Published in final edited form as:

Bioconjug Chem. 2011 August 17; 22(8): 1491–1502. doi:10.1021/bc100485f.

Fluorescent DNA Nanotags Featuring Covalently Attached Intercalating Dyes: Synthesis, Antibody Conjugation and Intracellular Imaging

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Abstract

We have synthesized fluorescent DNA duplexes featuring multiple thiazole orange (TO) intercalating dyes covalently attached to the DNA via a triazole linkage. The intercalating dyes stabilize the duplex against thermal denaturation and show bright fluorescence in the green. The emission color can be changed to orange or red by addition of energy-accepting Cy3 or Cy5 dyes attached covalently to the DNA duplex. The dye-modified DNA duplexes were then attached to a secondary antibody for intracellular fluorescence imaging of centrosomes in *Drosophila* embryos. Bright fluorescent foci were observed at the centrosomes in both the donor (TO) and acceptor (Cy5) channels, due to the fact that the energy transfer efficiency is moderate. Monitoring the Cy5 emission channel significantly minimized the background signal due to the large shift in emission wavelength allowed by energy transfer.

INTRODUCTION

A wide range of fluorescence technologies are available for biological imaging, allowing users to select virtually any color in the visible and near-IR region and a variety of orthogonal labeling strategies that permit imaging of multiple targets simultaneously.^{1,2} Both chemical approaches to fluorescence labeling (e.g. dye-conjugated antibodies) and biological fusion constructs based on inherently fluorescent proteins such as green fluorescent protein or other tags that can recognize dyes have enabled cell biologists to

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Supporting Information. Synthetic details for compound TO-N₃-2; fluorescence spectra of TO-modified DNA oligonucleotides ODN1-ODN3; fluorescence lifetime measurements of TO-modified ODN3 duplex ± Cy5 acceptor, details of R₀ calculation, MALDI-TOF mass spectrum of ODN4 single strand, UV-vis spectra of ODN4 single strand and duplex, UV melting curves of termolecular duplexes and spectral imaging analysis of individual *Drosophila* embryos. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

develop increasingly detailed understanding of the spatiotemporal patterns of molecular interactions occurring within cells and/or on cell surfaces.

While fluorescence technologies provide a palette of colors and labeling strategies, an area where there is still room for improvement is in the brightness of the labels. For stoichiometric labels such as fusion proteins, a single dye is attached to the protein of interest. If the protein is expressed in low amounts or is not strongly localized to a specific region, the resulting signal might not be sufficiently bright to detect, particularly in the complex environment of a cell.

The brightest fluorescent labels typically exhibit extraordinarily high molar extinction coefficients (ϵ). This includes semiconductor nanocrystals (i.e. quantum dots),³ inorganic^{4,5} and polymeric^{6,7} nanoparticles and phycobiliproteins⁸. These materials have found uses in certain labeling and detection applications. Nevertheless, one challenge that remains in adapting these high ϵ materials more broadly is installing surface chemistry that allows single-point attachment to molecules of interest.

In prior work, we created a new class of fluorescent labeling reagents based on DNA nanostructures and fluorogenic intercalating dyes.^{9,10} DNA can readily be designed to form 1-D, 2-D or 3-D nanostructures and intercalating dyes can insert into the helix at high densities, up to 1 fluorophore per two base pairs (Figure 1, top). Intercalating dyes of many fluorescence colors are commercially available as is DNA bearing a variety of end group modifications that can be used to attach the DNA to various surfaces or other molecules. Thus, a noncovalent *nanotag* can be assembled from readily available materials and can be easily applied to labeling of biomolecules via standard conjugation chemistries.

While assembly of noncovalent nanotags is facile, the lack of a stable linkage between the dye and the DNA template allows the fluorophore to dissociate from the DNA, leading to weaker fluorescence from the labeled molecule and, potentially unintended fluorescence from other molecules. For example, we observed that a noncovalent nanotag targeted to a cell-surface protein gave the intended peripheral fluorescence surrounding the cell, but also strong intracellular fluorescence from other cells.⁹ This was due to dissociation of the dye from the nanotag, uptake into (presumably dead) cells and staining of nucleic acids within those cells.

In order to enhance the utility of this class of fluorescent labels, we sought to develop covalent versions of our nanotags based on a robust “click” reaction.¹¹ In addition to providing stable conjugates between DNA and intercalating dyes, the resulting constructs have been attached to antibodies and used to stain intracellular proteins. Efficient Förster resonance energy transfer in these tags allows wavelength shifting of the emission to minimize background fluorescence.

EXPERIMENTAL PROCEDURES

General Materials and Methods

Reagents for the synthesis of thiazole orange azides were purchased from Sigma-Aldrich and Alfa-Aesar (USA). Solvents were HPLC grade. DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. (www.idtdna.com) as lyophilized powders unless specified. Unmodified and 5'-biotinylated oligonucleotides were purified by gel-filtration chromatography while Cy3- and Cy5-labeled oligonucleotides were purified by HPLC. Alkyne-modified DNA strands were synthesized in the Carell laboratory or by BaseClick GmbH. Streptavidin polystyrene beads (2 μ m diameter) were purchased from Spherotech, Inc. (Libertyville, IL). Intermediate **4** (2-methylthiobenzothiazole) was provided by Dr.

Brigitte Schmidt. ^1H NMR spectra were recorded at 300 MHz on a Bruker Avance instrument in either $\text{MeOD-}d_3$ or CDCl_3 as solvent, with TMS as internal standard. Electrospray ionization mass spectrometry (ESI-MS) experiments were run on a Finnigan LCQ quadrupole ion trap mass spectrometer in the positive ion mode using Xcalibur Ver. 1.2.

2-(2-Chloroethoxy)ethyl trifluoromethanesulfonate (1a)—Poly(vinylpyridine), PVPy, 1.10 g, was suspended in 60 mL of dry dichloromethane and 1.85 mL (11 mmol) of trifluoromethanesulfonate anhydride was added followed by 1.90 mL (9 mmol) of 2-(2-chloroethoxy)ethanol. The mixture was stirred at room temperature for two hours. The resin was gravity filtered and the reaction mixture was washed with 1% sodium bicarbonate followed by brine, dried with anhydrous sodium sulfate and the solvent was evaporated to dryness to give 1.28 g (5 mmol, 56% yield) of brown oil shown to be compound **1a**. ^1H NMR (300 MHz, CDCl_3) δ 4.67 (brt, 2H, $J = 4.5$ Hz); 3.88 (m, 2H); 3.82 (m, 2H); 3.68 (m, 2H).

2-[2-(2-Chloroethoxy)ethoxy]ethyl trifluoromethanesulfonate (1b)—PVPy, 550 mg, was suspended in 30 mL of dry dichloromethane, and 925 μL (5.5 mmol) of trifluoromethanesulfonate anhydride was added followed by 645 μL (4.5 mmol) of 2-[2-(2-chloroethoxy)ethoxy]ethanol. The mixture was stirred at room temperature for 2 hours. The resin was gravity filtered and the solvent was washed with 1% sodium bicarbonate, followed by brine and it was dried with sodium sulfate. Solvent evaporation yielded 1.13 g (3.8 mmol) of compound **1b** as an oil. Its identity was determined by ^1H NMR. Yield 84%. ^1H NMR (300 MHz, CDCl_3) δ : 4.66 (t, 2H, $J = 4.5$ Hz); 3.88 (m, 2H); 3.79 (m, 2H); 3.72 (m, 4H); 3.66 (m, 2H).

1-[2-(2-Chloroethoxy)ethyl]-4-methylquinolinium trifluoromethanesulfonate (2a)—Compound **1a**, 640 mg (2.5 mmol) was reacted with 298 μL (2.5 mmol) of 4-methylquinoline at 80°C overnight. The reaction mixture was washed several times with ethyl ether giving 814 mg (2.2 mmol) of a gray powder shown to be the product (**2a**) by ^1H NMR. Yield: 88%. ^1H NMR (300 MHz, $\text{MeOD-}d_3$) δ : 9.18 (d, 1H, $J = 6.1$ Hz); 8.60 (brd, 2H, $J \sim 8.8$ Hz, two overlapped protons); 8.27 (ddd, 1H, $J = 8.9$; 7.0; 1.4 Hz); 8.08 (ddd, 1H, $J = 8.9$; 6.9; 1.0 Hz); 7.99 (dd, 1H, $J = 6.1$; 0.6 Hz); 5.27 (t, 2H, $J = 4.8$ Hz); 4.10 (t, 2H, $J = 4.5$ Hz); 3.69 (m, 2H); 2.54 (m, 2H); 3.10 (s, 3H).

1-{2-[2-(2-chloroethoxy)ethoxy]ethyl}-4-methylquinolinium trifluoromethanesulfonate (2b)—Compound **1b**, 1.08 g (3.58 mmol) was reacted with 426 μL (3.22 mmol) of 4-methylquinoline at 90°C for 48 hrs. The reaction mixture was washed several times with ethyl ether and the residue purified by column chromatography using reversed phase C-18 silica gel and mixtures of water/methanol as eluents giving 1.69 g (2.8 mmol) of intermediate **2b**. The product showed to be pure by ^1H NMR. Yield: 78%. ^1H NMR (300 MHz, CDCl_3) δ : 9.30 (d, 1H, $J = 6.1$ Hz); 8.53 (d, 1H, $J = 9.0$ Hz); 8.37 (dd, 1H, $J = 8.5$; 1.3 Hz); 8.21 (ddd, 1H, $J = 9.0$; 7.1; 1.3 Hz); 7.99 (ddd, 1H, $J = 9.1$; 7.1; 0.8 Hz); 7.90 (d, 1H, $J = 6.1$ Hz); 5.30 (t, 2H, $J = 4.7$ Hz); 4.14 (t, 2H, $J = 4.7$ Hz); 3.62-3.67 (m, 4H); 3.53-3.60 (m, 4H); 3.04 (s, 3H).

3-{2-[2-(2-Chloroethoxy)ethoxy]ethyl}-2-(methylthio)-1,3-benzothiazol-3-ium trifluoromethanesulfonate (5)—2-Methylthiobenzothiazole (**4**), 362.6 mg (2.0 mmol), was reacted with compound **1b**, 600 mg (2.0 mmol) at 80°C . After a few minutes it formed a hard solid which was let stand for 3 hours before ethanol was added and refluxed with stirring overnight. The solvent was evaporated and the solid washed several times with ethyl ether to give 474.8 mg (1.0 mmol) of a white solid identified as **5** by ^1H NMR and ESI-MS

(50 % yield). ^1H NMR (300 MHz, CDCl_3) δ : 8.10 (d, 1H, $J = 8.2$ Hz); 8.06 (d, 1H, $J = 8.5$ Hz); 7.81 (ddd, 1H, $J = 8.2$; 7.2; 1.2 Hz); 7.69 (ddd, 1H, $J = 8.5$; 7.2; 0.9 Hz); 4.94 (t, 2H, $J = 5.0$ Hz); 4.11 (t, 2H, $J = 5.0$ Hz); 3.66-3.60 (m, 4H); 3.60-3.51 (m, 4H); 3.13 (s, 3H).

1,4-Dimethylquinolinium iodide (6)—4-Methylquinoline, 950 mg (6.6 mmol), was reacted with iodomethane 1.14 g (0.5 mL, 8.0 mmol), and potassium carbonate 46 mg (0.3 mmol) under reflux overnight. The yellow solid formed was crushed into a fine powder, washed several times with ethyl ether, boiled in ethylacetate and hot filtered. After cooling to room temperature the solid was filtered and dried under vacuum giving 1.85 g (6.5 mmol) of product **6**. Yield: 98%. ^1H NMR (300 MHz, CDCl_3) δ : 10.26 (d, 1H, $J = 6.0$ Hz); 8.40 (dd, 1H, $J = 8.5$; 1.4 Hz); 8.31 (br d, 1H, $J = 8.9$ Hz); 8.24 (ddd, 1H, $J = 8.9$; 7.0; 1.4 Hz); 8.04 (d, 1H, $J = 8.9$; 7.0; 1.4 Hz); 7.99 (br d, 1H, $J = 6.0$ Hz); 4.89 (s, 3H); 3.06 (s, 3H).

2-{1-[2-(2-Chloro-ethoxy)-ethyl]-1H-quinolin-4-ylidenemethyl}-3-methyl-benzothiazol-3-ium trifluoromethanesulfonate (TO-Cl-1)—Intermediate **2a** (125 mg, 0.25 mmol) was reacted with 3-methyl-2-(methylthio)-1,3-benzothiazol-3-ium iodide (**3**) (81 mg, 0.25 mmol) in 1 mL of anhydrous ethanol added with triethylamine (34.8 μL). The reaction mixture turned orange almost immediately and a solid appeared. After 90 min the solvent was evaporated under vacuum and the solid washed exhaustively with ethyl ether. The crude dye was purified by vacuum liquid chromatography on a reversed phase C18 column using a gradient of water/methanol and methanol containing 0.05% TFA. Further purification was done on a silica gel column using 10% MeOH in dichloromethane obtaining 54.7 mg of pure **TO-Cl-1** (0.11 mmol; 44% yield). ^1H NMR (300 MHz, CDCl_3) δ : 8.72 (d, 1H, $J = 7.1$ Hz); 8.58 (dd, 1H, $J = 8.5$; 1.0 Hz); 7.93 (dd, 1H, $J = 8.5$; 1.0 Hz); 7.82 (td, 1H, $J = 7.0$; 1.4 Hz); 7.70 (m, 2H); 7.54 (ddd, 1H, $J = 8.5$; 7.5; 1.2 Hz); 7.37 (m, 2H); 6.74 (s, 1H); 4.85 (br t, 2H, $J = 4.9$ Hz); 4.08 (br t, 2H, $J = 4.9$ Hz); 3.99 (s, 3H); 3.78 (m, 2H); 3.59 (m, 2H). ESI-MS (positive mode) m/z : 397.27 (M $^+$); calculated for $\text{C}_{22}\text{H}_{22}\text{ClN}_2\text{OS}$ m/z : 397.21.

2-{1-[2-(2-Iodo-ethoxy)-ethyl]-1H-quinolin-4-ylidenemethyl}-3-methyl-benzothiazol-3-ium trifluoromethanesulfonate (TO-I-1)—**TO-Cl-1** (15.0 mg, 0.029 mmol) was reacted with 4 equivalents of sodium iodide (60.0 mg) in dry acetone under reflux overnight. About 97% of the dye was converted into **TO-I-1** according to ^1H NMR integral of the triplet at δ : 3.2 ($-\text{CH}_2\text{I}$). The dye was isolated by evaporating the acetone and extracting with dichloromethane; after evaporation of the solvent, the dye was washed with water. The resulting oil was dissolved in a small volume of methanol and mixed with water to get a 10% methanol solution; it was put onto a RPC18 silica gel column and eluted with water and mixtures water/methanol of increasing strength. ^1H NMR (300 MHz, CDCl_3) δ : 8.85 (d, 1H, $J = 7.2$ Hz); 8.64 (br d, 1H, $J = 8.4$); 7.98 (br t, 1H, $J = 8.4$ Hz); 7.84 (br t, 1H, $J = 7.4$ Hz); 7.73 (br d, 1H, $J = 8.2$ Hz); 7.54 (ddd, 1H, $J = 8.4$; 7.2; 1.3 Hz); 7.44-7.31 (m, 4H); 6.77 (s, 1H); 4.98 (br t, 2H, $J = 4.8$ Hz); 4.10 (br t, 2H, $J = 4.8$ Hz); 4.04 (s, 3H); 3.77 (m, 2H); 3.20 (m, 2H). ESI-MS (positive mode) m/z : 489.13 (M $^+$); calculated for $\text{C}_{22}\text{H}_{22}\text{IN}_2\text{OS}$ m/z : 489.05.

2-{1-[2-(2-Azido-ethoxy)-ethyl]-1H-quinolin-4-ylidenemethyl}-3-methyl-benzothiazol-3-ium trifluoromethanesulfonate (TO-N₃-1)—**TO-I-1** (15.0 mg, 0.024 mmol) was reacted with sodium azide (15.0 mg, 0.23 mmol) in dimethylformamide (750 μL), overnight at room temperature. The DMF was eliminated by adding excess of water to the reaction mixture and washing the dye on a reversed phase C18 column with 1 L distilled water. The dye was finally eluted with pure methanol; **TO-N₃-1** (11.6 mg, 0.022 mmol) was obtained in 92.0 % yield. (Overall yield 39.2 %). ^1H NMR (300 MHz, CDCl_3) δ : 8.80 (d, 1H, $J = 7.5$ Hz); 8.48 (br d, 1H, $J = 8.5$; 1.2 Hz); 8.10 (br d, 1H, $J = 8.5$ Hz); 7.93 (td, 1H, J

= 7.5; 1.2 Hz); 7.79 (br d, 1H, J = 7.5 Hz); 7.72 (td, 1H, J = 8.5; 1.2 Hz); 7.57 (br t, 1H, J = 7.5 Hz); 7.41 (m, 2H); 6.77 (s, 1H); 4.97 (br t, 2H, J = 4.9 Hz); 4.10 (br t, 2H, J = 4.9 Hz); 3.99 (s, 3H); 3.69 (m, 2H); 3.31 (br t, 2H, J = 4.6 Hz). ESI-MS (positive mode) m/z : 404.20 (M^+); calculated for $C_{22}H_{22}N_5OS$ m/z : 404.15.

3-[2-(2-Chloroethoxy-ethoxy)-ethyl]-2-(1-methyl-1H-quinolin-4-ylidenemethyl)-benzothiazol-3-ium trifluoro-methanesulfonate (TO-Cl-3)—Intermediate **5** (385.6 mg, 0.8 mmol) and 1,4-dimethylquinolinium iodide (**6**) (228.0 mg, 0.8 mmol), were dissolved in 3 mL of warm absolute ethanol, and added with triethylamine, 80 mg (110 μ L, 0.8 mmol). The reaction mixture turned red and was allowed to stand overnight at room temperature. The red crystals formed were filtered and purified by column chromatography on silica gel using dichloromethane with increasing concentrations of methanol (2 % through 5% total) as the eluent. The fractions were analyzed by TLC and pooled together according to their composition; 125 mg (0.2 mmol) of intermediate dye **TO-Cl-3** was obtained. Yield: 26.4%. 1H NMR (300 MHz, $CDCl_3$) δ : 8.61 (d, 1H, J = 6.9 Hz); 8.59 (dd, 1H, J = 8.0; 1.5 Hz); 7.74 (m, 2H); 7.59 (br d, 1H, J = 8.0 Hz); 7.53 (dd, 1H, J = 8.2; 1.2 Hz); 7.42 (m, 2H); 7.24 (m, 2H); 6.87 (s, 1H); 4.70 (br t, 2H, J = 5.0 Hz); 4.08 (br t, 2H, J = 5.0 Hz); 4.05 (s, 3H); 3.60 (m, 2H); 3.50 (m, 4H); 3.39 (m, 2H). ESI-MS (positive mode) m/z : 441.27 (M^+), calculated for $C_{24}H_{26}ClN_2O_2S$ m/z : 441.14.

3-[2-(2-Iodoethoxy-ethoxy)-ethyl]-2-(1-methyl-1H-quinolin-4-ylidenemethyl)-benzothiazol-3-ium trifluoro-methanesulfonate (TO-I-3)—Intermediate **TO-Cl-3** (125.0 mg, 0.2 mmol) and a large excess of sodium iodide (3 g) were refluxed in 3 mL of dry acetone for 32 hours. Dichloromethane was then added to the round bottom flask and sodium chloride and excess sodium iodide precipitated. Dichloromethane washes continued until the precipitate was no longer pink. The isolated product **TO-I-3** was concentrated *in vacuo* giving a total of 120.0 mg (0.18 mmol). Yield: 90%. 1H NMR (300 MHz, $CDCl_3$) δ : 8.99 (d, 1H, J = 7.3 Hz); 8.58 (d, 1H, J = 8.2 Hz); 7.90 (br t, 1H, J = 7.7 Hz); 7.82-7.70 (m, 3H); 7.58-7.42 (m, 3H); 7.36 (br t, 1H, J = 7.5 Hz); 7.02 (s, 1H); 4.73 (br t, 2H, J = 4.9 Hz); 4.30 (s, 3H); 4.14 (br t, 2H, J = 4.8 Hz); 3.66-3.47 (m, 4H); 3.07 (t, 2H, J = 6.6 Hz). ESI-MS (positive mode) m/z : 533.13 (M^+), calculated for $C_{24}H_{26}IN_2O_2S$ m/z : 533.08.

3-[2-(2-Azidoethoxy-ethoxy)-ethyl]-2-(1-methyl-1H-quinolin-4-ylidenemethyl)-benzothiazol-3-ium trifluoro-methanesulfonate (TO-N₃-3)—The final azide substituted dye, **TO-N₃-3** prepared by reacting **TO-I-3** (120.0 mg, 0.18 mmol) with sodium azide (35.1 mg, 0.5 mmol) in dimethylformamide (2 mL), at room temperature with stirring overnight. The solution was diluted with 500 mL of distilled water and put onto a reversed phase C18 silica gel column, washed with 1.5 L of water, and after drying the column, the dye was eluted with methanol containing 0.1% TFA. Additional purification was done by reversed phase C18 silica gel column chromatography to yield pure **TO-N₃-3** (97.0 mg, 0.17 mmol) was obtained in 93.6 % yield. Overall yield = 22.2%. 1H NMR (300 MHz, $CDCl_3$) δ : 8.63 (d, 1H, J = 7.4 Hz); 8.44 (br d, 1H, J = 8.1 Hz); 7.92 (br t, 1H, J = 7.9 Hz); 7.83-7.70 (m, 3H); 7.58-7.50 (m, 2H); 7.46 (br d, 1H, J = 8.0 Hz); 7.38 (br t, 1H, J = 7.4 Hz); 7.00 (s, 1H); 4.65 (br t, 2H, J = 5.2 Hz); 4.25 (s, 3H); 4.11 (br t, 2H, J = 5.1 Hz); 3.71-3.56 (m, 4H); 3.52 (t, 2H, J = 4.9 Hz); 3.24 (t, 2H, J = 4.9 Hz). ESI-MS (positive mode) m/z : 448.20 (M^+), calculated for $C_{24}H_{26}N_5O_2S$ m/z : 448.18.

Conjugation of Azido Dyes to Alkyne-Modified DNA Oligonucleotides—To 25 μ L of 0.5mM alkyne-functionalized DNA solution (12.5nmol) in water, 6.25 μ L of 100mM dye-azide solution (625nmol, 50eq) in DMSO and 5 μ L of a freshly prepared solution containing 1:1 ratio of CuBr (Sigma) and TBTA ligand (Sigma) in degassed 3:1 DMSO:tBuOH (0.05M, 125nmol, 20eq) were added. The mixture was vortexed and shaken

at 20°C for 1 hour. A second 5 μ L portion of the CuBr-TBTA solution mixture was added, and the reaction was again vortexed and shaken at 20°C for another hour. The reaction was evaporated to almost complete dryness and was dissolved in 200 μ L of 0.3M NaOAc. The DNA was precipitated by adding 1mL of cold absolute ethanol and was left overnight at -20°C. The reaction was centrifuged at 14,000rpm and the supernatant was removed. The product pellet was washed 3 times with 70% ethanol, again centrifuged at 14,000rpm and supernatant removed. The final product was suspended in 200 μ L water (or 10mM Tris, pH 7.5 for enhanced solubility) and was desalted using a MicroSpin G-25 column (GE Healthcare) to remove any remaining unconjugated dye. MALDI-TOF mass spectrometry of ODN4, a 39mer bearing three TO dyes, gave a peak at $m/z = 13,607.3$ (calc: 13,596.8).

Characterization of Covalent Dye-DNA Single Strands and Duplexes—DNA solutions were prepared in 10 mM aqueous sodium phosphate (NaP_i) buffer (pH = 7.0) and stored at -4 °C. Concentrations were determined by UV absorbance at 260 nm using a Varian Cary 3 Bio UV-vis spectrophotometer. The extinction coefficient of ODN4 at 260 nm was calculated to be $375,500 \text{ M}^{-1}\text{cm}^{-1}$ using an online calculator developed by Owczarzy and coworkers¹² and available at www.idtdna.com. We accounted for the contribution of the three TO dyes to the absorbance at this wavelength using $\epsilon_{260}(\text{TO}) = 6,600 \text{ M}^{-1}\text{cm}^{-1}$.¹³ The molar extinction coefficient of the TO at 509 nm was then determined based on the absorbance and DNA strand concentration. The value obtained was $161,100 \text{ M}^{-1}\text{cm}^{-1}$ ($53,700 \text{ M}^{-1}\text{cm}^{-1}$ per TO).

Equal concentrations of each strand were used and the DNA strands were annealed by heating to 90 °C then slow cooling to 25 °C in 10 mM NaP_i . UV melting curves were measured by monitoring the absorbance at 260 nm while the temperature was increased at a rate of 1 °C/min. The molar extinction coefficient at the TO maximum (509 nm) was $178,900 \text{ M}^{-1}\text{cm}^{-1}$ ($59,600 \text{ M}^{-1}\text{cm}^{-1}$ per TO).

Fluorescence Measurements—The fluorescence quantum yield of the ODN4 duplex was determined to be 0.16, relative to fluorescein as the standard ($\phi_f = 0.95$ in 0.1 M NaOH). For FRET experiments 0.1 μ M solutions of duplexes were analyzed using either a Cary Eclipse or a Photon Technology International fluorimeter. All samples were excited at 485 nm and the fluorescence intensity was measured from 500-750 nm. The bandpass for both the excitation and emission monochromators was 5 nm. The FRET efficiency (E) for Cy5-labeled duplexes was measured based on the relative fluorescence intensity of the TO donor in the presence (F_{DA}) and absence (F_D) of the acceptor Cy5:

$$E = 1 - F_{DA}/F_D.$$

Fluorescence lifetimes of the bulk nanotags in buffer were obtained using a home-built spectrometer consisting of a pulsed diode laser emitting at 437 nm (LDP-PC-440, Picoquant GmbH, ca. 100 ps FWHM), 10 nm band pass filters to isolate specific emission frequencies, time correlated single photon counting electronics (PicoHarp 300, Picoquant), and detection by an actively quenched photodiode (SPD-5-CTC, Micro Photon Devices). Sample concentrations were all ca. 100 nM.

Bead-Based Confocal Microscopy—For bead labeling experiments DNA duplexes were biotinylated at the 3'-terminus of the DNA strand complementary to the TO-modified strand. (For FRET duplexes, Cy3 or Cy5 was attached to the 5'-terminus of this strand.) Cy3 and Cy5 labeled and unlabeled TO-modified duplexes were annealed by heating to 90 °C followed by cooling to 25 °C over a period of ca. 60 min. Nanotags were then added to aliquots of streptavidin beads in 0.02% Tween PBS free of calcium and magnesium. Samples were incubated in the dark for 20 minutes. Labeled beads were washed 2X with

PBS supplemented with 0.02% Tween-20 and placed onto a glass slide. Images of the nanotag-conjugated beads were collected following excitation at 488 nm with a Zeiss LSM 510 META confocal microscope. Fluorescence emission was collected with 500-550 nm bandpass (unlabeled duplex), 570-615 nm bandpass (duplex-Cy3), and 650 nm longpass (duplex-Cy5) filters, respectively.

Synthesis and Characterization of Antibody-DNA Conjugates—AffiniPure goat anti-rabbit IgG (H+L) antibody (Ab) with minimal cross-reaction to human, mouse, and rat serum proteins was purchased from Jackson ImmunoResearch Laboratories. Prior to modification, Ab was desalted using a MicroSpin G-50 column (GE Healthcare) and buffer exchanged (150mM NaCl, 100mM Na phosphate at pH 7.3) using YM-50 centrifugal filters (50kDa MWCO; Millipore Corporation). Hydralink SANH reagent (EMD Chemicals/Merck) in N,N-dimethylformamide (DMF) was added to the Ab in a 10:1 ratio (SANH:Ab). Separately, Hydralink SFB reagent (EMD Chemicals/Merck) in DMF was added to a 5'-aminated DNA oligonucleotide (Integrated DNA Technologies, Inc.) dissolved in modification buffer supplied by the manufacturer in a 10:1 ratio (SFB:DNA). Both Ab and DNA coupling reactions were done at room temperature for 2 h. Excess SANH and SFB were removed using MicroSpin G-50 and G-25 columns, respectively. Molar substitution ratio (MSR) assays using a standard protocol (SoluLink) were performed to determine the number of modifications per Ab and DNA molecule. A 2-fold excess of SFB-derivatized DNA was then combined with the SANH-derivatized Ab and allowed to react overnight at room temperature. Noncoupled DNA was removed using YM-50 centrifugal filters. Fractions from each filtration round were tested for presence of unbound free DNA (A260 using NanoDrop® spectrophotometer). The conjugation of Ab-DNA was verified spectrophotometrically, confirming the presence of hydrazone linkage ($\lambda = 354\text{nm}$) formed between SANH and SFB.

Ab:DNA Ratio Determination—To quantitatively determine the number of DNA strands conjugated per Ab molecule, a calibration experiment was done for each of the Ab-DNA conjugates. 20- μL solutions of various ratios of Ab and DNA separate components were mixed as follows: 1:0.25 (4 μM : 1 μM), 1:0.50 (4 μM : 2 μM), 1:1 (4 μM : 4 μM), 1:2 (4 μM : 8 μM), and 1:4 (4 μM : 16 μM). Using the Nanodrop® spectrophotometer, the A_{260}/A_{280} absorbance ratios were determined and recorded for each solution. The obtained calibration curve was then used to determine the number of DNA per Ab in the conjugate. This procedure accounts for the fact that the absorbance of the DNA nucleobases (260nm) overlaps with the absorbance of the Ab protein (280nm). For DNA-Ab conjugates reported here, 3 DNA strands (i.e. 9 TO dyes) were attached to each antibody.

Immunofluorescence Microscopy—Wild-type fly stocks (w^{1118}) were maintained at room temperature. Embryos were collected after 0-2 hrs at 27°C and fixed in 1:1 37% formaldehyde and heptane. Embryos were blocked in 1% goat serum and 0.1% TritonX-100 in phosphate buffered saline (PBS) for 1 hour. To label the centrosomes, embryos were incubated with anti-Centrosomin (1:4000) (gift from Timothy Megraw, Florida State University) in PBS supplemented with 1% goat serum and 0.1% TritonX-100 overnight at 4°C. Secondary antibody staining was performed with either a Goat anti-Rabbit Alexa Fluor 488 antibody (2 mg/ml) (1:1000) (Invitrogen) incubated in PBS or a Goat anti-Rabbit-nanotag conjugate (~0.5 mg/ml) (1:1000) incubated in supplemented with 1% goat serum and 0.1% TritonX-100 at 4°C overnight. Images were collected on a Zeiss LSM 510 META confocal microscope with a 63X 1.40 NA Plan-Achromat objective using ZEN 2009 software. Fluorescence emission was collected for Alexa488 and TO (505-550 nm) and FRET ($\lambda > 650\text{ nm}$) channels after excitation at 488 nm. Fluorescence emission profiles were collected at a spectral bandwidth of 10.7 nm. Images were analyzed with ImageJ (ver.

1.44). The emission profiles of the Ab-nanotag conjugate were obtained by utilizing the average pixel intensity within a $0.84\ \mu\text{m} \times 0.84\ \mu\text{m}$ field encompassing each centrosome, and the average pixel intensity of 3-4 embryos is reported.

RESULTS AND DISCUSSION

1. Rationale

To expand the potential utility of fluorescent DNA nanotags, we pursued a strategy by which the intercalating dyes could be attached covalently to the DNA (Figure 1). In our approach, intercalation is a key element since this minimizes self-quenching when multiple dyes are held within close proximity.⁹ Intercalated dyes are also well known to stabilize DNA duplexes and higher-order nanostructures against both thermal denaturation and enzymatic degradation.¹⁰

Conjugation of unsymmetrical cyanine dyes to nucleic acid strands has been motivated by the tendency of these dyes to exhibit enhanced fluorescence when constrained by end-stacking or intercalation. These dyes have been useful noncovalent stains for nucleic acids^{14,15} and have recently found additional applications as covalent conjugates to DNA or PNA oligonucleotides. For example, Ishiguro and coworkers first attached oxazole yellow to DNA termini as a hybridization reporter.¹⁶ Kubista and Svanvik reported similar properties for PNA “light-up” probes in which thiazole orange (TO) was attached to the PNA terminus.¹⁷ A variety of other TO-conjugates with DNA and PNA have been reported in which the TO was attached to internal or terminal positions.^{13,18-25}

We relied on a Cu(I)-mediated Huisgen cyclization (“click”) reaction to covalently attach the dye molecules to the DNA.^{26,27} As shown in Scheme 1, an alkyne group linked through a flexible spacer to C5 of a uracil residue can react with an azide-substituted intercalator dye (thiazole orange, TO) to covalently attach the two via a newly formed triazole linkage. A variety of methods have been reported for conjugating TO to DNA,^{13,18-22,24,28-30} but we chose this reaction since Carell and coworkers recently reported that it can be used to incorporate six consecutive fluorescein dyes onto a DNA single strand in high yield.^{11,31} while Seela and Pujari have reported successful conjugation of coumarin dyes to alkyne-functionalized DNA.^{31,32}

In contrast to cases where the cyanine dye was attached to DNA terminus, we expect the TO in our systems to intercalate into the helix. This is distinct from earlier work where TO was used as a nucleobase replacement, as originally reported by Seitz and coworkers for PNA conjugates³³ and subsequently by Wagenknecht and coworkers for DNA conjugates.^{25,30} In our approach, the TO residues should intercalate into fully base paired helices, leading to higher thermal stabilities for the dye-modified duplexes.

TO is readily functionalized at multiple positions, allowing optimization of the linkage to the DNA. Three TO-azides were synthesized: **TO-N₃-1**, **TO-N₃-2** and **TO-N₃-3** (Chart 1). These dyes feature two points of attachment and variable linker lengths. (We also synthesized a version with a shorter linker on the benzothiazole side, but did not pursue DNA conjugation with it.)

2. Dye Synthesis and DNA Conjugation

The synthetic method for the azido-substituted thiazole orange dyes is outlined in Scheme 2. Quaternization of the 2-methylheterocyclic ammonium salts is generally performed by heating the corresponding heteroaromatic base with a molar equivalent or an excess of an alkylating agent such as an alkyl iodide, bromide, sulphate or tosylate.^{34,35} This alkylation reaction requires high temperatures and long reaction times to achieve acceptable yields. We

chose an alternative route in which the corresponding alcohols were first converted to trifluoromethanesulfonic acid esters via reaction with triflic anhydride in the presence of the polymer bound non-nucleophilic base poly(vinylpyridine).³⁶ This reaction proceeds rapidly at room temperature and the alkyl triflates (**1a** and **1b**) are obtained in high yield after filtration. Quaternization of the heterocycle to give **1a**, **1b** and **5** then proceeded smoothly at room temperature as demonstrated by the formation of white powdery solids. To ensure complete alkylation the reaction was refluxed overnight in anhydrous ethanol. Previously reported methods were used to condense the two dye halves.³⁷ The purified chloro-dyes (**TO-Cl-1**, **TO-Cl-2** and **TO-Cl-3**) were refluxed in dry acetone and subjected to iodo-exchange with a large excess of sodium iodide. The iodo-dyes were isolated by multiple washes with dichloromethane and then underwent efficient azide exchange at room temperature in the presence of a 3-fold excess of sodium azide in DMF. The reaction mixture was diluted in water and loaded onto a C-18 reversed phase column to remove the DMF and isolate the final dyes **TO-N₃-1**, **TO-N₃-2** and **TO-N₃-3**.

The TO-azide dyes were conjugated to a 16mer DNA oligonucleotide bearing a single alkyne-modified uracil residue at position 8:

ODN1-ODN3: 5'-GCG CTG TXC ATT CGC G-3' where X = TO-Uracil conjugate

The DNA-TO conjugates were purified by HPLC and exhibited a single band on a polyacrylamide gel. The modified strands are labeled **ODN1-ODN3** corresponding to attachment of **TO-N₃-1**, **TO-N₃-2** or **TO-N₃-3**.

3. Characterization of Click-DNA Duplexes

UV Melting Curves—DNA strands **ODN1-ODN3** were hybridized to their complementary strand to form 16 base pair duplexes, which were then subjected to UV melting analysis. For comparison, we included a duplex formed from the alkyne-DNA precursor, i.e. without a clicked dye. As shown in Figure 2A, the **ODN1** and **ODN2** duplexes exhibited higher, but less cooperative, melting transitions than the unmodified duplex control ($T_m = 51\text{ }^{\circ}\text{C}$ vs $49\text{ }^{\circ}\text{C}$). This suggests that the linker between the TO intercalator and the DNA is too short since the higher T_m could result from partial intercalation of the dye into the base pair stack, but a distortion of the helix due to the linker length could diminish the cooperativity. In contrast, the duplex formed by **ODN3** exhibits an even higher melting transition ($T_m = 56\text{ }^{\circ}\text{C}$) and the cooperativity is similar to that of the unmodified duplex.

Fluorescence Spectroscopy—We also compared the fluorescence intensities of the three TO-DNA conjugates in single- and double-stranded forms. In the absence of a complementary strand, the order of fluorescence intensity is **ODN2** > **ODN1** > **ODN3** (Figure S1). The fluorescence in all three cases is much higher than for the individual unconjugated **TO-N₃** dyes in aqueous solution (data not shown). However, after hybridization to form duplexes, **ODN3** exhibits the strongest fluorescence (Figure 2B). The higher fluorescence and higher/more cooperative UV melting transition for the **ODN3** duplex perhaps reflect better intercalation of the dye into the helix, facilitated by the longer linker connecting the dye to the uracil and the attachment through the benzothiazole side of the dye. Based on these results, further experiments were performed using DNA functionalized with **TO-N₃-3**.

Photophysics—The fluorescence lifetimes of TO-conjugated nanotags were measured using time-correlated single photon counting to probe the binding properties of the dyes and the effects of dye-dye interactions in the multiply-substituted nanotags. The **ODN3** duplex was found to exhibit a minimum of two resolvable lifetime components of 4.4 ns and 2.1 ns

in a 1:1.4 ratio (Figure S2). The multi-exponential decay suggests multiple binding orientations for this tethered dye and is consistent with prior work by Netzel and coworkers on noncovalently bound intercalating TO dyes.³⁸ We also determined the fluorescence quantum yield for the **ODN3** duplex to be $\phi_f = 0.2$ (relative to fluorescein in 0.1N NaOH), which is comparable to the value reported for a noncovalently intercalated TO derivative in calf thymus DNA ($\phi_f = 0.25$, reference 30). Thus, covalent attachment to the DNA does not significantly alter the photophysics of the dye.

Energy Transfer in the Clicked Duplex—In our noncovalent nanotags, Förster resonance energy transfer (FRET) from the intercalated dyes to covalently attached acceptor dyes placed at one or more of the DNA strand termini was exceptionally efficient, allowing shifting of the emission wavelength by nearly 300 nm. We investigated FRET in the covalent construct by attaching a single Cy5 acceptor dye to the 5'-end of the complementary strand.²⁸ As shown in Figure 3, excitation of the TO intercalator in the presence of the Cy5 results in 82% FRET, as calculated from the apparent quenching of the TO fluorescence. This result is supported by lifetime measurements, showing that the TO lifetime components are reduced from 4.4 and 2.1 ns (1:1.4 ratio) to 4.0 and 1.1 ns (1:1.5 ratio) in the presence of the Cy5 (Figure S2).

4. Labeling of Polymer Beads

An important application of fluorescent labels is in bead-based assays, where the beads are tagged with a variety of different colors and intensities for both encoding and sensing strategies.³⁹⁻⁴¹ We synthesized duplexes in which **ODN3** was hybridized to a complementary strand having a biotin at the 3'-terminus and no dye, a Cy3 or a Cy5 at the 5'-terminus. These duplexes were then mixed with streptavidin-coated polystyrene beads and imaged in a fluorescence microscope. As shown in Figure 4, the three sets of beads can be excited at the same wavelength, but fluoresce green, orange or red, based on whether the duplexes were tagged with no acceptor, Cy3 or Cy5, respectively. Not only can the beads be distinguished by emission wavelength, variation of the amount of DNA used for labeling would allow the beads to be distinguished by intensity, all while exciting at the same wavelength.

5. Synthesis and Characterization of a Tri-Functionalized DNA Strand

Our ultimate goal is to label the DNA with many TO dyes in order to further increase the fluorescence intensity compared to a label bearing a single fluorophore. As a first step, we designed a DNA 39mer (**ODN4**) having three alkynes at positions 7, 20 and 33. The alkyne-modified DNA was then conjugated to **TO-N₃-3** as described above. After removal of unincorporated dye, MALDI-TOF mass spectrometric analysis was consistent with three dyes/DNA strand. UV-vis spectra of **ODN4** in single- and double-stranded contexts are provided in Supporting Information (Figure S3).

ODN4—5'-TAC TAA XTA CAT TTG CTA GXC CAG ATC GGC AGX GCA GGC-3'

The trifunctionalized DNA was hybridized to the complementary 39mer and gave a satisfactory UV melting curve and fluorescence spectrum (Figures 5A and 5B, respectively). The molar extinction coefficient and fluorescence quantum yield of the trifunctionalized DNA duplex were $\epsilon_{509} = 184,850 \text{ M}^{-1}\text{cm}^{-1}$ ($61,600 \text{ M}^{-1}\text{cm}^{-1}$) and $\phi_f = 0.16$, respectively. The molar extinction coefficient and quantum yield values are comparable to values determined for PNA-conjugated TO after hybridization to complementary DNA.⁽²³⁾ Time-resolved experiments on the **ODN4** duplex yielded values of 3.6 and 1.8 ns (ca. 1:2 ratio), similar to the monofunctionalized **ODN3** duplex (data not shown).

Hybridization of the trifunctionalized **ODN4-TO₃** to a complementary strand having a 3'-Cy5 label gave a similar melting curve (Figure 5A). The corresponding duplexes exhibited 44% FRET efficiency, based on TO quenching (Figure 5B). Although this is considerably lower efficiency than for **ODN3**, this is not a surprising result since the Cy5 is approximately 109 Å, 65 Å and 24 Å away from the three TO intercalators and the critical transfer distance at which FRET is 50% efficient (R_0) for TO/Cy5 is estimated to be 41 Å based on the $\phi_F = 0.2$ determined for the **ODN3** duplex.

We were interested in determining whether an internal Cy5 might provide better FRET due to closer proximity on average to all three TO intercalators. We separated the complementary strand into 19 and 20 nucleotide fragments that can be combined with the triclick DNA strand to form a ternary duplex (Figure 6). This design provides greater flexibility in the synthesis of antibody conjugates as described in the next section and is also considerably less expensive than purchasing a full-length DNA oligonucleotide with an internal Cy5 modification. UV melting curves verified assembly of the ternary duplexes (Figure S3). Duplex A contains no Cy5 acceptor and provides the maximum TO fluorescence. Duplexes B and C place a single Cy5 at an internal position, closer on average to all three TO dyes than in the case of a terminal Cy5 (duplex D). Interestingly, duplex D exhibited the greatest TO quenching, but also the lowest Cy5 fluorescence, while duplex B exhibits the least TO quenching (ca. 25%) but the brightest Cy5 fluorescence. Clearly, the different environment of the Cy5 in the internal versus terminal configurations has a significant effect on the quantum yields. This was verified by direct excitation of the Cy5, which resulted in similar relative fluorescence intensities as observed due to energy transfer from TO (data not shown).

6. Antibody Conjugation and Fluorescence Labeling in Cells

To test the ability of TO-functionalized DNA strands for cellular imaging, we designed an antibody construct depicted in Scheme 3. Starting with an anti-rabbit antibody IgG, we conjugated a 19mer DNA strand bearing a 5'-amino group and a 3'-Cy5 dye using a commercially available HydraLinK™ kit. (This chemistry involves conjugating the amino groups from the DNA terminus and antibody side chains to succinimidyl ester reagents bearing aldehyde and hydrazine groups. The DNA and antibody can then be reacted with one another to produce a stable hydrazone linkage.) UV-vis analysis indicated that 3-4 DNA strands were conjugated per antibody. The trifunctionalized DNA 39mer was then hybridized to the antibody along with a 20mer to complete the duplex assembly. The tripartite design of this nanotag (design B in Figure 5) leaves open the option to add a second acceptor dye to the 20mer component, leading to more efficient energy transfer, although we have not yet explored this.

The utility of the Ab-nanotag conjugate as a secondary antibody for intracellular labeling was demonstrated by immunofluorescence microscopy. Centrosomin, a protein that localizes to the centrosomes, was selected as a target protein. Centrosomes are the microtubule-organizing center of cells and localize in well-characterized punctate foci allowing easy visualization when stained with a conventional Alexa-tagged fluorescent secondary antibody (Fig 7A).⁴² Thus, Centrosomin provides a convenient marker to test the ability of the Ab-nanotag conjugate to recognize and report the localization of a specific primary Ab.

Syncytial (0-2 hr) wild-type *Drosophila* embryos were fixed and stained for Centrosomin localization first using a Centrosomin-specific primary antibody followed by the Ab-nanotag conjugate as a secondary Ab (Scheme 3) and visualized with fluorescence microscopy (Figure 7B and 7C). Imaging the TO intercalator donor signal from the duplex reports Centrosomin localization (Figure 7B). This indicates that the secondary antibody is still functional after conjugation of multiple DNA duplexes to increase the fluorescence signal.

The observation of donor TO fluorescence is consistent with the fact that energy transfer to the Cy5 acceptor is only ca. 25% efficient in solution (Figure 6, design B), i.e. there are unquenched TO intercalators in the assemblies.

The centrosomes are clearly evident as bright foci, however some background fluorescence is present in the TO channel even after utilizing a zwitterionic buffer (0.5M PIPES/0.5M HEPES) to minimize non-specific binding of the oligonucleotides.⁴³ The excess background fluorescence could be due to (i) autofluorescence from the embryo, (ii) excess TO-conjugated strand from the initial assembly, and/or (iii) partial dehybridization of the TO-conjugated strand from the antibody reagent during the washing step prior to imaging. Importantly, TO fluorescence is not observed in the nuclei showing that the conjugated TO dyes do not stain the cellular DNA. (Compare Figure 7B with 7D, where genomic DNA is stained with DAPI.) In our prior work with noncovalent nanotags, the dissociation of dyes from the DNA framework led to nonspecific staining of cellular DNA, compromising the quality of cellular images acquired with these reagents.⁽⁹⁾ Covalent conjugation of the TO dyes to the DNA eliminates genomic DNA staining.

The background fluorescence is significantly reduced upon collection of the FRET signal (exciting the nanotag donor TO at 488 nm and collecting the emission of the Cy5 acceptor (Figure 7C). Thus, the emission wavelength of the nanotags can be red-shifted by >100 nm by FRET. In addition, the acquisition of the FRET signal indicates that the nanotag remains at least partially hybridized, since dissociation of the TO-conjugated strand would prevent sensitized emission from the Cy5, which is covalently attached to the antibody. Line profiles of both the TO and FRET signals show an improved signal-to-noise ratio (Figures 7E and 7F).

Spectral imaging with a confocal microscope was utilized to acquire the emission spectra of the Ab-nanotag conjugates in fixed syncytial embryos (Figure 8). The emission spectrum shown is the average pixel intensity of Centrosomin-Ab-nanotag foci of 4 different embryos ($n = 30$ -35 centrosomes per embryo) (Figure 8A). The emission spectrum of the Ab-nanotag conjugates containing both TO and Cy5 fluorophores is similar to the solution data of the duplex in Figure 6 (duplex B). Emission profiles collected without the presence of the Cy5 acceptor molecule within the Ab-nanotag do not show an emission peak at 663 nm (Figure 8B). In addition, the emission spectrum of the Ab-nanotag construct without the TO donor shows only a minimal amount of Cy5 fluorescence following excitation at 488 nm. It is difficult to compare the immunofluorescence results directly to the FRET efficiency results in solution (Figure 6), as the amount of protein at the centrosome can vary during the cell cycle, differences in immunolabeling between embryos can occur (Figure S4), and different in fluorescence emission filters used. However, a similar emission spectrum of the Ab-nanotag conjugate is observed in both the *in vitro* and the *in vivo* spectral imaging, illustrating efficient energy transfer to the Cy5 acceptor.

Conclusion

The results described above illustrate the value of DNA as a scaffold on which to assemble multifluorophore arrays for fluorescence imaging. Alkyne-functionalized nucleotides based on uracil¹¹ or 8-aza-7-deazaadenine³¹ allow click chemistry to be used to attach fluorescent or fluorogenic dyes to internal positions of a DNA strand without interfering with hybridization. Our results demonstrate that a trifunctionalized DNA strand labeled with TO intercalators provides sufficiently bright fluorescence, efficient energy transfer and facile hybridization to antibodies conjugated to complementary strands to create useful immunofluorescence reagents. While there have been numerous other examples of DNA-antibody conjugates, most prior work has relied on the DNA component to either hybridize to a target DNA or RNA strand in solution, as in proximity-based ligation assays⁴⁴, or to a

complementary DNA immobilized on a surface for preparation of antibody microarrays⁴⁵⁻⁴⁷. A recent report demonstrated DNA-based labeling of antibodies, but the DNA was labeled noncovalently with a cationic fluorescent polymer and antibody conjugation was achieved using biotinylated DNA and Ab, with streptavidin crosslinking the two.⁴⁸

Ongoing work is directed toward increasing the number of dyes attached to a given DNA strand in order to increase the brightness of a given DNA-functionalized antibody and/or reduce the number of DNA strands that need to be attached to an antibody to achieve a given level of brightness. (The brightness of our trifunctionalized ODN4 duplex label is lower than that of a single Cy5 dye, due primarily to the lower quantum yield of TO, although this would be offset by a much higher extinction coefficient in systems that incorporated more and/or brighter intercalator dyes.) We are also working on developing nanotags based on shorter wavelength dyes, such as the coumarin recently reported by Seela and Pujari, which emits at 420 nm.⁽³¹⁾ Based on the efficient energy transfer in our nanotags, even in the absence of large spectral overlap, excitation with the increasingly common violet laser line (405 nm) will allow emission across the visible spectrum, allowing for simultaneous multicolor imaging of different targets, provided orthogonal recognition components (e.g. antibodies) are used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to the NIH (R01GM080994) and the donors of the American Chemical Society's Petroleum Research Fund (44470-AC4) for financial support of this research. NMR instrumentation at Carnegie Mellon University was partially supported by NSF (CHE-0130903). Mass spectrometers were funded by NSF (DBI-9729351).

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Figure 1. Schematic of noncovalent (top) and covalent (bottom) fluorescent DNA nanotags. A simple linear nanotag is shown, but multidimensional versions are readily assembled.

Figure 2. UV melting curves (A) and fluorescence spectra (B) for DNA duplexes based on **ODN1-ODN3**. Samples contained 1.0 μ M duplex (A) or 0.1 μ M duplex (B) in 10 mM NaP_i, 100 mM NaCl, 0.1 mM EDTA.

Figure 3.

Fluorescence emission spectra recorded for **ODN3** duplex in the absence or presence of Cy5 energy acceptor dye on complementary strand. Samples contained 0.1 μ M duplex in 10 mM NaP_i, 100 mM NaCl, 0.1 mM EDTA. Samples were excited at 488 nm.

Figure 4.

Confocal fluorescence imaging of streptavidin-coated polystyrene microspheres (2 μm diameter) conjugated with biotinylated **ODN3** duplex (left) or **ODN3** duplex labeled with Cy3 (center) or Cy5 (right) FRET acceptor dyes. The following bandpass (BP) or longpass (LP) filters were used: for TO (left) 500-550nm BP, for Cy3 (center) 570-615nm (BP) and for Cy5 (right) 650nm LP. Scale bar is 10 μm .

Figure 5. (A) UV melting curve of trifunctionalized **ODN4-TO₃** duplex with and without Cy5 label. The initial absorbance ($T = 20^{\circ}\text{C}$) was subtracted from the rest of the curve to set both curves to zero. (B) Fluorescence emission spectra of **ODN4-TO₃** duplex with and without Cy5 label. Duplex concentration = 50 nM in both cases.

Figure 6.
Fluorescence emission spectra of various ternary duplexes based on trifunctionalized ODN4-TO₃ 39mer. Duplex concentration = 50 nM in both cases.

Figure 7.

Localization of Centrosomin (Cnn) in fixed 0-2 hr syncytical *Drosophila* embryos. A) Centrosomin localization within the embryo probed with a conventional Alexa488 labeled secondary antibody. Centrosomin stained with an Ab-nanotag conjugate containing TO and Cy5. Images were collected following excitation at 488 nm and collection of either the TO (B) or FRET (C) emission. D) Nuclei are shown by DNA staining (DAPI). The same embryo was imaged for micrographs B-D. Intensity profiles collected along the lines shown in B and C for the TO (E) and FRET (F) emission show that the FRET emission has improved brightness and signal-to-noise ratio compared to the TO emission. Scale bar is 20 μm.

Figure 8.

The average fluorescence emission spectra of the Ab-nanotag conjugates in fixed syncytical *Drosophila* embryos. The emission spectra were acquired after excitation at 488. A) The average pixel intensity at the centrosome of an Ab-nanotag conjugate containing both TO and Cy5 at a given emission wavelength ($n = 120 - 140$ centrosomes in 4 embryos). B) The average pixel intensity of Ab-nanotag conjugates containing only the TO donor (orange) or Cy5 acceptor (blue).

Scheme 1.

Scheme 3.

TO-N₃-1: R₁ = CH₃, R₂ = CH₂CH₂OCH₂CH₂N₃

TO-N₃-2: R₁ = CH₃, R₂ = (CH₂CH₂O)₂CH₂CH₂N₃

TO-N₃-3: R₁ = (CH₂CH₂O)₂CH₂CH₂N₃, R₂ = CH₃

Chart 1.