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Near-Infrared Heavy-Atom-Modified Fluorescent Dyes for Base-Calling in DNA-Sequencing Applications Using Temporal Discrimination

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A series of near-IR fluorescent dyes were prepared which contained an intramolecular heavy atom for altering the fluorescence lifetimes to produce a set of probes appropriate for base-calling in a single-lane DNA sequencing format. The heavy-atom modification consisted of an intramolecular halogen situated on a remote section of the chromophore in order to minimize the perturbation on the lifetimes and fluorescence quantum yields. In addition, the dye series possessed an isothiocyanate functional group to allow facile attachment to sequencing primers. The unconjugated dyes showed similar absorption and emission maxima (λ_{abs} = 765−768 nm; λ_{em} = 794−798 nm) as well as fluorescence quantum yields that were invariant, within experimental error, with the heavy atom. However, the lifetimes of these dyes were found to vary with the identity of the halogen substitution (I, τ_f = 947 ps; F, τ_f = 843 ps, measured in methanol), with an average variation within the dye series of 35 ps. The spectroscopic properties of the free dyes and the dyes conjugated to sequencing primers on the 5′-end of the oligonucleotide were determined in a DNA-sequencing matrix (denaturing gels containing formamide). The results indicated slight differences in the fluorescence properties of the free dyes compared to those of the dye/primer conjugates in this particular matrix. Inspection of the ground-state absorption spectra showed significant aggregation for the free dyes in this solution, but the conjugated dyes exhibited no sign of aggregation due to the highly anionic nature of the oligonucleotide. The fluorescence lifetimes of the dye/primer conjugates demonstrated lifetimes which ranged from 735 to 889 ps, with an average variation of 51 ps, an adequate difference to allow facile discrimination of these dyes in DNA-sequencing conditions. In addition, the free solution electrophoretic mobilities of the native heavy-atom-modified dyes were found to be very similar. When the dye/primer conjugates were electrophoresed in a cross-linked polyacrylamide gel electrophoresis capillary column, they comigrated, indicating that, in single-lane sequencing applications, when utilizing these dyes, no postrun corrections would be required to correct for dye-dependent mobility shifts.

The use of fluorescence detection in DNA sequencing has become the preferred method due to the fact that it can provide on-line analysis, possesses limits of detection comparable to those of radiography, is easily integrated to the microgel separation techniques, and can potentially allow the base-calling to be performed in a single separation lane using spectral discrimination.[1−3] The ability to perform the separation and base-calling in a single lane has become critically important, in light of the Human Genome Project, where throughput issues are a prime concern.

The commonly used approach for single lane base-calling in DNA sequencing applications using fluorescence is spectral discrimination, where a set of four spectrally distinct chromophores, which can be attached to either the sequencing primer or the dideoxynucleotide, are identified on the basis of unique emission maxima.[1] The dyes typically used are the fluorescein and/or rhodamine derivatives, which contain structural modifications to alter the absorption and emission maxima to allow efficient discrimination.[1,3,4] An ideal property of the chromophore set is similar absorption maxima, to allow excitation with a single excitation source, but widely spaced emission maxima, to permit efficient sorting onto the appropriate detection channel. A set of commercial dyes are available (6-FAM, NED, TAMRA, and ROX) which nearly possess the aforementioned characteristics.[5] These dyes can be excited with the 488- or 514-nm lines from an argon ion laser and then spectrally isolated using a series of optical filters onto appropriate photodetectors.[3,6] Some of the potential difficulties with spectral discrimination include the need for multiple

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excitation sources in some cases and multiple detection channels, cross-talk between detection channels due to the broad emission profiles associated with the chromophores, and dye-dependent electrophoretic mobilities of the labeled oligonucleotides.

A method to eliminate the need for multiple detection channels has been to use rotating filter wheels.[3] Here, only one detection channel is required, but, due to decreases in the duty cycle, losses in the signal-to-noise ratio can result, producing errors in base-calling. Losses in signal-to-noise ratio can be particularly troublesome when utilizing micro-CE-based systems for DNA sequencing, where the injection volume can be on the order of several tens of picoliters.[7−9] In addition, this method still may require the need for multiple excitation sources, and dye-dependent electrophoretic mobilities are present. Another approach to eliminate the need for multiple detection channels has been to incorporate only one or two dyes and then use intensity variations to identify the terminal base.[3,10,11] The intensities of the resulting electrophoretic peaks are controlled by adjusting the molar concentration of the ddNTP during DNA polymerization. The difficulties associated with this approach arise from nonuniformities in the incorporation efficiencies of the ddNTPs during polymerization and the inability to use dye-labeled terminators.

To eliminate the need for multiple excitation sources, the use of fluorescence-energy-transfer probes have been utilized in single-lane DNA-sequencing applications.[12−14] In this approach, a donor (FAM) is attached to the 5′-end of a sequencing primer, and an acceptor (FAM, JOE, TAMRA, ROX) is covalently bound to a modified thymidine residue eight or nine bases down the sequence. Since the primers utilize Förster resonance energy transfer, only a single excitation source is required (488 nm), with the emission sorted onto appropriate detectors. Due to the structural requirements for efficient energy transfer and the need for two dyes per primer, this method will be difficult to adapt in primer walking strategies, and the use of dye-labeled dideoxynucleotides can be problematic. In addition, concerns with dye-dependent mobility shifts are present.

Recently, a set of electrophoretically uniform fluorescent dyes for DNA sequencing have been reported.[15] The dyes, which are BODIPY derivatives, are attached to sequencing primers (5′-end) via a unique linker structure that produces excellent sequencing data without software correction for dye-dependent mobility shifts. In addition, the dye−primer set yields narrower spectral emission bandwidths compared to those of conventional dye−primer sets, resulting in smaller amounts of cross-talk between detection channels. However, as with the energy-transfer dyes, this approach will not easily be amenable to primer walking strategies or dye-labeled dideoxynucleotide DNA sequencing.

As an alternative to spectral discrimination, various groups have suggested that fluorescence lifetimes can potentially serve as a viable method for base-calling in DNA-sequencing applications.[16−18] The principal advantages associated with lifetime discrimination for base-calling are the following: (1) since the calculated lifetime is immune to concentration differences, dye-labeled terminators can potentially be used as well as dye−primers, with a wide choice in polymerase enzymes to suit the particular sequencing application; (2) lifetime values can be determined with higher precision than fluorescence intensities under appropriate conditions, improving the accuracy in base-calling; (3) lifetime determinations do not suffer from broad emission profiles associated with spectral discrimination; and (4) the fluorescence can potentially be processed on a single detection channel without the need for spectral sorting to multiple detection channels. Several problems do arise when considering lifetime discrimination for DNA sequencing, especially when utilizing microseparation techniques such as capillary gel electrophoresis. The most pervasive problem is associated with the complex instrumentation required for lifetime determinations. For example, in time domain techniques, a pulsed laser is required with a fast detector, typically a microchannel plate photomultiplier tube, and sophisticated counting electronics. In addition, poor photon statistics (low number of photocounts in decay profile) produced from low loading masses and the transient nature of the signal, determined by the width of the electrophoretic band (1−10 s), can produce poor precision in the measurement. The poor precision would also be compounded by the presence of large amounts of scattering and impurity photons included in the decay profile. Finally, complex algorithms are required for abstracting the lifetime from the decay profile, making on-line determinations during electrophoresis difficult.

Many of these concerns associated with lifetime determinations for base-calling in DNA sequencing have been addressed using near-IR fluorescence. For example, several groups have demonstrated that semiconductor diode lasers, which can be operated in a pulsed mode and lase between 680 and 800 nm in conjunction with single-photon avalanche diodes (SPADs), can produce a time-correlated single-photon-counting apparatus that is simple to operate, with performance characteristics comparable to those of visible devices, using mode-locked Nd:YAG lasers and microchannel plates.[19−22] In addition, we have recently demonstrated that lifetime measurements can be acquired in the near-IR using a solid-state device and counting electronics situated on a PC

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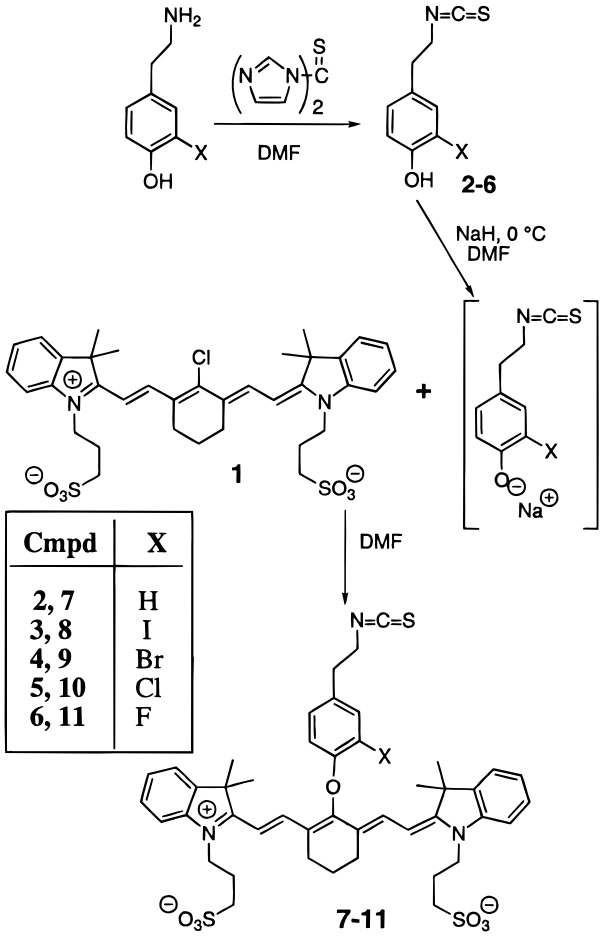
board.[23] We have also shown that simple algorithms can be used to calculate fluorescence lifetimes on-line during free solution or gel capillary electrophoresis using near-IR time-resolved fluorescence.[17] The standard deviation in the lifetime measurement of C-terminated fragments labeled with a near-IR dye was found to be approximately ±9 ps with decay profiles constructed from as few as 5000 photocounts. The high precision resulted primarily from the fact that, in the near-IR, the low scattering cross sections and the minimal number of intrinsically fluorescent components produced low numbers of interfering photocounts in the decay.

Recently, a set of rhodamine chromophores have been prepared which possess appropriate characteristics for lifetime-based base-calling in DNA-sequencing applications.[24−26] These dyes absorb radiation in the deep red (~650 nm) and have lifetimes which range from 1.7 to 4.0 ns. However, the individual dyes in the series are structurally unique, resulting in slightly different absorption and emission maxima as well as unique mobilities, requiring postrun correction in the electrophoresis for dye-dependent mobility shifts on the oligonucleotide.

In this work, we report on the preparation, photophysical characterization, and electrophoretic mobility studies of a set of unique fluorophores appropriate for single-lane base-calling for DNA sequencing using lifetime discrimination. The dyes developed for this application are near-IR tricarbocyanine dyes which possess an intramolecular heavy-atom modification. The heavy-atom modification consisted of covalently inserting a halogen (I, Br, Cl, F) into the molecular framework of the dye. The intramolecular heavy atom results in perturbations in the singlet-state photophysics (fluorescence quantum yield, Φ_f, and fluorescence lifetime, τ_f) due to enhanced intersystem crossing into the triplet state resulting from spin−orbit coupling.[27−29] The heavy atom, however, does not alter the absorption or emission maximum of the base chromophore, allowing the fluorescence to be excited with a single source and processed on a single detection channel. In addition, we will show that the heavy-atom modification to the base chromophore for these dyes produces uniform electrophoretic mobilities in capillary gel conditions, negating the need for postrun corrections due to dye-dependent mobility shifts.

EXPERIMENTAL SECTION

Chemicals. Aniline, cyclohexanone, *N*,*N*-dimethylformamide (DMF), propane sultone, 2-iodophenol, 2-bromophenol, 2-chlorophenol, 2-fluorophenol, 1,1-thiocarbonyldiimidazole, *m*-chloroperbenzoic acid, tetrabutylammonium tribromide, 1-(chloromethyl)-4-fluoro-1,4-diazoniabicyclo[2.2.2]octane bis(tetrafluoroborate), and sodium hydride were purchased from Aldrich Chemical Co. (Milwaukee, WI). Phosphorous oxychloride and 2,3,3-trimethylindoline were purchased from Kodak Co. (Rochester, NY). Riboflavin and tyramine were obtained from Sigma Chemical Co. (St. Louis, MO). All other solvents were purchased from Fisher Scientific Co. (Pittsburgh, PA) and used as received.



Cmpd	X
2, 7	H
3, 8	I
4, 9	Br
5, 10	Cl
6, 11	F

Figure 1. Synthetic scheme for the preparation of the isothiocyanate phenol intermediates (**2**−**6**) and the near-IR heavy-atom-modified dyes (**7**−**11**).

Dye Synthesis and Purification. The structures of the near-IR heavy-atom-modified dyes are shown in Figure 1, along with a general scheme for their synthesis. The synthesis of dye **1** was carried out using previously published procedures.[30−32] The heavy-atom-modified near-IR dyes containing the isothiocyanate functional group were synthesized by first preparing the appropriate phenol and adding this adduct to **1**. The synthesis of each heavy-atom-modified *p*-isothiocyanatoethylphenol along with the characteristic MS and NMR data are outlined below.

*Synthesis of 2-(p-Hydroxyphenyl)ethyl Isothiocyanate (**2**).* Tyramine (400 mg, 2.9 mmol) was dissolved in anhydrous DMF (10 mL) under an N_2 atmosphere. 1,1-Thiocarbonyldiimidazole (520 mg, 2.9 mmol) was added, and the pale yellow solution turned an

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amber red. After 30 min, the solvent was taken off by rotary evaporation at 40 °C to produce an orange oil. The oil was dissolved in CH_3OH/H_2O (1:3, 4 mL) to precipitate the isothiocyanate. The product was filtered and washed with cold water, to afford 320 mg of **2** (62%). GC/MS: calcd for C_9H_9NOS, 180.0 (M^+), found, 180.0. ^{1}H NMR (DMSO-d_6, 250 MHz): δ 7.67 (s, 1H), 7.05 (d, 2H, J = 8.1 Hz), 6.70 (d, 2H, J = 8.6 Hz), 3.80 (t, 2H, J = 6.6 Hz), 2.81 (t, 2H, J = 6.6 Hz). IR: 2081 cm^{-1} (N═C═S).

*Synthesis of 2-(3-Iodo-4-hydroxyphenyl)ethyl Isothiocyanate (**3**).* According to a procedure outlined by Evangelatos et al.,[33] tyramine (1.34 g, 0.01 mol) was dissolved in NH_4OH (200 mL) with stirring. A 0.96 N solution of I_2 (21 mL, 0.02 mol) was slowly added into the reaction solution from a pressure-equalizing addition funnel. After 1.5 h, the solvent was concentrated to 50 mL, and the solution was kept at 4 °C overnight to precipitate a white powder. The precipitate was filtered and dried in vacuo to give 2-(3-iodo-4-hydroxyphenyl)ethylamine. A 750-mg sample of this compound (2.8 mmol) was dissolved in anhydrous DMF (5 mL) under an N_2 atmosphere. 1,1-Thiocarbonyldiimidazole (606 mg, 3.4 mmol) was then added, and the pale yellow solution turned amber. After 24 h, H_2O (20 mL) was added to the mixture, and the product was extracted into CH_2Cl_2 (7 × 50 mL). The extract was dried with $MgSO_4$ and filtered, and the solvent was removed in vacuo to produce an orange, oily mixture which was purified by flash chromatography (solvent, CH_2Cl_2). Pure fractions were pooled and dried in vacuo. The oily residue of **3** was crystallized at 4 °C overnight. Yield: 282 mg (33%). GC/MS: calcd for C_9H_8INOS, 304.9 (M^+), found, 305.0. ^{1}H NMR (CD_3OD, 250 MHz): δ 7.59 (s, 1H), 7.08 (d, 1H, J = 8.2 Hz), 6.78 (d, 1H, J = 8.4 Hz), 3.67 (t, 2H, J = 6.8 Hz), 2.85 (t, 2H, J = 6.6 Hz).

*Synthesis of 2-(3-Bromo-4-hydroxyphenyl)ethyl Isothiocyanate (**4**).* Following a modification of the procedure of Kajigaeshi et al.,[34] tyramine (1.03 g, 7.5 mmol) and $CaCO_3$ (830 mg, 8.3 mmol) were dissolved in CH_2Cl_2/CH_3OH (7:3, 60 mL). After the tyramine had completely dissolved, tetrabutylammonium tribromide (4.0 g, 8.3 mmol) was added with stirring. Immediately, the orange solution turned brown. The reaction was followed by TLC (silica, CH_2Cl_2/CH_3OH 7:3; plates were stained with a ninhydrin solution (1% in butanol)) and was complete after 5 min. The reaction was then filtered and the filtrate washed with Et_2O (5 × 20 mL). The ether was removed, and the residue, 2-(3-bromo-4-hydroxyphenyl)-ethylamine, was used without further purification. This amine was dissolved in anhydrous DMF (5 mL) under an N_2 atmosphere with 1,1-thiocarbonyldiimidazole (1.44 g, 7.5 mmol) added, turning the orange solution an amber color. The reaction was followed by TLC (silica, CH_2Cl_2/CH_3CN 4:1). After 30 min, the solvent was removed on a rotary evaporator at 40 °C. The residue was repeatedly washed with Et_2O (7 × 10 mL). The Et_2O was removed to afford an orange oil which was purified by flash chromatography (silica, CH_2Cl_2/hexane 3:1). The pure fractions were collected, pooled, and dried in vacuo to yield **4**. Yield: 400 mg (21%). GC/MS: calcd for C_9H_8BrNOS, 257.0 (M^+), found, 257.0. ^{1}H NMR (CD_3OD, 250 MHz): δ 7.38 (s, 1H), 7.05 (d, 1H, J = 8.5 Hz), 6.85 (d, 1H, J = 8.6 Hz), 3.69 (t, 2H, J = 6.7 Hz), 2.86 (t, 2H, J = 6.6 Hz).

*Synthesis of 2-(3-Chloro-4-hydroxyphenyl)ethyl Isothiocyanate (**5**).* Following a modification of a procedure outlined by Chung et al.,[35] tyramine hydrochloride (1.0 g, 5.8 mmol) was dissolved in a solution of 1 M hydrochloric acid in anhydrous DMF (9 mL) with stirring. *m*-Chloroperbenzoic acid (1.09 g, 6.3 mmol) was added slowly with stirring, and the solution turned a pale yellow color. After 30 min, the reaction was poured into CH_2Cl_2 (50 mL) with stirring. The resulting precipitate was collected by filtration and washed with CH_2Cl_2 (3 × 10 mL). The resulting white product was dried at 70 °C to produce 2-(3-chloro-4-hydroxyphenyl)-ethylammonium chloride. This product was dissolved in anhydrous DMF (3 mL) under N_2, and 1,1-thiocarbonyldiimidazole (644 mg, 3.6 mmol) was added with stirring. After 30 min, the DMF was removed in vacuo, and the residue was dissolved in CH_2Cl_2 (20 mL). This solution was washed with H_2O (3 × 50 mL) in a 250-mL separatory funnel. The CH_2Cl_2 layer was collected, dried with $MgSO_4$, and filtered. The CH_2Cl_2 was removed in vacuo to afford an oil which was purified by flash chromatography (silica, CH_2Cl_2/CH_3CN 10:1). Pure fractions from the column were collected, pooled, and concentrated in vacuo. Pure product **5** was crystalized at 4 °C. Yield: 167 mg (33%). GC/MS: calcd for C_9H_8ClNOS, 213.0 (M^+), found, 213.0. ^{1}H NMR (CD_3OD, 250 MHz): δ 7.20 (s, 1H), 7.00 (d, 1H, J = 7.9 Hz), 6.87 (d, 1H, J = 8.5 Hz), 3.67 (t, 2H, J = 6.8 Hz), 2.84 (t, 2H, J = 6.7 Hz).

*Synthesis of 2-(3-Fluoro-4-hydroxyphenyl)ethyl Isothiocyanate (**6**).* Following a modification of a procedure outlined by Banks et al.,[36] tyramine hydrochloride (1.0 g, 5.8 mmol) was dissolved in H_2O/CH_3OH (1:1, 26 mL) with stirring. 1-(Chloromethyl)-4-fluoro-1,4-diazoniabicyclo[2.2.2]octane bis(tetrafluoroborate) (2.06 g, 5.8 mmol) was added, and the solution turned a dark brown color. After 18 h, the solvent was taken off in vacuo, and the residue was dissolved in CH_3OH (20 mL) and filtered. The solvent was then removed from the filtrate, and the crude product, 2-(3-fluoro-4-hydroxyphenyl)ethylammonium chloride, was precipitated from CH_3CN (10 mL). This was dissolved in anhydrous DMF (3 mL) under N_2 with stirring, and 1,1-thiocarbonyldiimidazole (286 mg, 1.6 mmol) was added. After 30 min, the reaction was removed from the stirrer, and H_2O (20 mL) was added. The product was extracted from the solution with CH_2Cl_2 (3 × 50 mL). The CH_2Cl_2 layer was collected, dried with $MgSO_4$, and filtered. The CH_2Cl_2 was then removed from the filtrate to give an orange oil. The oil was purified by flash chromatography (silica, CH_2Cl_2/CH_3OH 10:1). The pure fractions were collected, pooled, and dried in vacuo to afford **6** as an oil. Yield: 127 mg (40% of crude). GC/MS: calcd for C_9H_8FNOS, 197.0 (M^+), found, 197.0. ^{1}H NMR (CD_3OD, 250 MHz): δ 6.98 (d, 1H, J = 11.9 Hz), 6.88 (m, 2H), 3.69 (t, 2H, J = 6.6 Hz), 2.87 (t, 2H, J = 6.7 Hz).

Synthesis of Near-IR, Heavy-Atom-Modified Dyes 7−11. Following a procedure outlined by Narayanan and Patonay,[37] a 60% oil dispersion of NaH (14 mg, 0.3 mmol) was added to anhydrous DMF (4 mL) under an N_2 atmosphere at 0 °C. In a separate flask, the appropriate phenol (**2**−**6**, 58 mg, 0.3 mmol) was dissolved in anhydrous DMF (1 mL) under an N_2 atmosphere at 0 °C with this solution added to the slurry of NaH. After 30 min, the

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phenoxide isothiocyanate solution was added to chlorodye **1** (100 mg, 0.14 mmol) dissolved in anhydrous DMF (4 mL) under N_2. The reaction was followed by HPLC. After 18 h, the reaction was quenched with dry ice, and the solvent was removed on a rotary evaporator at 40 °C. The residue was dissolved in CH_3OH/H_2O (1:1, 3 mL), filtered, purified by preparatory HPLC, and dried in vacuo overnight to give **7**−**11**. Typical yield: 30−50 mg (25−45%). The compounds were characterized and checked for purity using FAB-MS, NMR, and FT-IR. The following data were collected for the FAB-MS results: molecular weight calculated for **8**, 966.2 (protonated form, M^+), found, 965.8; calcd for **9**, 920.2, found, 920.8; calcd for **10**, 876.3, found, 876.7; calcd for **11**, 860.3, found, 860.4. The data obtained from the proton NMR characterization were as follows. **8** (CD_3OD, 250 MHz): δ 7.92 (s, 1H), 7.86 (D, 2H, J = 14.1 Hz), 7.37 (m, 5H), 7.19 (m, 4H), 6.73 (d, 1H, J = 8.7 Hz), 6.35 (d, 2H, J = 14.3 Hz), 4.32 (t, 4H, J = 7.3 Hz), 3.71 (t, 2H, J = 5.9 Hz), 2.95 (m, 6H), 2.77 (br t, 2H), 2.21 (m, 4H), 2.04 (br t, 2H), 1.66 (s, 6H), 1.12 (s, 6H). **9** (CD_3OD, 250 MHz): δ 7.89 (d, 2H, J = 14.1 Hz), 7.71 (s, 1H), 7.36 (m, 5H), 7.20 (m, 4H), 6.83 (d, 1H, J = 8.0 Hz), 6.36 (d, 2H, J = 14.0 Hz), 4.32 (t, 4H, J = 7.5 Hz), 3.74 (t, 2H, J = 5.9 Hz), 2.95 (m, 6H), 2.80 (br t, 4H), 2.21 (m, 4H), 2.05 (br t, 2H), 1.63 (s, 6H), 1.13 (s, 6H). **10** (CD_3OD, 250 MHz): δ 7.89 (d, 2H, J = 14.2 Hz), 7.56 (s, 1H), 7.37 (m, 5H), 7.20 (m, 4H), 6.86 (d, 1H, J = 8.1 Hz), 6.36 (d, 2H, J = 14.3 Hz), 4.33 (t, 4H, J = 7.5 Hz), 3.75 (t, 2H, J = 5.8 Hz), 2.95 (m, 6H), 2.81 (br t, 4H), 2.21 (m, 4H), 2.04 (br t, 2H), 1.29 (br s, 12 H). **11** (CD_3OD, 250 MHz): δ 7.96 (d, 2H, J = 14.0 Hz), 7.37 (m, 5H), 7.23 (m, 4H), 7.00 (d, 1H, J = 8.7 Hz), 6.90 (d, 1H, J = 8.1 Hz), 6.36 (d, 2H, J = 14.0 Hz), 4.33 (t, 4H, J = 7.5 Hz), 3.76 (t, 2H, J = 6.5 Hz), 2.94 (m, 6H), 2.80 (br t, 4H), 2.21 (m, 4H), 2.04 (br t, 2H), 1.35 (s, 12H). The isothiocyanate near-IR dyes were stored as dry powders at −20 °C in the dark until required for labeling reactions.

Labeling and Purification of Sequencing Primers with Near-IR, Heavy-Atom-Modified Dyes. The M13mp18 universal sequencing primers (17mer) containing a 6-carbon alkyl linker terminated with an amino group on the 5′-end were derivatized with the near-IR dyes according to procedures outlined by Li-COR.[38] Briefly, 50 nmol of DNA (2× precipitated from NaOAc buffer in cold ethanol) was added to 25 μL of carbonate buffer (400 mM, pH 9.5), 25 μL of EDTA (2 mM), and 100 μL of the near-IR dye (5 mM) dissolved in DMF, giving a 10-fold molar excess of dye over DNA. After the reaction was allowed to proceed at room temperature for approximately 4 h, 10 μL of NaOAc and 480 μL of cold ethanol were added to the reaction mixture. The solution was centrifuged for 20 min at 10 °C and 15 000 rpm. The supernatant was discarded and the ethanol precipitation step repeated again. The DNA/dye conjugate was then dried, and 200 μL of water was added to the pellet. The DNA/dye conjugate was finally purified using preparatory HPLC under the following conditions: column, C_{18} (10 cm × 4.6 mm, Brownlee); flow rate, 1.7 mL/min; mobile phase A, 0.1 M triethylammonium acetate, 4% CH_3CN, 96% H_2O; mobile phase B, 0.1 M triethylammonium acetate, 80% CH_3CN, 20% H_2O. The gradient conditions were 90/10 to 55/45 A/B over 5 min, 55/45 to 0/100 A/B over 20 min, hold at 0/100 A/B for 5 min. The collected fractions were pooled and taken to dryness using a centrifugal evaporator and stored in the dark at −20 °C. The yield of dye-labeled primer was estimated to be 30%.

Spectroscopic Analysis. The absorbance spectra were acquired on a Perkin-Elmer Lambda 3 spectrophotometer (Perkin-Elmer, Norwalk, CT). The uncorrected fluorescence spectra were collected on a Spex 3000 fluorometer (Spex, Edison, NJ). The spectrofluorometer contained a red-sensitive photomultiplier tube (R636, Hamamatsu Corp.) and emission gratings blazed for 750 nm. The fluorescence quantum yields were calculated relative to IR-125 in DMSO (Φ_f = 0.13) according to the procedure outlined by Demas and Crosby.[39]

Time-resolved fluorescence measurements were performed using a near-IR time-correlated single-photon-counting instrument built in-house, which has been described previously.[40] The system basically consisted of a mode-locked Ti:sapphire laser pumped by the all-lines output of an Ar ion laser (Coherent Lasers, San Jose, CA) and a passively quenched single-photon avalanche diode (EG&G Optoelectronics, Vaudreuil, Canada). The dye concentration used for lifetime determinations was 1×10^{-8} M in the appropriate solvent system. The fluorescence lifetimes were calculated using a reiterative nonlinear least-squares algorithm written in-house, with decay profiles accumulated until approximately 10 000 photocounts were present in the channel with the maximum number of counts.

Capillary Electrophoresis. Capillary zone electrophoresis was performed on a Waters Quanta 4000 CE System (Millipore, Marlborough, MA), with the output signals integrated on a Perkin-Elmer LCI-100 laboratory computing integrator (Norwalk, CT). Free solution separations were performed using a 75-μm-i.d. capillary column (Polymicro Technologies, Phoenix, AZ) with a total length of 58 cm and a detection window 50 cm from the injection end. The running buffer consisted of 5 mM sodium borate buffer (pH 9.3) dissolved in 50:50 water/methanol. Dye concentrations of 5×10^{-5} M dissolved in the running buffer were electrokinetically injected onto the column for 20 s with an applied voltage of 30 kV (517 V/cm). The separations were performed at an applied voltage of 25 kV (431 V/cm). The analytes were detected on-column using absorbance at 254 nm. Free solution mobilities were calculated relative to the mobility of riboflavin (neutral marker) in order to correct for the electroosmotic flow.

Capillary gel electrophoresis was performed in a 6%T/5%C polyacrylamide gel column (75 μm i.d., J&W Scientific, Folsom, CA) with a total length of 33 cm and a detection window 26 cm from the injection end. A mixture of the dye (**8**−**11**)/oligonucleotide conjugates was electrokinetically injected onto the column for 3 s at an applied voltage of −5 kV, with separations performed in reverse mode at an applied voltage of −8.25 kV (250 V/cm). The detection was performed using laser-induced fluorescence, incorporating the system described above for lifetime measurements, except that the laser was operated in a continuous wave mode of operation.

RESULTS AND DISCUSSION

The absorption and emission spectra of the heavy-atom-modified near-IR dyes measured in methanol are shown in Figure 2. As can be seen from this figure, the introduction of the heavy-

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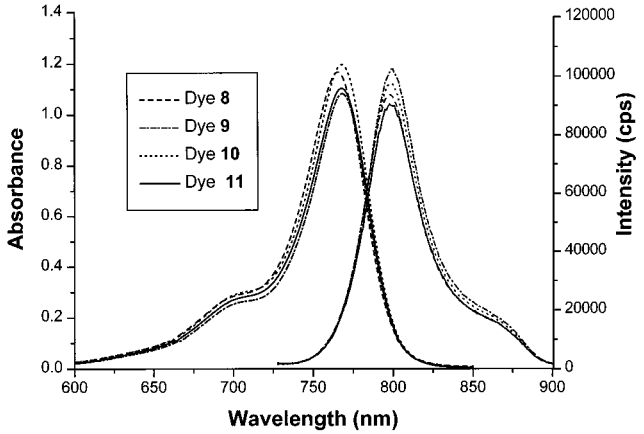

Figure 2. Absorption and emission spectra of the near-IR heavy-atom-modified dyes dissolved in methanol at a concentration of 1 μM. In the case of the fluorescence spectra, the data were collected on a red-sensitive photon-counting spectrofluorometer using an excitation wavelength of 710 nm, which allowed collection of the entire emission spectrum without interference from the Rayleigh line.

Table 1. Absorption and Emission Maxima, Extinction Coefficients and Fluorescence Quantum Yields for the Heavy-Atom-Modified Near-IR Fluorescent Dyes[a]

dye	λ_{abs} (nm)	λ_{em} (nm)	ϵ (M^{-1} cm^{-1})	Φ_f[b]
1	778	802	181 000	0.05
7	765	794	230 000	0.07
8	766	796	216 000	0.15
9	768	798	254 000	0.14
10	768	797	239 000	0.14
11	768	797	221 000	0.14

[a] All values were measured in methanol. [b] Precision in Φ_f measurements was ~15%.

atom modification onto these dyes produced only minor changes in the absorption (λ_{abs} = 765−768 nm) and emission (λ_{em} = 794−798 nm) maxima. Table 1 lists the absorbance and emission maxima as well as the molar absorptivities and fluorescence quantum yields (Φ_f) for dyes **1**, **7**−**11**. The addition of the intramolecular heavy atom produced only minor differences in the extinction coefficients (ϵ_S), with all extinction coefficients >200 000 cm^{-1} M^{-1}, typical values for these types of tricarbocyanine dyes.[40] In Table 1 are also shown the fluorescence quantum yields that were calculated for the dye series; as can be seen, the introduction of the heavy-atom modification had no appreciable effect on Φ_f within the precision of the measurement, with the quantum yields for the heavy-atom dyes ranging between 0.14 and 0.15. In the case of dye **7**, it can be seen that it had a smaller quantum yield (0.07) compared to those of the heavy-atom-modified dyes (**8**−**11**). Also, for dye **1**, where the chloro substituent was attached directly to the chromophore, the fluorescence quantum yield was smaller than for those dyes where the heavy-atom modification was spatially removed from the base chromophore. In addition, its absorption and emission maxima were red-shifted from those seen for dyes **7**−**11**. If the intramolecular heavy atom affected only the rate of intersystem crossing, then it would be expected that the dyes containing a halogen would show fluorescence quantum yields less than those for dye **7**, with the quantum yields decreasing in the order **8** < **9** < **10** < **11**, with dye **1** showing the smallest quantum yield since the heavy atom (Cl) is attached directly to the chromophore and, in **8**−**11**, it is spatially removed from the chromophore.

Table 2. Fluorescence Lifetimes of Near-IR Dyes (**1**, **7**−**11**) in Pure Methanol[a]

dye	τ_f (ps)[b]	χ^2	SD (ps)
1	529	1.1	6
7	873	1.1	8
8	947	1.3	10
9	912	1.2	10
10	880	1.1	9
11	843	1.2	8

[a] The lifetimes were measured at a dye concentration of 1 nM and excited with 1 mW of average laser power tuned to 765 nm. [b] Fluorescence lifetime data were collected in methanol and calculated using a reiterative nonlinear least-squares deconvolution method. The decay profiles were collected until approximately 10 000 counts were accumulated into the channel with the maximum number of counts.

The fluorescence lifetime data for these dyes in pure methanol are shown in Table 2, along with the χ^2 values and the standard deviation in the measurements. Relative to dye **1**, the insertion of the heavy atom directly onto the chromophore (**1**) perturbs the lifetime of the singlet state to a much larger degree than in those cases where the heavy atom was spatially removed from the chromophore (**8**−**11**). In the latter cases, spin−orbit coupling would be expected to occur predominately through-space and not through-bond (as in **1**) and, thus, exert a smaller influence on the singlet-state lifetime, consistent with our data.[41] Interestingly, we found that τ_f increased with increasing molecular weight of the intramolecular heavy-atom modification, contrary to what is typically observed.[24−26,38−41] We have recently carried out photophysical measurements of the triplet state using flash photolysis techniques to understand the observed trend.[42] The results of these studies indicated that the heavy-atom modification does increase the efficiency of crossing into the triplet state, with the heavier atom showing a larger rate of intersystem crossing. However, the major nonradiative manifold in these dyes was not intersystem crossing but internal conversion, which is typically observed in these tricarbocyanine dyes.[43] Both the intersystem crossing and internal conversion rates are affected by the presence of the heavy-atom modification, but to differing degrees, producing the observed trend in the lifetimes of these dyes.[42] However, it should be pointed out that, for the present application (base-calling in DNA sequencing), the order of the effect is inconsequential. The only important criterion is that the lifetimes can be discriminated with high precision to affect high accuracy in the base-calling. In the case of methanol, the average variation in this series of dyes was found to be 35 ps (±3 ps).

We next carried out a spectroscopic analysis of the heavy-atom-modified near-IR dyes and the dye−primer conjugates in a DNA sequencing matrix. In this case, the absorption and fluorescence spectra were measured in a nonpolymerized acrylamide solution (6%T/5%C) containing 1× TBE and 40% formamide. The motivation for using formamide was to keep the organic content high,

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Table 3. Fluorescence Properties of the Heavy-Atom-Modified Near-IR Dyes (**8**–**11**), Both Free and Conjugated to a 17mer Sequencing Primer, Measured in a Nonpolymerized Acrylamide Solution Containing 40% Formamide and 6%T/5%C[a]

	absorbance maxima (nm)		emission maxima (nm)		fluorescence lifetime (ps)	
dye	free	conjugate	free	conjugate	free	conjugate
8	764	773	792	799	760	889
9	764	773	793	797	735	821
10	765	774	793	800	719	759
11	766	774	794	800	688	735

[a] The absorption and emission spectra were obtained using a dye concentration of 1 μM, while the lifetimes were measured using a dye concentration of 10 nM. The free dyes and dye–primer conjugates were excited with 1 mW of average laser power tuned to 765 nm.

since the tricarbocyanine near-IR dyes have been shown to possess improved photophysical properties in high organic content solutions.[40] While urea gels are commonly used for sequencing, formamide gels have been shown to offer some unique advantages, such as improved stability when operating under high electric field conditions.[44–46] The absorbance and fluorescence emission maxima are shown in Table 3 for both the free dyes and the dye–primer conjugates. The covalent attachment of the dye to the oligonucleotide resulted in minor perturbations to the absorption and emission maxima. The absorption maxima for the native dyes were found to be ~765 nm, while the maxima for the dye–primer conjugates were ~773 nm, an 8-nm shift. We also observed aggregation effects in the ground-state absorption spectra for the free dyes in this matrix, while the dye–primer conjugate spectra showed only monomeric species (see Figure 3). The peak seen at 765 nm for the free dyes can be assigned to the monomer, since it was present in the case of methanol, where it is expected that little, if any, aggregation should occur. The broad peak centered at approximately 689 nm is most likely due to a dimer or other higher order aggregate that forms at this dye concentration (1 μM) in this particular solvent system but was absent in the case of methanol. It can also be seen from these data that the absorption of the monomeric form decreases in the order **8** < **9** < **10** < **11**. In the case of the dye–primer conjugates, there is an absence of this broad, blue-shifted band arising from the dimer or other higher order aggregate. The lack of aggregation for the dye–primer conjugates is likely due to the increased solvation expected for the conjugate due to the highly anionic nature of the oligonucleotide.

In the fluorescence spectra, the emission maxima for the dye–primer conjugates showed an approximate 6-nm shift compared to the native dyes in this solvent system. In addition, the emission intensities of the dye–primer conjugates were found to be somewhat higher than those associated with the free dyes in this gel matrix (data not shown). This effect was attributed to the increased absorption of the monomeric or fluorescing state of the dye due to the lack of aggregation effects observed for the conjugates at this dye concentration.

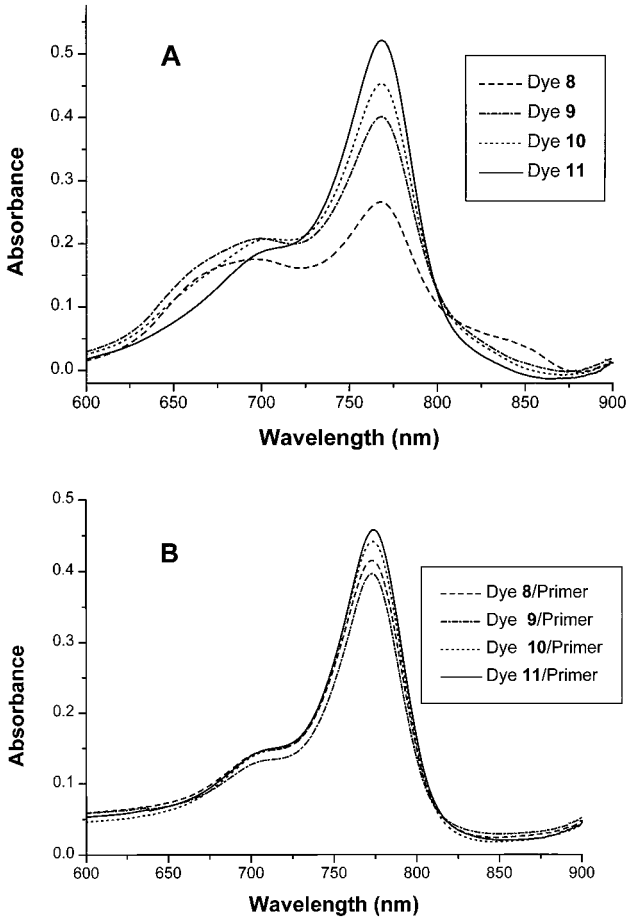

Figure 3. Absorption spectra of the free dyes (A) and the dye–primer conjugates (B) measured in the nonpolymerized acrylamide solution containing 40% formamide. The absorption spectra were acquired using a dye concentration of approximately 1 μM.

The fluorescence lifetimes for the heavy-atom-modified dyes and the dye–primer conjugates were next measured in this nonpolymerized acrylamide solution with the data listed in Table 3. In Figure 4 is shown a prompt peak and decay profiles for dyes **8** and **11** conjugated to the sequencing primer. All decays were found to be adequately fit to a single-exponential function with χ^2 values which ranged from 1.1 to 1.7. As can be seen from the data in Tables 2 and 3 for the free dyes, the lifetimes were shorter in the nonpolymerized gel solution compared to those of methanol, but the general order in the lifetime values was preserved. In the case of methanol, dye **8** had a lifetime of 947 ps, while in the gel matrix it was found to be 760 ps. The differences in lifetimes between the two solvent matrixes are consistent with our previous work, which has shown that the tricarbocyanines display solvent-dependent photophysics, with generally poorer photophysics observed in more aqueous media.[40] It was also noticed that the lifetime variation for the free dye series was found to be 24 ± 8 ps, while in the case of methanol, it was 35 ± 3 ps.

If these dyes are to be effectively used in a single-lane DNA sequencing format for base-calling, both the absolute magnitude and the variation in the lifetimes for the dye series are important. When the dyes possess short lifetimes, the criteria imposed on

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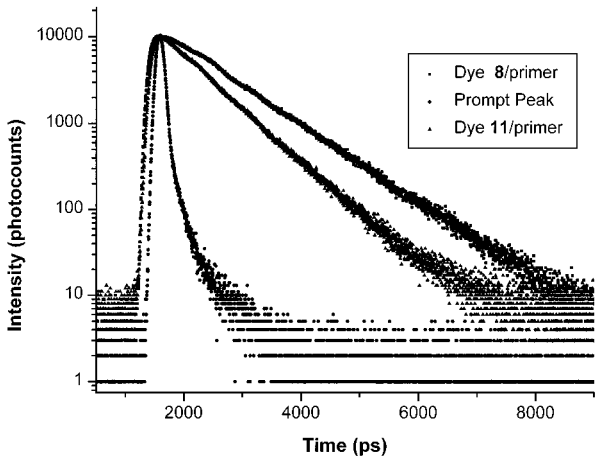

Figure 4. Fluorescence decay profiles for dyes **8** and **11** conjugated to the M13mp18 sequencing primers measured in a nonpolymerized acrylamide gel solution containing 40% formamide and 1× TBE along with the prompt peak (instrument response function). In this case, the dye concentration was set at 10 nM with approximately 1 mW of laser power at 765 nm used for excitation. To minimize photobleaching effects during accumulation of the decay profiles, the dye solution was flowed through the laser beam at a volumetric flow rate of 0.1 mL/min.

the instrument become critical: namely, a short instrument response function becomes necessary in order to adequately determine the lifetime. This will be particularly important under ultradilute conditions and short residence times (poor photon statistics), since it may be necessary to gate out scattered photons to perform the lifetime calculation over a time interval where the scattered photons contribute insignificantly. In the present case, our instrument showed a response function of 165 ps, significantly less than the lifetime of dye **11** (688 ps) measured in this nonpolymerized acrylamide solution. The variation or spread in the lifetimes for the dye series will determine the accuracy in the base-calling, since the bases are called by the lifetime calculated for each band in the electropherogram. Basically, the lifetime variation between dyes must be larger than the discrimination criterion, which is based on the standard deviation in the measurement. Using maximum likelihood estimators for determining fluorescence lifetimes, the standard deviation (σ) in the measurement can be determined from[47]

$$\sigma = \tau_f(N)^{-(1/2)} \quad (1)$$

where N is the total number of photocounts comprising the decay profile. If each electrophoretic band consists of ~5000 counts and the lifetime of the base chromophore is 760 ps, then a lifetime difference of approximately 31 ps is required for discrimination at 3σ. If the number of counts in each band is doubled, then the variation required in this case is only 22 ps (3σ). Therefore, the average variation observed for the free dyes is well within the criterion for facile discrimination when the number of photocounts exceeds 10 000. However, it should be noted that interferences, such as scattering and/or fluorescent impurities, can degrade the relative precision in the measurement. Our recent results have indicated that, with the use of near-IR fluorescence monitoring, these effects are minimal, and the precision in the measurement is determined primarily by photon statistics.[17]

To evaluate the effects on the fluorescence lifetimes of these dyes by conjugating them to sequencing primers, the lifetimes of the dye−primer conjugates were determined in the nonpolymerized matrix containing formamide, the results of which are shown in Table 3. As can be seen from these data, the lifetime values between the free dyes and the dye−primer conjugates were found to differ, with the conjugates showing a longer lifetime than the free dye. For example, in the case of dye **8**, the free dye showed a lifetime of 760 ps, while the conjugate of dye **8** possessed a lifetime of 889 ps. It was expected that the observed differences were not due to aggregation effects, since the dye concentration used (10 nM) was significantly less than those used to collect the absorption and emission spectra and little aggregation would be expected at this low dye concentration. Also, the average variation in the lifetimes for this dye series was found to be 51 ± 23 ps for the dye−primer conjugates, significantly larger than in the case of the free dyes. However, the variation between the dye−primer conjugates of **10** and **11** was only 24 ps, while those for the other pairs, **8**/**9** and **9**/**10**, were 68 and 62 ps, respectively. Therefore, for base-calling applications, the photocounts necessary to achieve the required precision in the measurement will be set by the smallest variation within the series, which, in this case, is 24 ps. This means that at least 10 000 photocounts will be required per decay profile or electrophoretic band to achieve discrimination at 3σ.

We noticed that the observed lifetimes were sensitive to the amount of formamide present in the acrylamide solution. For example, when the amount of acrylamide and bisacrylamide was kept constant (6%T/5%C) and the percentage of formamide was reduced to 10%, the lifetimes were determined to be 621 ps for dye **8**, 608 ps for dye **9**, 534 ps for dye **10**, and 513 ps for dye **11**. In addition, the χ^2 values were also higher (>2.0). Attempts to fit the data to double-exponential functions did not reduce the value of χ^2 (i.e., improve the goodness of the fit). We also measured the lifetimes of these dyes in a nonpolymerized denaturing solution containing 7 M urea and found that these lifetimes were smaller than those found in the 40% formamide solutions. Dye **8** possessed a lifetime of 586 ps in 7 M urea, and dye **10** was found to have a lifetime of 483 ps in this same medium. These data suggest that the lifetime values will be sensitive to the composition of the fractionating medium. However, even though the actual lifetime values of the heavy-atom-modified dyes did change with changes in the composition of the medium, the relative order of the lifetime values for each dye in the series was retained in any particular solvent matrix.

Effective use of these dyes in a DNA-sequencing protocol requires the electrophoretic mobilities (μ_{em}) be uniform in order to minimize base-calling errors arising from dye-dependent mobility shifts. The apparent mobilities (μ_{app}) of the heavy-atom-modified chromophores were calculated in free solution capillary zone electrophoresis using

$$\mu_{app} = L_{eff}/(t_m E) \quad (2)$$

where L_{eff} is the length of the capillary column from the injection

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Table 4. Electrophoretic Mobilities of Heavy-Atom-Modified Near-IR Fluorescent Dyes (**7**−**11**) in Free Solution[a]

dye	μ_{em} (cm^2/(V·s), × 10^5)[b]
7	−5.5 ± 0.2
8	−5.1 ± 0.1
9	−5.0 ± 0.2
10	−4.9 ± 0.3
11	−5.2 ± 0.1

[a] The carrier buffer consisted of borate (pH 9.3) and methanol, with the electrophoresis carried out at a field strength of 431 V/cm. [b] The negative sign indicates that the mobility is associated with anionic species that run counter to the direction of the electroosmotic flow.

to the detection window (cm), t_m is the migration time of the analyte (s), and E is the field strength (V/cm). The electrophoretic mobilities (μ_{em}) were determined from the expression

$$\mu_{em} = \mu_{app} - \mu_{eo} \quad (3)$$

where μ_{eo} is the electroosmotic flow. The calculated mobilities are shown in Table 4. As can be seen from these data, the heavy-atom-modified dyes **8**−**11** demonstrated rather uniform electrophoretic mobilities (within experimental error), with these values ranging between −4.9 and −5.2 × 10^{-5} cm^2/(V·s). In the case of the near-IR dye not containing the heavy-atom modification (dye **7**), it showed a mobility that was larger than those of the heavy-atom dyes, most likely a result of the smaller frictional contribution to the mobility produced by H compared to the heavy-atom modifications (I, Br, Cl, F), since all of these dyes possess similar charges (monoanionic at this pH).

The heavy-atom dyes **8**−**11** conjugated to the 17mer sequencing primer were then electrophoresed in a gel-filled capillary column to investigate mobility differences under these electrophoretic conditions. The resulting electropherogram is shown in Figure 5. As can be seen from this figure, a single peak was observed which migrated from the column at 1752 s. Shown in the inset of this figure is an expanded view of the resulting electrophoretic peak, which clearly shows only one peak, indicating that the heavy-atom-substituted dyes comigrated; therefore, they possessed uniform mobilities under these gel conditions. Due to the lack of dye-dependent mobilities observed for this dye series, postrun corrections in the electropherogram will not be required during DNA sequencing applications, simplifying the base-calling and improving the accuracy in sequence reconstruction.

CONCLUSION

We have synthesized and characterized a series of chromophores that contain an intramolecular heavy atom (halogen) which does not perturb the absorbance or emission maximum of the base chromophore but induces a difference in τ_f resulting from influences on both the intersystem crossing and internal conversion rates on the base chromophore. These dyes were conjugated to sequencing primers, and the conjugates were observed to have

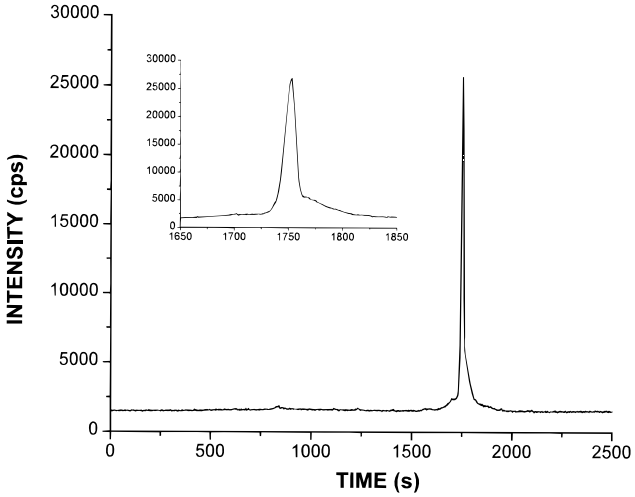

Figure 5. Capillary gel electropherogram of the heavy-atom-modified near-IR dyes (**8**−**11**) conjugated to a 17mer M13mp18 sequencing primer. In this case, the dye−primer concentration was 1 nM, and the column consisted of a 6%T/5%C cross-linked polyacrylamide sieving matrix with 7 M urea as the denaturant. The sample was injected onto the column electrokinetically for 10 s at 5 kV, and the electrophoresis was carried out at a field strength of 250 V/cm.

photophysical properties which differed from those of the free dyes in a formamide/acrylamide medium. Electrophoretic mobility studies in free solution carried out on these heavy-atom-modified near-IR dyes indicated that they possess uniform mobilities in free solution and, when conjugated to sequencing primers, comigrate under gel electrophoresis conditions.

For DNA-sequencing applications, these dyes will serve as excellent labels using lifetime discrimination, since only one excitation source and one detection channel will be required to process the fluorescence data since they possess similar absorption and emission maxima. In addition, the uniform mobilities will eliminate the need for postrun corrections, typically necessary for multidye approaches. While performing a time-resolved measurement can be somewhat instrumentally prohibitive, the dyes we have prepared absorb light in the near-IR, allowing the use of solid-state pulsed-diode lasers to serve as excitation sources. In conjunction with avalanche photodiodes, a simple time-correlated single-photon-counting apparatus can be constructed to dynamically measure fluorescence lifetimes on-line during capillary gel electrophoresis.[23] In addition, the use of near-IR fluorescence monitoring will significantly reduce matrix interferences, improving the precision in the lifetime measurement.

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