

DNA hybridization assays using metal-enhanced fluorescence

Joanna Malicka,* Ignacy Gryczynski, and Joseph R. Lakowicz

*Department of Biochemistry and Molecular Biology, Center for Fluorescence Spectroscopy, University of Maryland at Baltimore,
725 West Lombard Street, Baltimore, MD 21201, USA*

Received 28 April 2003

Abstract

We describe a new approach to DNA hybridization assays using metal-enhanced fluorescence. Thiolated oligonucleotides were bound to silver particles on a glass substrate. Addition of a complementary fluorescein-labeled oligonucleotide resulted in a dramatic time-dependent 12-fold increase in fluorescence intensity during hybridization. Proximity to silver particles resulted in a decreased fluorescence lifetime. This effect is thought to be the result of enhanced fluorescence from fluorescein near metallic silver particles. Hybridization could thus be measured from the decay kinetics of the emission, which can be measured independently from the emission intensity. These results suggest the use of silver particles as a general approach to measure DNA hybridization as a method to increase the sensitivity of DNA detection.

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Keywords: DNA hybridization; Metal-enhanced fluorescence; Silver nanoparticles

Detection of DNA hybridization is the basis of a wide range of biotechnology and diagnostic applications [1]. DNA hybridization is measured on gene chips [2,3], during PCR [4,5] and for fluorescence in situ hybridization [6], to name a few. In all these applications increased sensitivity is desirable, particularly for detection of a small number of copies of biohazard agents. Also, it would be valuable to have a general approach to detect the changes in fluorescence intensity upon hybridization. In general, the detectability of a fluorophore is determined by two factors, the extent of background emission from the sample and the photostability of the fluorophore. A highly photostable fluorophore undergoes about 10^6 excitation–relaxation cycles prior to photobleaching, yielding about 10^3 – 10^4 measured photons per fluorophore [7,8]. Background emission from the samples can easily overwhelm weak emission signals.

In the present report we describe a simple approach which should provide a readily measurable change in fluorescence intensity in many DNA hybridization formats. Additionally, our approach increases the intensity relative to the background and increases the number of

detected photons per fluorophore molecule by a factor of 10-fold or more. This method is based on our recent studies of metal-enhanced fluorescence (MEF) which showed that proximity of fluorophores to metallic silver particles increases the brightness and photostability of many commonly used fluorophores [9–13]. We now show how this technology can be applied to DNA hybridization assays.

Materials and methods

Sample preparation. All oligonucleotides (Fig. 1) were obtained from the Biopolymer Core Facility at the University of Maryland, School of Medicine. Nanopure H_2O ($>18.0\text{ M}\Omega$), purified using Millipore Milli-Q Gradient System, was used for all experiments. All other compounds were purchased from Sigma–Aldrich (St. Louis, MO) and used without additional purification.

The quartz slides used for silver deposition were first cleaned overnight by soaking in 10:1 (v/v) mixture of H_2SO_4 (95–98%) and H_2O_2 (30%). Use of this cleaning solution requires extreme caution to avoid organic materials. After washing with ultrapure water, the quartz surface was coated with amino groups by dipping the slides in a 1% aqueous solution of 3-aminopropyltriethoxysilane (APS) for 30 min at room temperature. The slides were washed extensively with water and air-dried. Silver island film (SIF) deposition was accomplished as described previously [10–13]. Briefly, ten drops of fresh 5% NaOH solution were added to a stirring silver nitrate solution (0.25 g

* Corresponding author. Fax: 1-410-706-8408.

E-mail address: aska@cfs.umbi.umd.edu (J. Malicka).

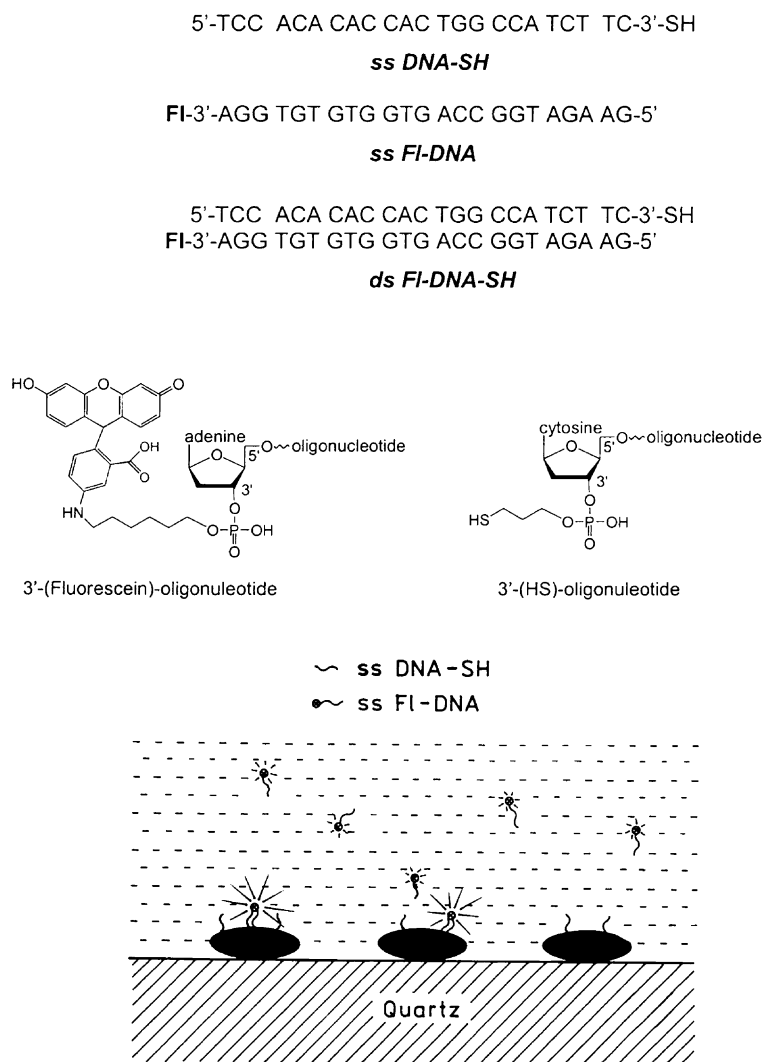


Fig. 1. Structures of DNA oligomers. The lower panel shows a schematic of the oligomers bound to silver particles.

in 35 ml of water). One ml of ammonium hydroxide was added drop by drop to redissolve the dark-brown precipitate. The solution was cooled to 5 °C in an ice bath and a fresh solution of D-glucose (0.36 g in 10 ml of water) was added, followed by placing four pairs of APS-coated quartz slides into the solution. The mixture was stirred for 2 min in ice bath and then allowed to warm up to 30 °C for the next 5 min. As the color of the mixture turned from yellow-greenish to yellow-brown the color of the slides became greenish. The slides were removed from the beaker, rinsed with water, and bath sonicated for 1 min at room temperature. Only one side of each slide was coated with silver islands. All free amino groups remaining on the quartz surface were blocked with succinic anhydride placing them into the freshly prepared solution of 0.111 g succinic anhydride in 7 ml of 1-methyl-2-pyrrolidone and 0.77 ml of 0.2 M sodium borate buffer, pH 8. After 15 min incubation at room temperature slides were washed in three changes of water.

The quartz slides described above formed one side of a 0.2 mm demountable cuvette (12.5 mm × 45 mm; Starna Cells, Atascadero, CA). Monolayers of thiol-derivatized oligonucleotides deposited on silver islands were prepared by placing each slide in 3 ml of 5 nM solution of ss DNA-SH in 5 mM Hepes (pH 7.5), 0.1 M KCl, and 0.25 mM EDTA buffer in 1 cm² cuvette for 48 h at 5 °C, followed by washing three times using the same buffer. The concentrations of thiol-derivatized oligonucleotides and the fluorescein-labeled oligonucleotide were determined

using $\epsilon(260 \text{ nm}) = 225,000 \text{ M}^{-1} \text{ cm}^{-1}$ for ss DNA-SH and $\epsilon(495 \text{ nm}) = 76,000 \text{ M}^{-1} \text{ cm}^{-1}$ for ss FI-DNA (pH 9). To estimate the oligo surface coverage on silver island area we incubated one of the slides in 3 ml of 5 nM solution of previously hybridized FI-DNA-SH in above buffer. By measuring the decrease in emission of double-stranded FI-DNA-SH in solution we calculated the surface coverage is about 0.36 pmol/cm². We prepared a 18 nM solution of complementary oligonucleotide ss FI-DNA in hybridization buffer (5 mM Hepes (pH 7.5), 0.1 M KCl, and 0.25 mM EDTA). This 18 nM concentration of ss FI-DNA in 0.2 mm cuvette could result in maximum surface coverage 0.36 pmol/cm². For kinetic hybridization measurements we filled the 0.2 mm demountable cuvette with 18 nM solution of complementary oligomers.

Fluorescence measurements. Emission spectra from DNA-silver island layers were collected using front face geometry on a SLM 8000 spectrofluorometer with 470 nm excitation from a Xenon lamp. For photostability measurements we used 514 nm excitation from argon ion laser. Lifetimes were measured on 10 GHz frequency-domain fluorometer [14] using mode-locked argon ion laser 514 nm, 76 MHz repetition rate. The emission was selected with a combination of 520 nm long pass liquid chromate filter (CrO₄²⁻/Cr₂O₇²⁻, 0.3 M, pH 8) and an interference filter centered at 540 nm. For frequency-domain measurements the excitation was vertically polarized and the emission was observed through a polarizer oriented at 54.7° from the vertical

position. The FD intensity decays were analyzed in terms of the multi-exponential model

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i),$$

where τ_i are the lifetimes with amplitudes α_i and $\sum \alpha_i = 1.0$. The contribution of each component to the steady-state intensity is given by

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j}.$$

The amplitude-weighted lifetime is given by

$$\langle \tau \rangle = \sum_i \alpha_i \tau_i.$$

The value of $\langle \tau \rangle$ is proportional to the area under an intensity decay curve. If the α_i values are not normalized, and the data are collected under identical conditions, then the value of $\langle \tau \rangle$ should be proportional to the relative steady-state intensities.

Results

Description of studied system

Fig. 1 (top) shows the sequence and structure of the oligomers used in these experiments. The thiolated oligonucleotide ss DNA-SH was used as the capture sequence which bound spontaneously to the silver particles. The sample containing the silver-bound DNA was positioned in a fluorometer (Fig. 2, bottom) fol-

lowed by addition of 18 nM ss FI-DNA, which is an amount approximately equal to the amount of silver-bound capture DNA.

Emission intensity and spectra

The fluorescence intensity began to increase immediately upon mixing and leveled off after about 20 min (Fig. 2, top). We believe this increase in intensity is due to localization of ss FI-DNA near the silver particles by hybridization with the capture oligomer (Fig. 1, bottom). Since metallic silver particles can increase the emission intensity of many fluorophores [10–13] this result suggests that localization of labeled oligomers near silver particles can be used in a wide range of hybridization assays. In control experiments we hybridized ss DNA-SH with ss FI-DNA prior to deposition on silver particles. We found a similar 12-fold increase in intensity upon immobilization on silver as compared to an equivalent amount of ds FI-DNA-SH in solution.

We examined the emission spectra of ss FI-DNA before and after hybridization to form ds FI-DNA-SH (Fig. 3). The fluorescence intensity was found to be 12-fold higher for the bound form.

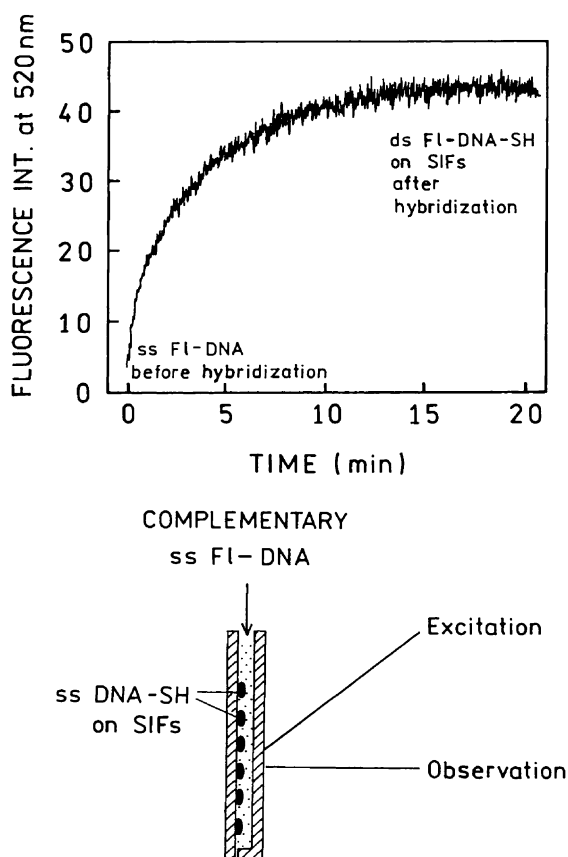


Fig. 2. Time-dependent hybridization of ss FI-DNA to ss DNA-SH. The lower panel shows the sample configuration.

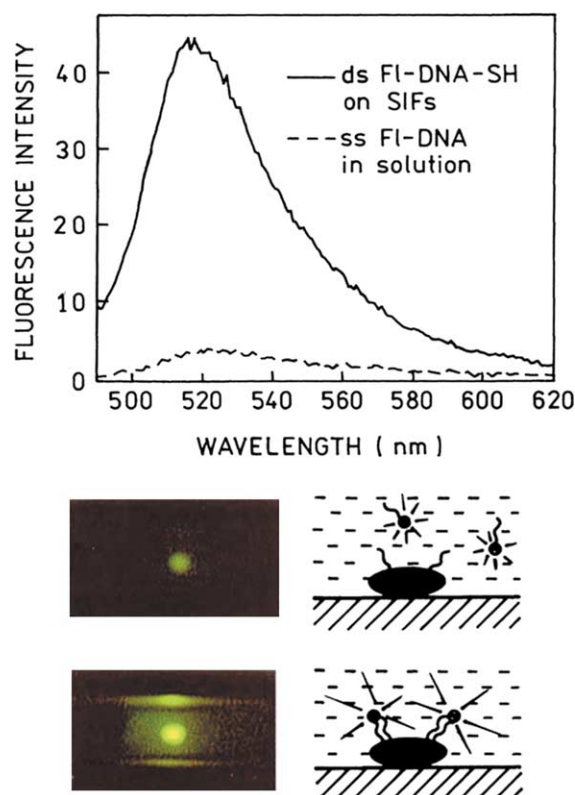


Fig. 3. Emission spectra of ss FI-DNA in solution (dashed line) and bound (solid line) to silver particles. Roughly the same number of molecules of ss FI-DNA and ds FI-DNA-SH was in the illuminated area. The lower panel shows photographs of ss FI-DNA in solution (top) and ds FI-DNA-SH on SIFs (bottom).

This dramatic increase can be seen visually by the photographs in Fig. 3 bottom. There was no detectable shift in the emission spectra. The intensity increase was reversed by melting the DNA at 80 °C (Fig. 4) and increased once again upon cooling and presumed rehybridization. The intensity did not recover completely upon slow cooling, which may be due to loss of capture DNA from the silver surfaces.

Time-resolved study

It is well known that fluorescent intensities can change as a result of numerous factors such as the local

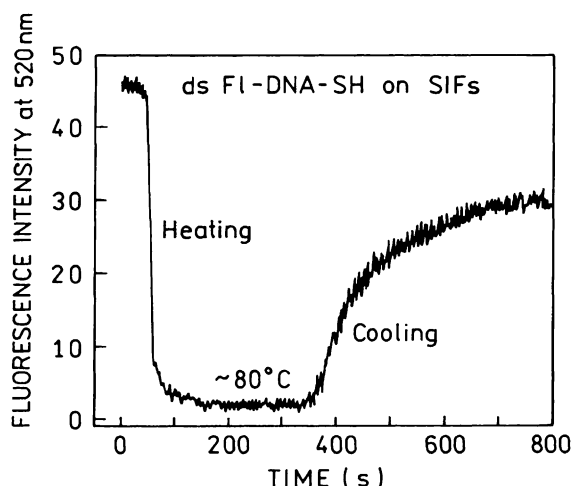


Fig. 4. Thermal melting of ds FI-DNA-SH on a SIF near 80 °C and rehybridization of FI-DNA-SH on SIFs.

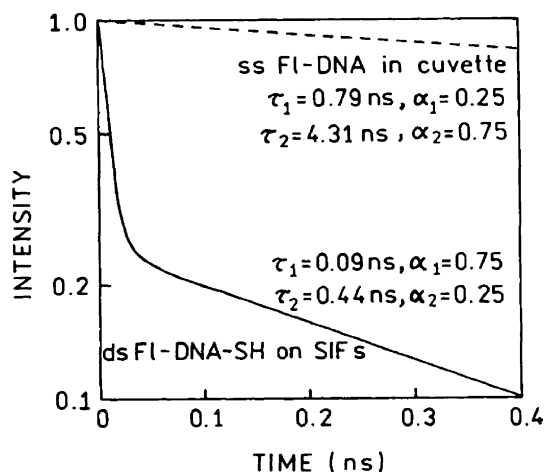
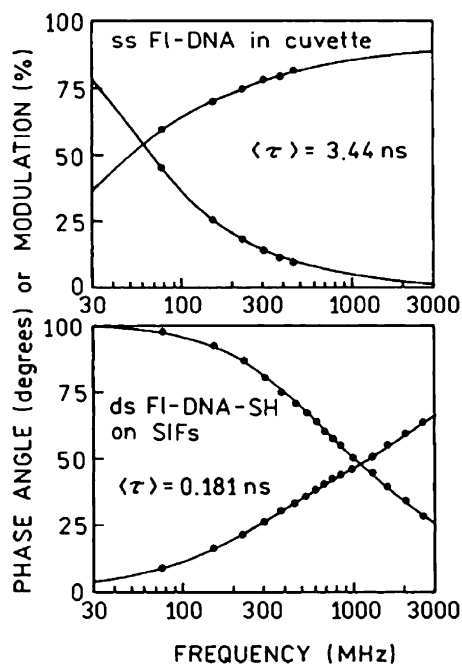


Fig. 5. Intensity decays of ss FI-DNA in solution and ds FI-DNA-SH on SIFs measured in the frequency-domain (left) and reconstructed in the time-dependent decays (right).

polarity or presence of nearby quenching groups [15]. Typically the intensities and lifetimes change in the same direction, that is, both increased or decreased in unison. In contrast, for MEF, the lifetime decreases as the intensity increases [9,10]. This unusual effect is due to a change in the radiative decay rate of the fluorophores, which depends on the photonic mode density near the fluorophore [16,17]. Fig. 5 shows the frequency-domain intensity decays of the single-stranded fluorescein-labeled oligomer in solution and the double-stranded oligomer when bound to silver particles. The lifetime is dramatically shortened for the silver-bound oligomer, which strongly supports our conclusion that the intensity increase is due to localization of the fluorophore near the silver surfaces. In control experiments we found that emissions of fluorescein in the single- and double-stranded oligos were similar to within 10%. The double-stranded form displayed an approximate 10% smaller intensity. Hence the differences in intensity and lifetime between the solution and silver-bound forms are not due to effects of hybridization on the fluorescein probe. It is interesting to note that there is no detectable 4 ns component for the sample with silver particles, indicating that all the emission is due to the silver-bound DNA.

Phase-modulation study of hybridization

The differences in lifetime of fluorescein between the solution and silver-bound form suggested an alternative approach to measuring hybridization. We used the frequency-domain instrument [14] to measure the emission phase angle and modulation. These values depend on

the fluorescence lifetime and were thus expected to change upon hybridization. These measurements revealed a rapid decrease in phase angle and increase in modulation following addition of ss FI-DNA to the silver-bound capture oligomer (Fig. 6). These changes are due to the decrease in lifetime upon binding to the capture oligomers on the silver particles. The changes in phase and modulation (Fig. 6) occur somewhat more rapidly than the change in intensity (Fig. 2). This difference occurs because the phase and modulation are intensity-weighted parameters [15]. It is important to note that phase angle and modulation measurements are mostly independent of total intensity, to within the limitations of the instrumentation and the extent of background fluorescence in the sample [18]. This is important because the extent of hybridization can be measured using optical components such as optical fibers where the intensity may vary, or in micro well plates where the well-to-well intensity may vary due to the plate or adsorbing species in the sample.

Photostability

It is known that proximity to silver particles can result in increased rates of excitation [19] as well as in-

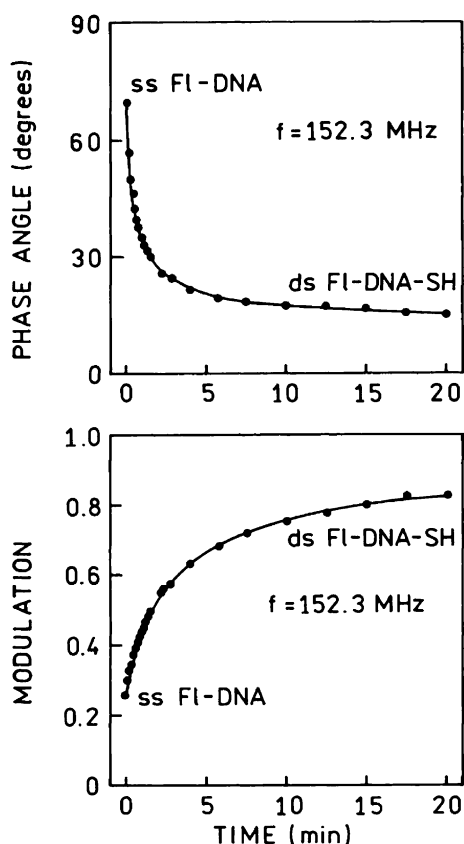


Fig. 6. Phase angle (top) and modulation (bottom) of ss FI-DNA upon hybridization to silver-bound ss DNA-SH. The light modulation frequency and detection frequency were 152.3 MHz.

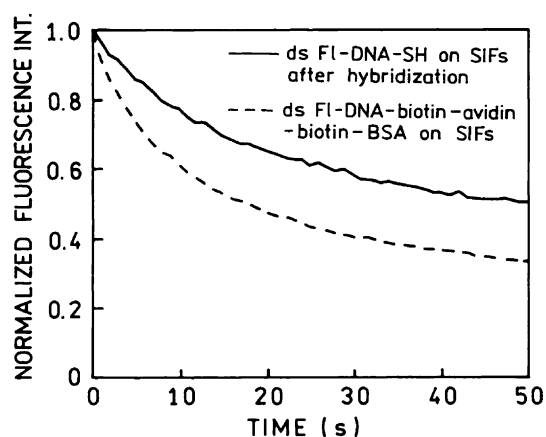


Fig. 7. Photostability of ds FI-DNA-SH bound to SIFs (solid line). For comparison we show the photostability of a similar biotinylated oligomer bound to a protein monolayer of BSA-biotin-avidin uniformly deposited on SIFs (dashed line). In this case some of FI-DNA-biotin are distant from and not affected by the silver particles.

creased quantum yields. An increased rate of excitation may not be useful if the fluorophore photobleaches more rapidly. We examined the intensity of FI-DNA-SH on the silver surfaces with continuous illumination (Fig. 7). As a comparison we examined a similar biotinylated oligomer bound to a silvered quartz slide using biotinylated bovine serum albumin and avidin [12]. We found that fluorescein in our samples deposited on silver particles photobleaches more slowly than when deposited on a protein monolayer. It should be noted that with the protein monolayer the FI-DNA was uniformly deposited on entire silvered slide, that is, also between silver particles. The lifetimes measured for sample on protein monolayer contained a long, about 4 ns component [12]. This component indicates that there is some fluorescein bound to the glass surface between the silver particles. If the increased intensity was mostly due to an increased rate of excitation near silver particles then we expect slower photobleaching with the protein monolayers when there are regions without an increased rate of excitation.

Discussion

In our opinion, metal-enhanced fluorescence is useful for a wide range of DNA analysis formats. A metal-bound oligomer could serve as a capture probe. A labeled oligomer could bind to both the capture DNA and the target sequence. Presence of the target sequence would bring the labeled oligomer closer to the silver and enhance its fluorescence. Alternatively, hybridization of the target and labeled oligomers could be performed in solution followed by hybridization to a capture oligomer bound to the silver particles. Depending upon the needed sensitivity the target DNA could be at its natural

abundance or amplified with PCR. Silver is non-toxic and insensitive to thermal cycling. Silver may tarnish with long term storage, but fresh surfaces can be created by simple chemical treatments, or the surfaces may be coated to minimize reactions with air. It appears that the use of silver and metal-enhanced fluorescence can be rapidly introduced into DNA arrays, PCR, and similar technologies.

Acknowledgments

This work was supported by the NIH National Center for Research Resources, RR-08119, HG-002655, EB-000682, and EB-00981. The authors thank Dr. Zygmunt Gryczynski for taking photographs used in Fig. 3.

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