Acta 1997;1331:117–129. [PubMed: 9325438]

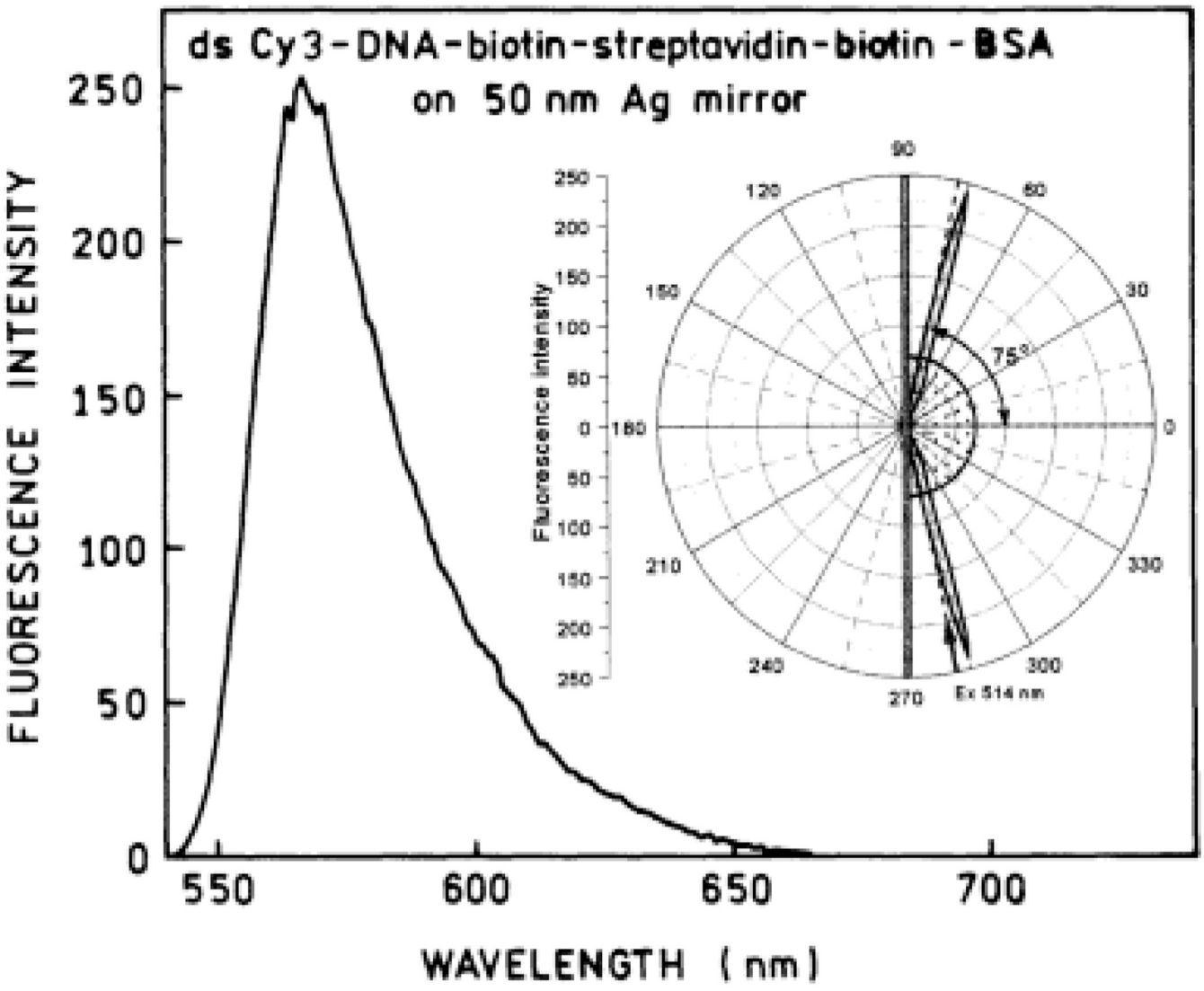


Figure 1.
Fluorescence spectrum of dsCy3–DNA–biotin directional emission (SPCE). The insert shows an angular distribution of the fluorescence observed at 565 nm upon SP excitation at 514 nm.

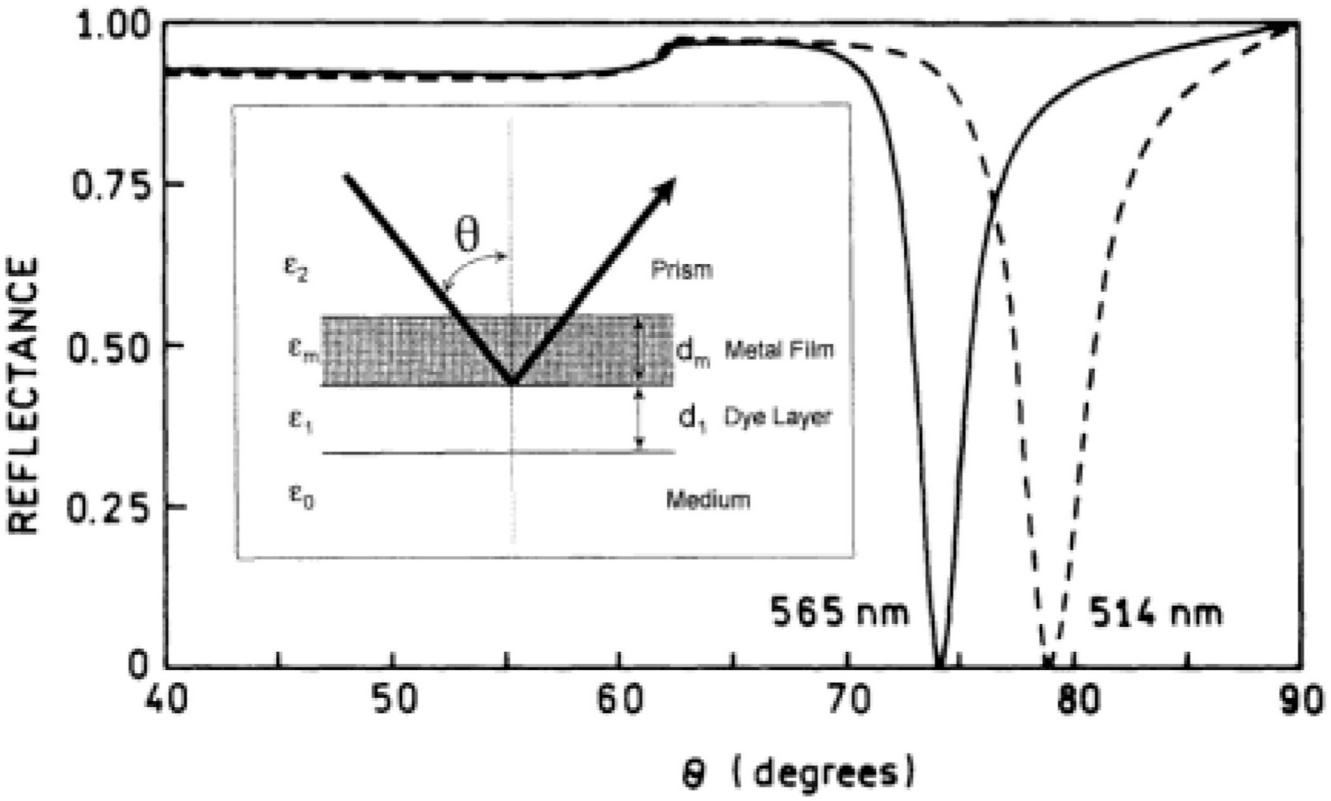


Figure 2.

Angle-dependent reflectivity of a silver film calculated according to ref [17]. This assumed values were dielectric constants of $\varepsilon_2 = 2.3$, $\varepsilon_m = -13.5 + 0.5i$ for 565 nm, and $\varepsilon_m = -10.7 + 0.33i$ for 514 nm, $d_m = 50$ nm, $\varepsilon_1 = 2.07$, $d_1 = 15$ nm, and $\varepsilon_0 = 1.79$.

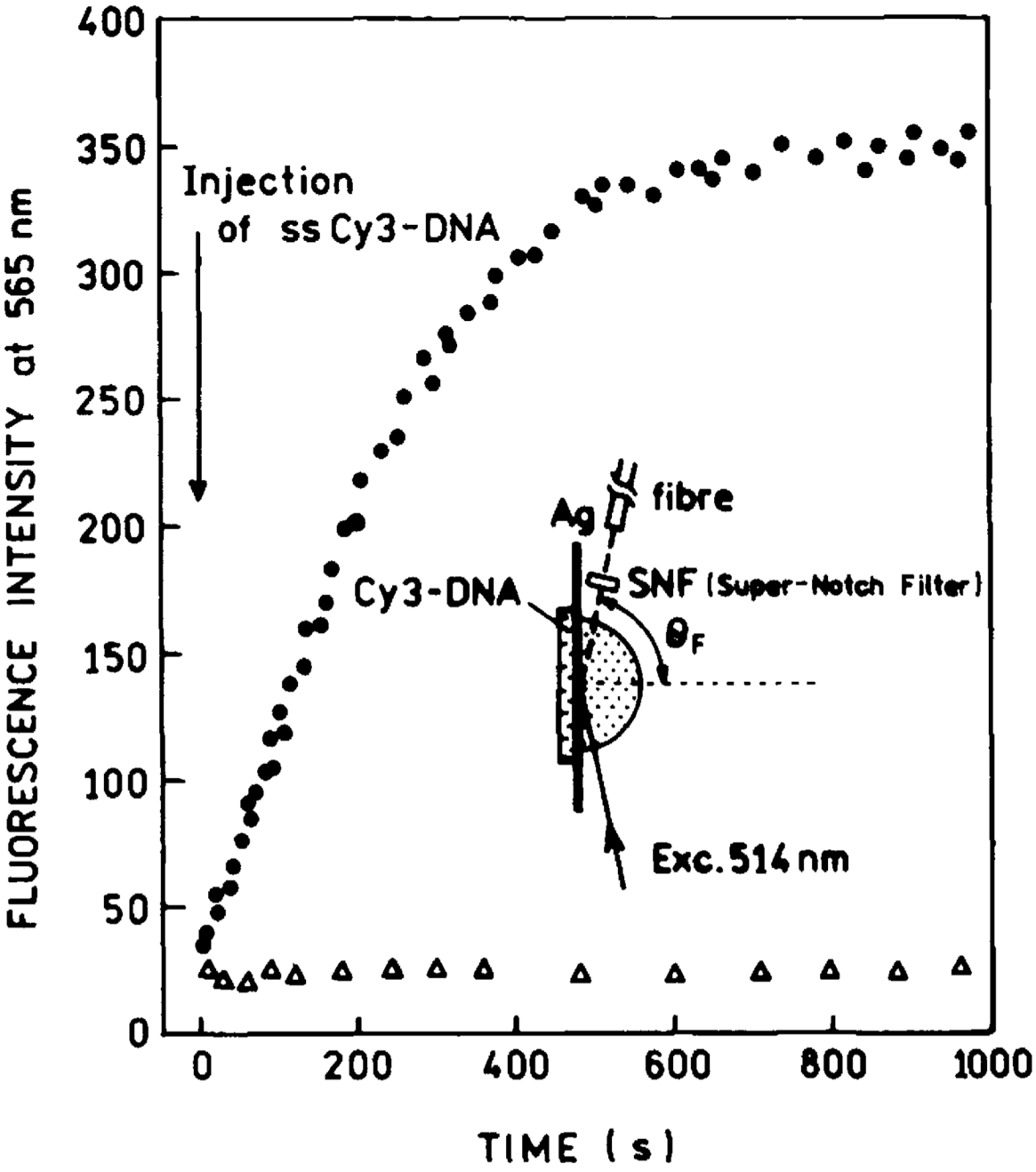


Figure 3.
SPCE fluorescence observed at 565 nm (Cy3–DNA emission) upon injection of a ssCy3–DNA in the presence (●) and absence (△) of a complementary ssDNA–biotin deposited on the protein-coated Ag 50 nm mirror.

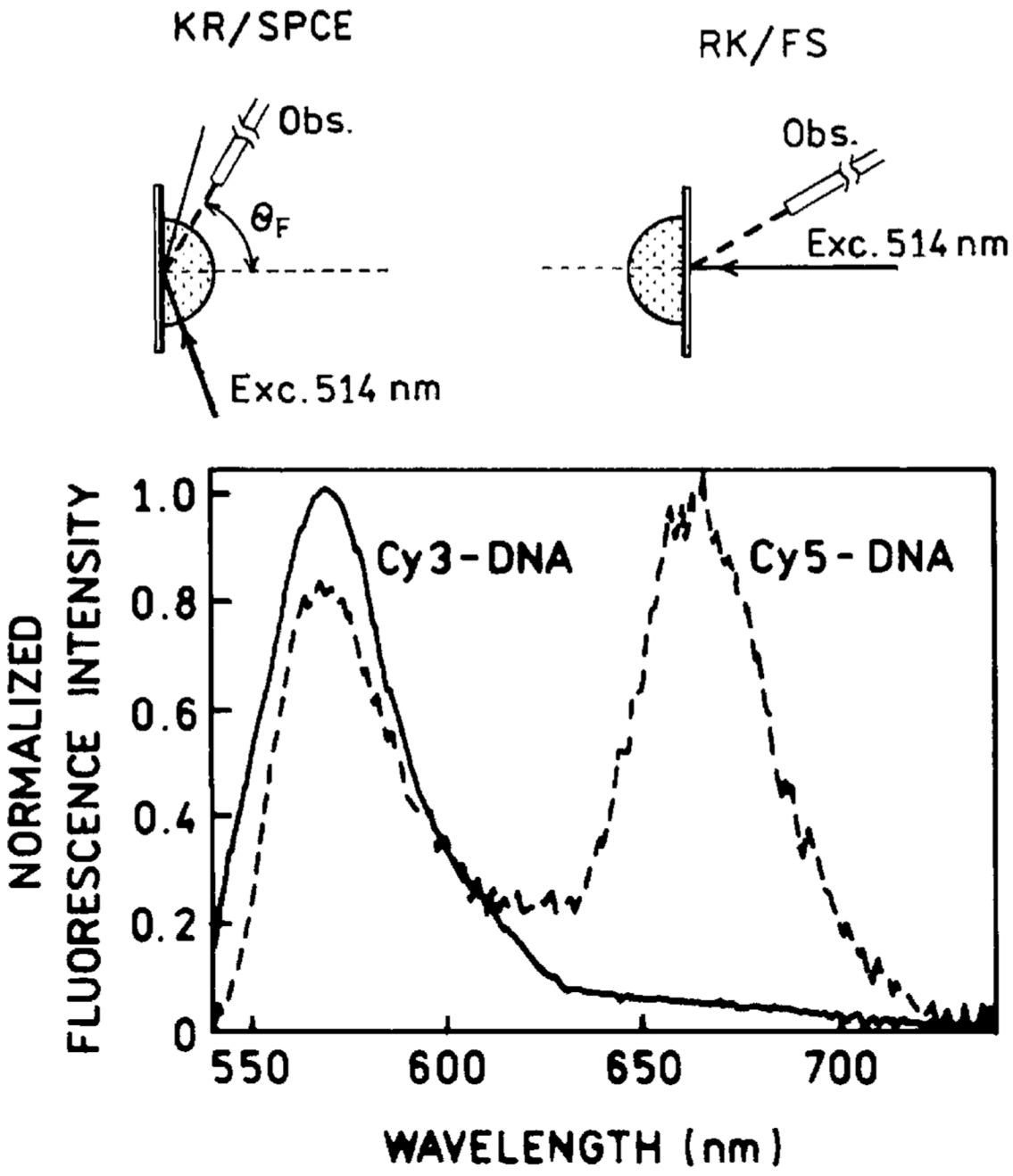
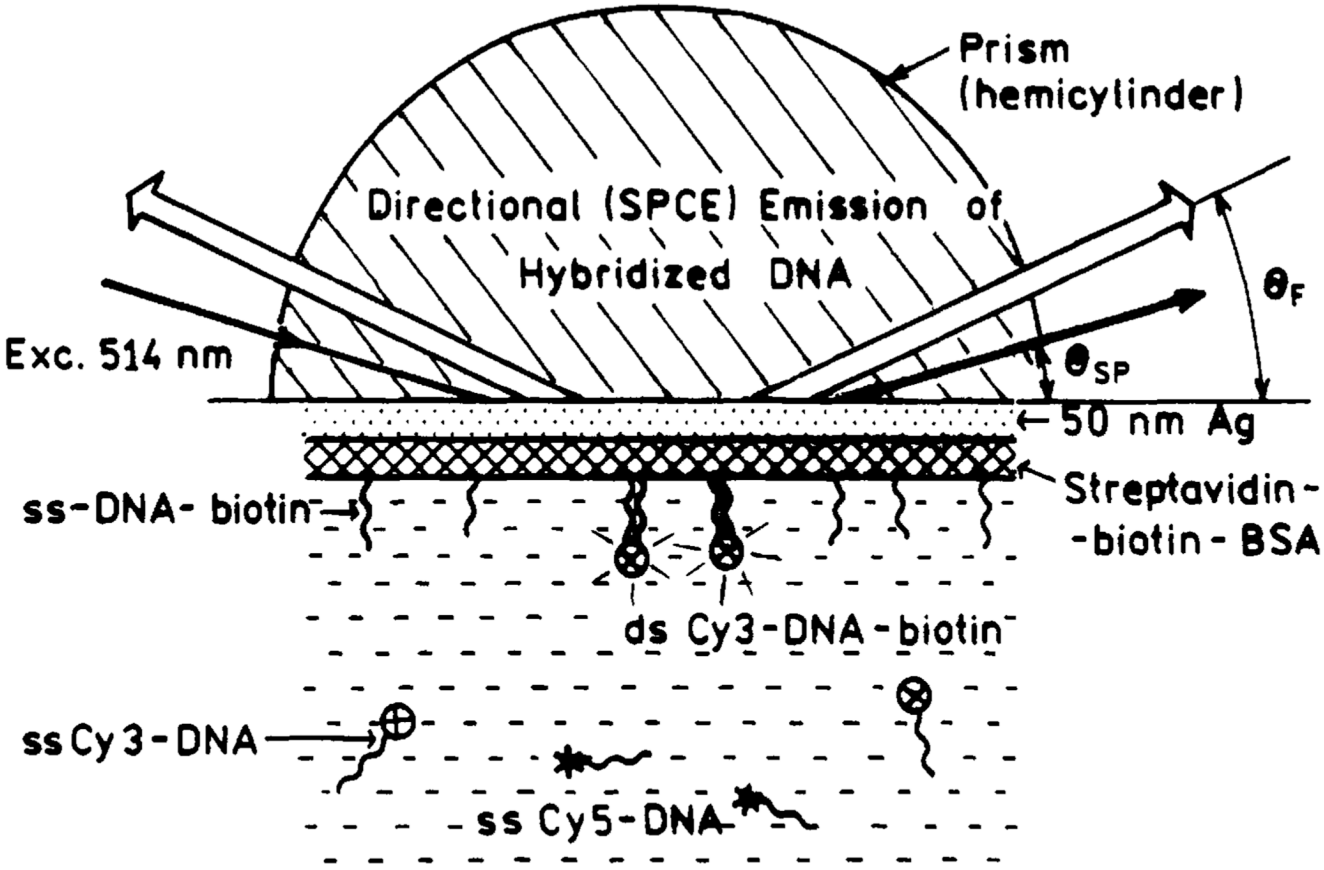


Figure 4.

SPCE spectrum of dsCy3–DNA in the presence of excess of ssCy5–DNA with surface plasmon (KR) excitation (−). Also shown is a free-space emission observed in RK configuration. [dsCy3–DNA] = 5.4×10^{-9} M, and [ssCy5–DNA] = 150×10^{-9} M.



Scheme 1.

Intuitive Description of Directional Fluorescence Emission from Hybridized DNAa

[a] Figure not drawn to scale. BSA–streptavidin = 90 Å, and ssCy3–DNA = 70 Å.

Cy3-5'-GAA GAT GGC CAG TGG TGT GTG GA-3'

ss Cy3-DNA

3'-CTT CTA CCG GTC ACC ACA CAC CT-5'-**biotin**

ss DNA-biotin

Cy3-5'-GAA GAT GGC CAG TGG TGT GTG GA-3'
3'-CTT CTA CCG GTC ACC ACA CAC CT-5'-**biotin**

ds Cy3-DNA-biotin

Cy5-5'-ATT GTT AT-3'

ss Cy5-DNA

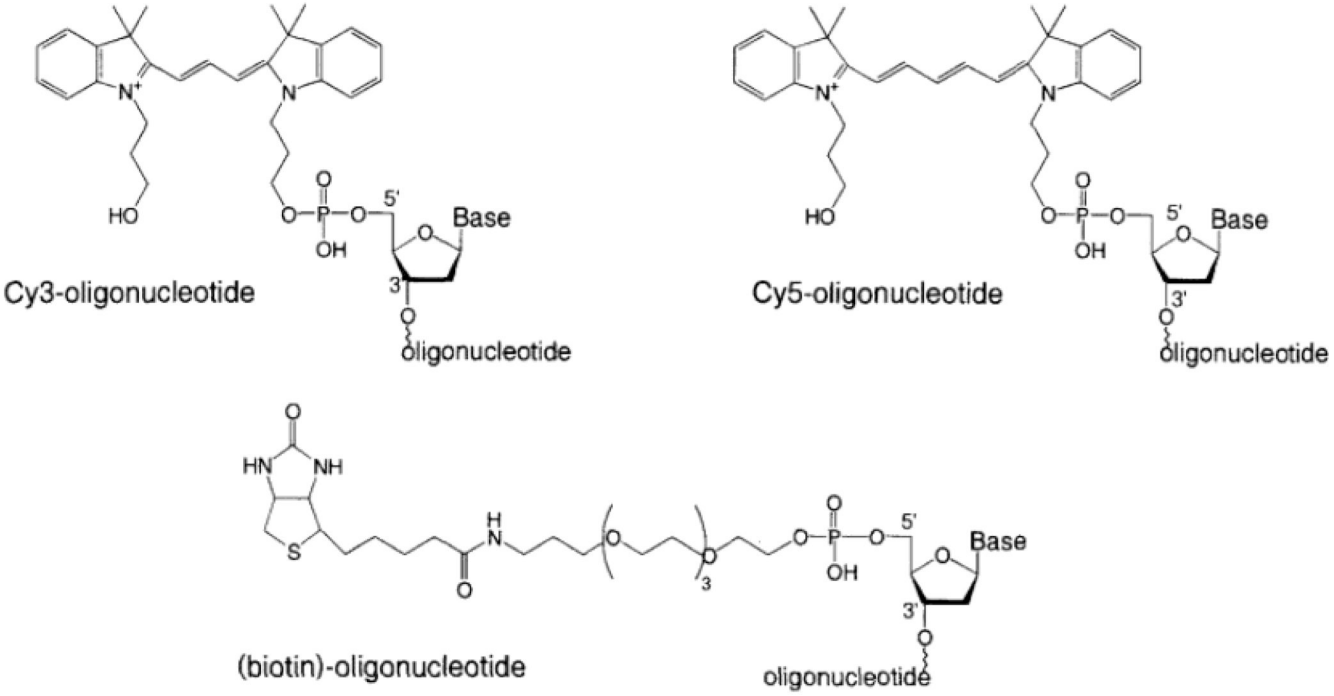


Chart 1.
Structures of Cy3–DNA and Cy5–DNA