Electronic Supplementary Information

Molecular Fluorescent Dye for Specific Staining and Imaging of RNA in Live Cells: a Novel Ligand Integration from Classical Thiazole Orange and Styryl Compounds

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List of contents:

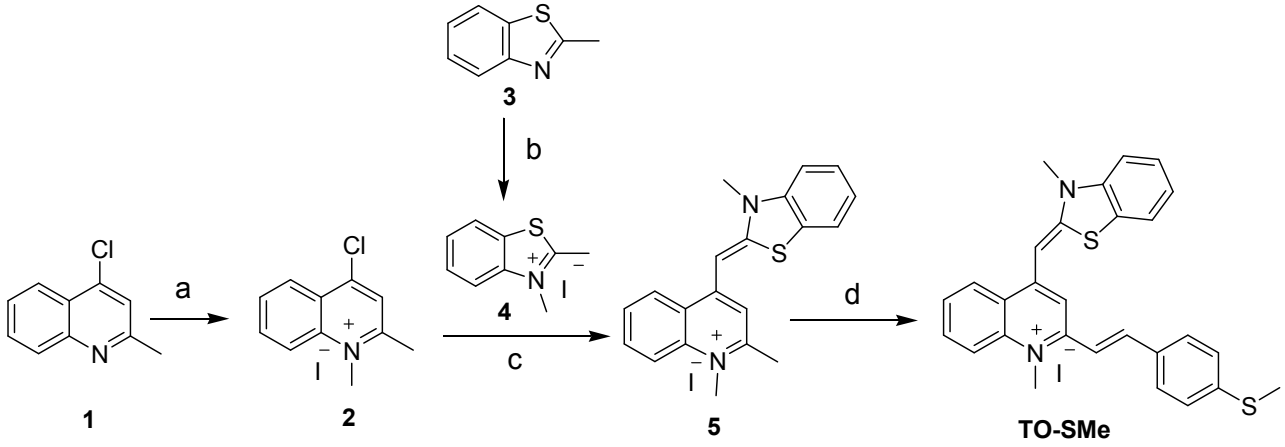
1. Materials and Instruments

RNA (16S- and 23S-Ribosomal from E. coli, Roche) and St-DNA (from salmon testes, Sigma) were used in a solution response study. Other oligonucleotides used in this study were purchased from Invitrogen (Shanghai, China). SYTO RNASelect, DNase-free RNase and DNase were purchased from Sigma-Aldrich. All the oligonucleotides, st-DNA, and RNA were dissolved in relevant buffer. To obtain G-quadruplex (G4) formation, highly concentrated solutions of oligonucleotides were annealed in relevant buffer containing KCl by heating to 95 °C for 5 min, followed by gradual cooling to room temperature and incubation at 4 °C overnight. The oligonucleotides were engaged in G-4 formation, as determined by circular dichroism (CD) measurements. Stock solutions of **Styryl-TO** (5 mM) were dissolved in DMSO and stored at -20 °C. Further dilutions of samples and SYTO RNAselect to working concentrations were made with relevant buffer immediately prior to use. PC3 cells were used in the cell staining study. Unless otherwise noted, chemical materials and solvents were purchased from commercial suppliers (Sigma-Aldrich and Aladdin) and were used without further purification. The pictures and images shown in both ESI and the manuscript are captured from a Leica DMRB fluorescence microscope and are not artificial.

Table S1. Sequences of oligonucleotides used in the present study

Name	Oligonucleotide Sequence	Type/Origin
ds26	5'-d(CAATCGGATCGAATTCGATCCGATTG)-3'□	Duplex
da21	5'-d[AAAAAAAAAAAAAAAAAAAAA]-3'□	Single-Strand
Oxy28	5'-GGGGTTTTGGGGTTTTGGGGTTTTGGGG-3'	Hybrid-Type G4
random	ATGTACCGATCA	Single-Strand
RNA	16S-and 23S-Ribosomal from E.coli,Roche	Single-Strand
htg21	5'-GGGTTAGGGTTAGGGTTAGGG-3'	Hybrid-Type G4
St-DNA	from salmon testes	Duplex

2. Synthesis and Characterization



Scheme S1. Synthesis route of **Styryl-TO.** Reagents and conditions: (a) iodomethane, tetramethylene sulfone, 52 °C, reflux; (b) iodomethane, absolute ethanol, 80°C, reflux; (c) $NaHCO_3$, methanol, room temperature; (d) p-methylthiobenzaldehyde, 4-methylpiperidine, n-butanol, 135°C, reflux.

^1H and ^{13}C NMR spectra were recorded using TMS as the internal standard in DMSO-d6 with a Bruker BioSpin GmbH spectrometer at 400 MHz, respectively. Mass spectra (MS) were recorded on Bruker amaZon SL mass spectrometer with an ESI or ACPI mass selective detector and high resolution mass spectra (HRMS) were recorded on Shimadzu LCMS-IT-TOF. **Styryl-TO** was identified by HPLC (Shimadzu Technologies) by using a C18 column (4.6×150mm) with a 4 min Elution and a gradient of 5%–95% CH_3CN -H_2O (containing 0.5% acetic acid). The HPLC contained a UV detector at λ = 250nm, 350 nm, 430 nm, and 500 nm.

Synthesis of 4-chloro-1,2-dimethylquinolin-1-ium iodide (2): To the solution of 4-Chloro-2-methylquinoline (0.2 g, 1.12 mmol) in sulfolane (10 mL) was added iodomethane (0.42 mL, 6.74 mmol). The reaction mixture was stirred at 50 °C for 18 h, cooled and anhydrous ether is added after the shock, suction filtration, the solid was washed with anhydrous diethyl ether, dried, and weighed in vacuum to give of Compound 2 (0.35 g, 96%): ^1H NMR (400 MHz, DMSO-d_6) δ 8.56 (d, J = 8.4 Hz, 1H), 8.46 (d, J = 8.3 Hz, 1H), 8.22 (t, J = 8.1 Hz, 1H), 8.01 (t, J = 7.9 Hz, 1H), 7.55 (s, J = 7.4 Hz, 1H) , 4.20 (s, 3H), 3.74 (s, 1H), 2.68 (s, 3H) . ESI-MS m/z: 192.3 $[M-I]^+$.

Synthesis of 1, 2-dimethyl–benzothiazol-1-ium iodide (4): A mixture of 2-Methylbenzothiazole (0.25 g, 1.68 mmol**)**, iodomethane (0.63 mL, 10.08 mmol) and anhydrous ethanol (10 ml) was stirred at reflux temperature for 15 h. After cooling, the mixture was dried over anhydrous ethanol and chloroform oscillating suction filtered. The precipitate was washed with chloroform and with a small amount of ethanol, and vacuum dried to give compound 4 (0.45 g, 91.7%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.44 (d, J = 8.1 Hz, 1H), 8.30 (d, J = 8.4 Hz, 1H), 7.90 (t, J = 7.8 Hz, 1H), 7.81 (t, J = 7.7 Hz, 1H), 4.20 (s, 3H), 3.54 (s, 1H), 3.17 (s, 3H). ESI-MS m/z: 164.2 $[M-I]^+$.

Synthesis of (Z)-1,2-dimethyl-4-((3-methylbenzo[d]thiazol-2(3H)-ylidene)methyl) quinolin-1-ium iodide (5): Compounds **2** (0.5 g, 1.60 mmol), **4** (0.5 g, 1.75 mmol) and aqueous sodium bicarbonate solution (0.5 mol/L, 2 ml) was added to a solution containing 10 ml of methanol, and stirred at room temperature for about 1 hour. To the reaction solution was added to 4 ml of saturated KI solution was stirred for about 15 minutes after filtration, washing with water and acetone, and dried to give compound 5 (0.49 g, 92.5%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.77 (d, J = 8.3 Hz, 1H), 8.18 (d, J = 8.7 Hz, 1H), 8.02 – 7.96 (m, 2H), 7.74 (d, J = 8.2 Hz, 2H), 7.59 (t, J = 7.7 Hz, 1H), 7.39 (t, J = 7.5 Hz, 1H), 7.34 (s, 1H), 6.85 (s, 1H), 4.07 (s, 3H), 3.98 (s, 3H), 2.87 (s, 3H). ESI-MS m/z: 318.9 $[M-I]^+$.

Synthesis of N-methyl-2-((E)-4-(methylthio)styryl)-4-((Z)-(3-methylbenzo[d]thiazol-2(3H)-ylidene)methyl)quinolin-1-ium iodide (6): A mixture of 5 (0.072 g, 0.16 mmol), p-methylthiobenzaldehyde (0.049 g, 0.32 mmol), 4-methylpiperidine (0.5 ml) and n-butanol (10 mL) was stirred at reflux temperature for 3 h. After the mixture was cooled, suction filtered, the solid washed with n-butanol, and dried to give Styryl-TO (0.068 g, 71%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.73 (d, J = 7.8 Hz, 1H), 8.14 (d, J = 8.4 Hz, 1H), 8.06 – 8.02 (m, 1H), 8.00 – 7.95 (m, 1H), 7.87 (d, J = 8.5 Hz, 2H), 7.76 – 7.68 (m, 3H), 7.64 (s, 1H), 7.61 – 7.55 (m, 2H), 7.42 – 7.35 (m, 3H), 6.87 (s, 1H), 4.13 (s, 3H), 3.97 (d, J = 3.7 Hz, 3H), 2.56 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 159.81, 152.50, 148.16, 142.08, 141.04, 140.86, 139.51, 133.71, 132.08, 129.41, 128.54, 126.94, 126.07, 125.67, 124.68, 124.26, 123.98, 123.44, 120.95, 119.01, 113.11, 108.42, 38.53, 34.11, 14.77. HRMS (ESI): m/z calcd for $C_{28}H_{25}N_2S_2^+$ ($[M - I]^+$) 453.1430; found 453.1416.

Synthesis of (E)-2-(2-(1H-indol-3-yl)vinyl)-1-methylquinolin-1-ium iodide (E36):

For detailed synthetic methods, please refer to Li's article. [1] ^{1}H NMR (400 MHz, DMSO) δ 12.32 (s, 1H), 8.83 (d, *J* = 9.1 Hz, 1H), 8.63 (dd, *J* = 17.8, 12.3 Hz, 2H), 8.49 – 8.36 (m, 2H), 8.24 (s, 2H), 8.09 (t, *J* = 7.9 Hz, 1H), 7.85 (t, *J* = 7.5 Hz, 1H), 7.57 (d, *J* = 15.2 Hz, 2H), 7.32 (d, *J* = 2.9 Hz, 2H), 4.48 (s, 3H). (ESI) m/z +1 for $C_{20}H_{17}N_2^+$ calcd 285.14 found 285.09.

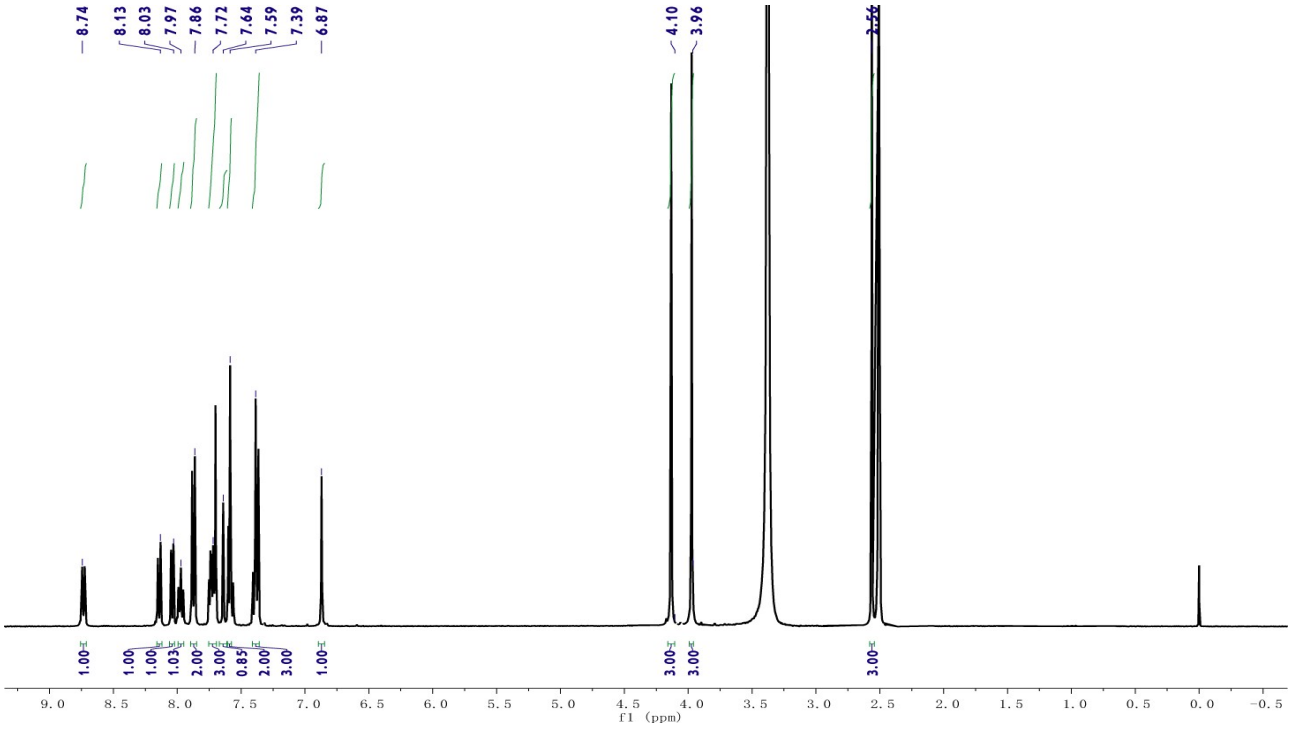


Fig.S1. ^{1}H NMR spectrum of **Styryl-TO**

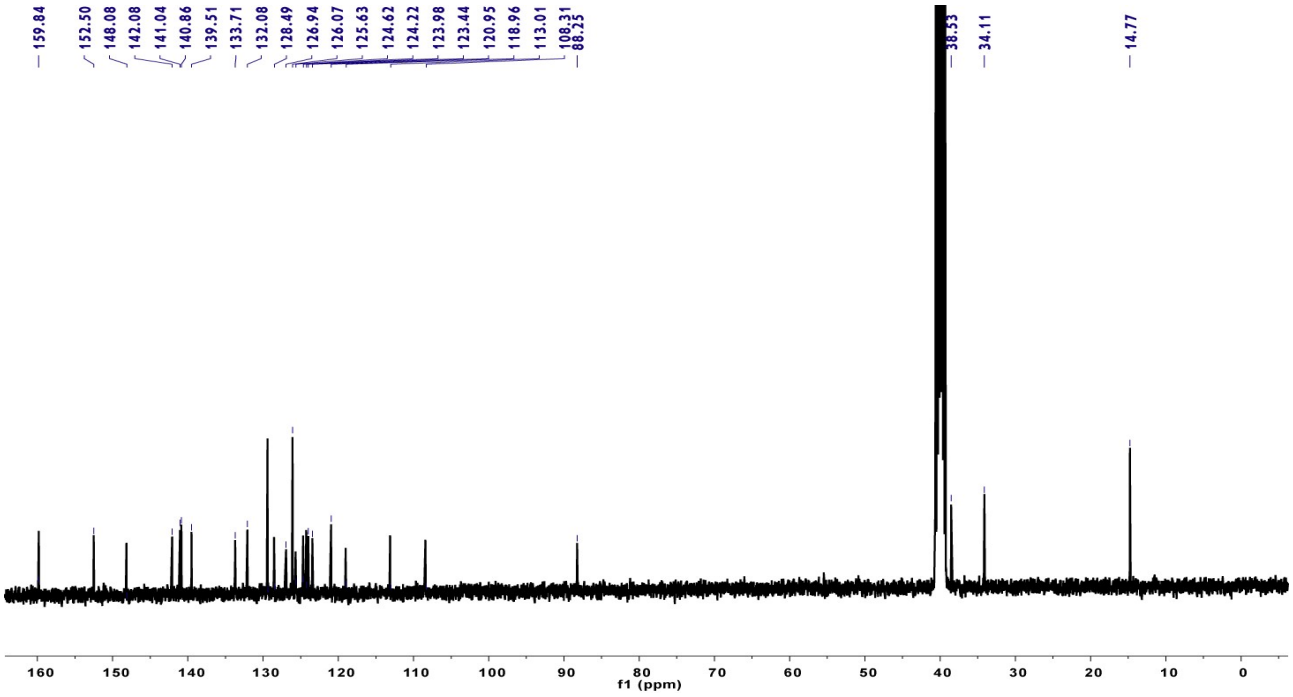


Fig. S2.[13]C NMR spectrum of **Styryl-TO**

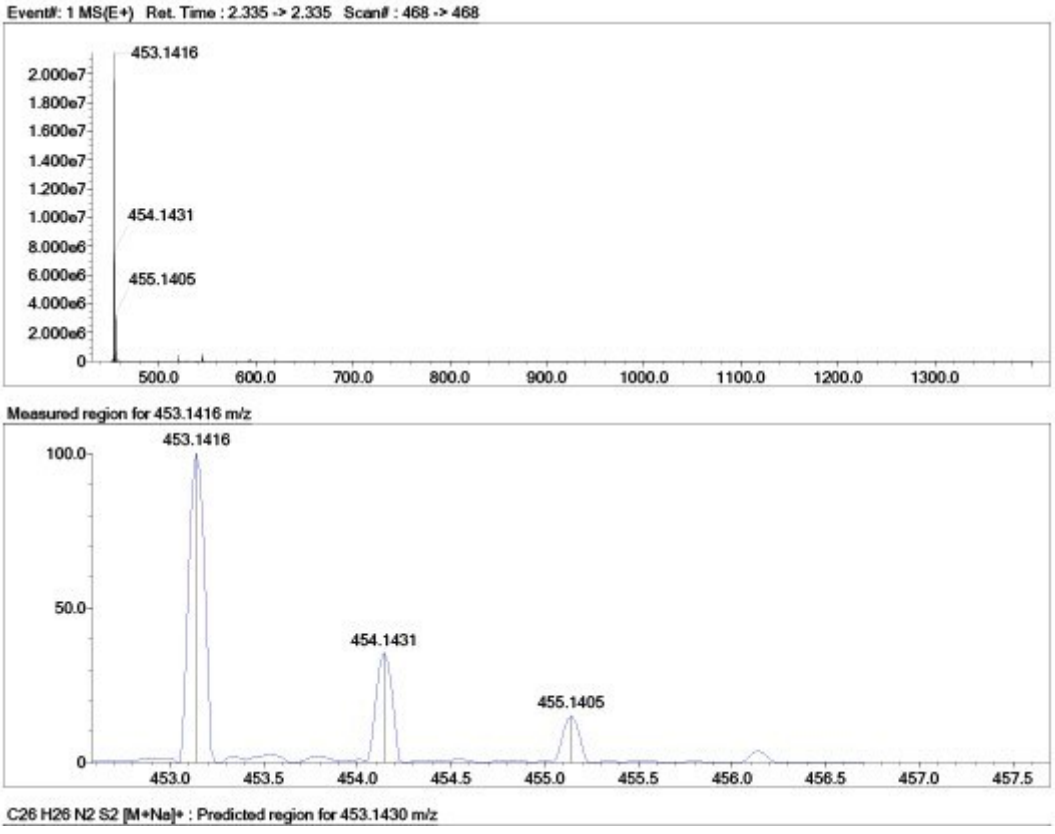


Fig. S3. MS analysis of **Styryl-TO**

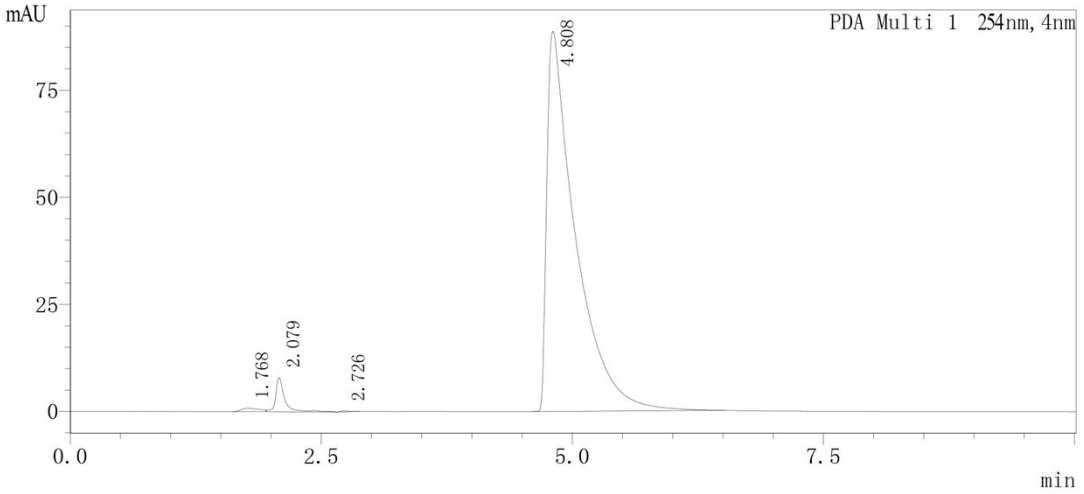


Fig. S4. HPLC analysis of **Styryl-TO**

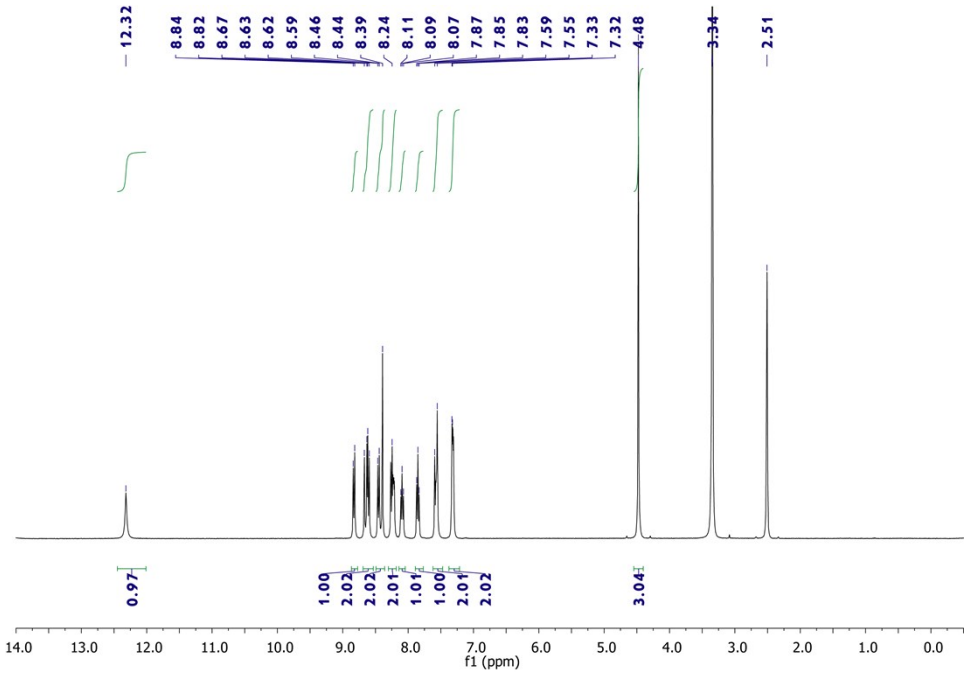


Fig.S5. ^{1}H NMR spectrum of E36

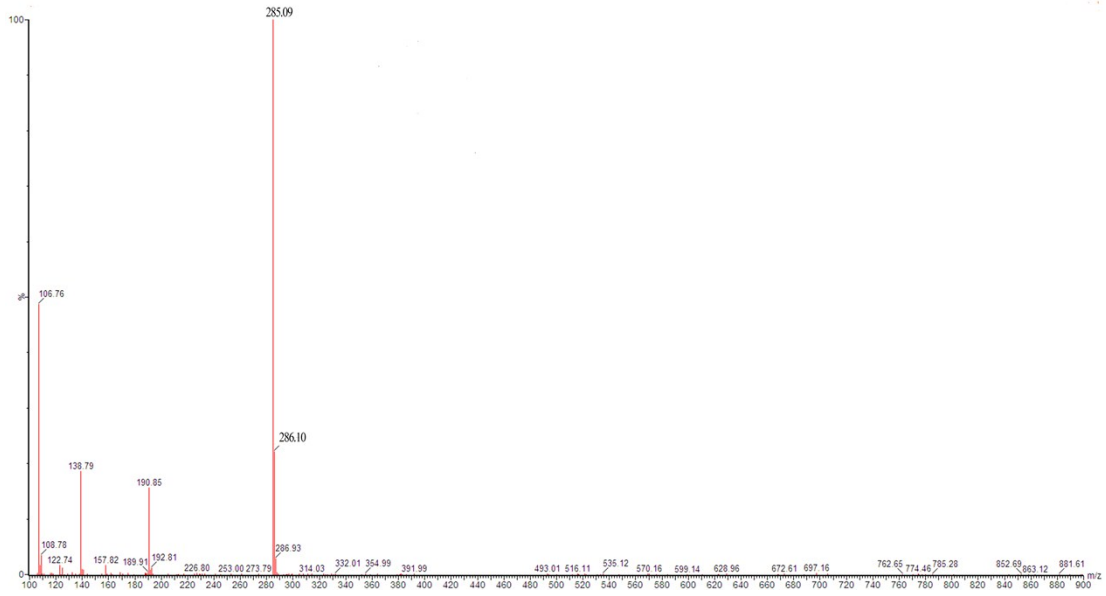


Fig. S6. MS analysis of **E36**

3. Photo-physical properties of Styryl-TO

All UV-visible spectra were obtained with a LAMBDA 25 UV/vis spectrometer (Perkin Elmer). Fluorescence emission /spectra were obtained with a Fluorescence spectrometer LS245 (Perkin Elmer), and all samples were excited at the isosbestic point between the corresponding absorbance spectra. The solubility of **Styryl-TO** in 10 mM Tris-HCl buffer containing 60 mM KCl buffer solution was 45 µM. The fluorescence quantum yields (Φ_f) of the RNA-bound dye were determined relative to fluorescein in 1.0 mol/L NaOH aqueous solution ($\Phi_f = 0.85$) as a reference.

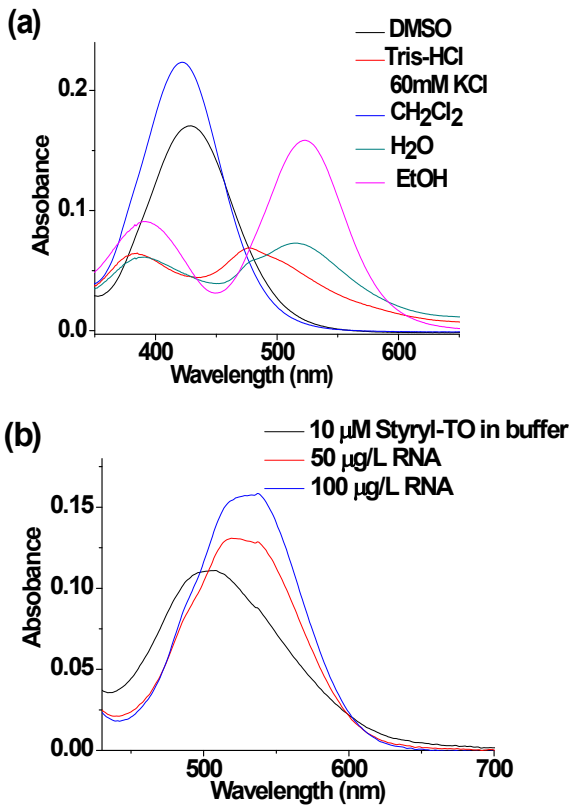


Fig. S7 (a) UV-vis spectra of 5 µM **Styryl-TO** in DMSO (black curve), 10 mM Tris-HCl buffer containing 60 mM KCl (red curve), CH_2Cl_2 (blue curve), water (green curve), and EtOH (pink curve); (b) UV-vis spectra of 5 µM **Styryl-TO** in the presence of RNA in 10 mM Tris- HCl buffer containing 60 mM KCl.

Table S2. Photophysical properties of **Styryl-TO**.

	λ_{max} (nm)		Φ_f
	Absorption	Emission [a]	
H_2O	520	528	0.0016
CH_2Cl_2	515	529	0.0053
RNA	520	531	0.0562

[a] Emission wavelength was determined by excitation at 527 nm

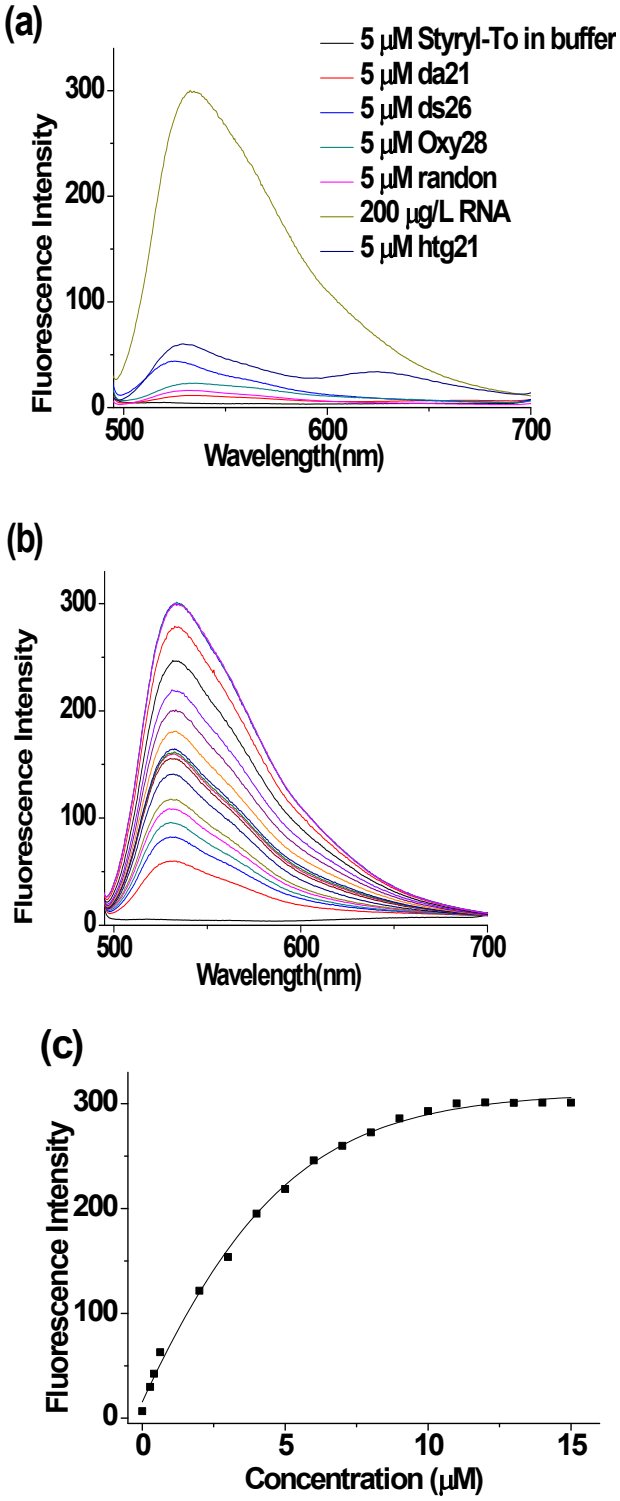


Fig. S8 (a) Fluorescence spectra (λ_{ex} = 475nm) of **Styryl-TO** (5 μM) in the absence (black curve) or presence of 1 equivalents of da21 (red curve), ds26 (blue curve), Oxy28 (green curve), randon (pink curve), htg21 (blue curve) or RNA (straw yellow curve). (b) Fluorescence titration of RNA (100 μg/L in Tris-HCl buffer, 60 mM KCl, λ_{ex} = 476 nm) upon

addition of increasing amount (0-15 μM) of **Styryl-TO**. (c) The plot of fluorescence intensity at 535nm versus the amount of **Styryl-TO** added.

4. Fluorescence titration with DNA

The change in fluorescence intensity of **Styryl-TO** with increasing amount of G-quadruplex DNA, double-stranded DNA, single-stranded DNA (see Table S1), and total DNA from salmon testes were recorded.

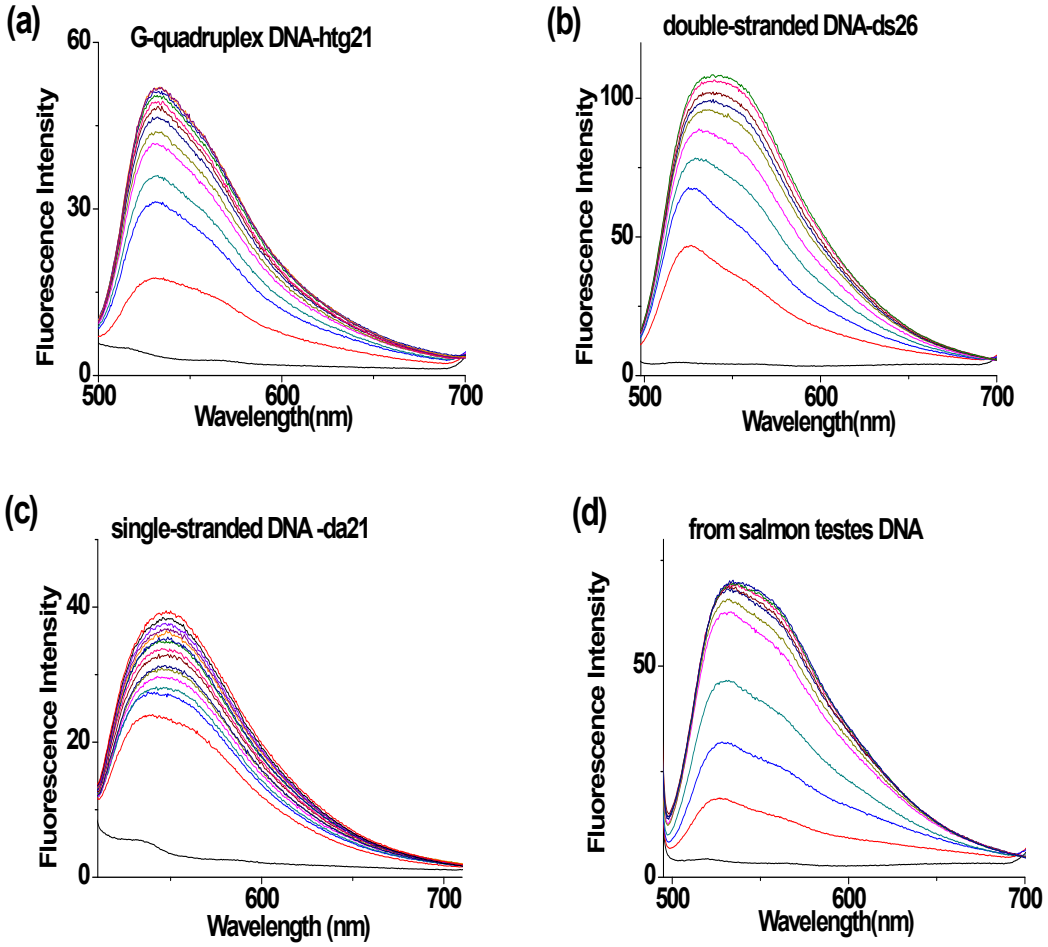


Fig. S9 Fluorescence titration of **Styryl-TO** (5 μM in Tris-HCl buffer, 60 mM KCl, λ_{ex} =476 nm) upon addition of increasing amount of G-quadruplex DNA –htg21 (a); double-stranded DNA-ds26 (b); single-stranded DNA da21(c); and total DNA from salmon testes (d).

With the data obtained through fluorimetric titrations, the binding constants were analyzed according to the independent site model [2] by nonlinear fitting to eq. (1) [3]. The

parameters P and M, Q and N were found via the Levenberg−Marquardt fitting routine in the Origin 8.5 software, whereas n was varied to obtain a better fit. $K_d = K_a^{-1}$.

$$F/F_0=1+(Q-1)/2\{N+1+X-[(X+1+N)^2 4X]^{1/2}\} \quad (1)$$

Where F_0 is the fluorescence intensity of **Styryl-TO** in the absence of DNA, F_{max} is the fluorescence intensity upon saturation of DNA, $Q=F_{max}(F_0)^{-1}$, $N=(K_aC_{dye})^{-1}$, and $X=nC_{DNA}(C_{dye})^{-1}$.

5. Cell culture and microscopy

5.1 Fixed cell staining

The human prostate cancer line PC3 was purchased from ATCC and grown in Dulbecco's modified Eagle's media (DMEM, Gibco) with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin (Invitrogen) at 37 °C with 5% CO_2 atmosphere. Cells were seeded in Lab-Tek chambered cover glass slide (Nunc) and grew overnight. Cells were fixed with 4% paraformaldehyde in PBS, stained with 5 μM of **Styryl-TO** in PBS for 15 minutes at room temperature. The cells were subsequently stained with 2 μM **DAPI** for 5 minutes at room temperature. The cells stained with **Styryl-TO** and **DAPI** were imaged with a Leica DMRB fluorescence microscope, equipped with a UV filter (330-380 nm) for observation of **DAPI** signal and a FITC filter (420-495 nm) for observation of **Styryl-TO** signal. 1000× magnification was utilized in the imaging. The brightness and contrast of all images were adjusted to improve picture quality.

5.2 Living cell staining

The PC3 cell line was grown in DMEM media with 10% fetal bovine serum, 1% penicillin/streptomycin at 37°C with 5% CO_2 atmosphere. Cells were seeded in Lab-Tek chambered cover glass slide (Nunc) and grew overnight. Cells were incubated with 5 μM of **Styryl-TO** in media for 15 minutes at room temperature and imaged directly without washing with a Leica DMRB fluorescence microscope, equipped with a FITC filter (420-495 nm) for observation of **Styryl-TO** signal.

The HUVEC (human umbilical vein endothelial cells), NIH-3T3 (mouse embryonic fibroblast cell line), and L929 (mouse fibroblasts cell line) were grown in DMEM media with 10% fetal bovine serum, 1% penicillin/streptomycin at 37 °C with 5% CO_2 atmosphere. Cells were seeded in Lab-Tek chambered cover glass slide (Nunc) and grew overnight. Cells were incubated with 5 μM of **Styryl-TO** in media for 15 minutes at room temperature and imaged directly without washing with a Leica DMRB fluorescence microscope, equipped with a FITC filter (420-495 nm) for observation of **Styryl-TO** signal.

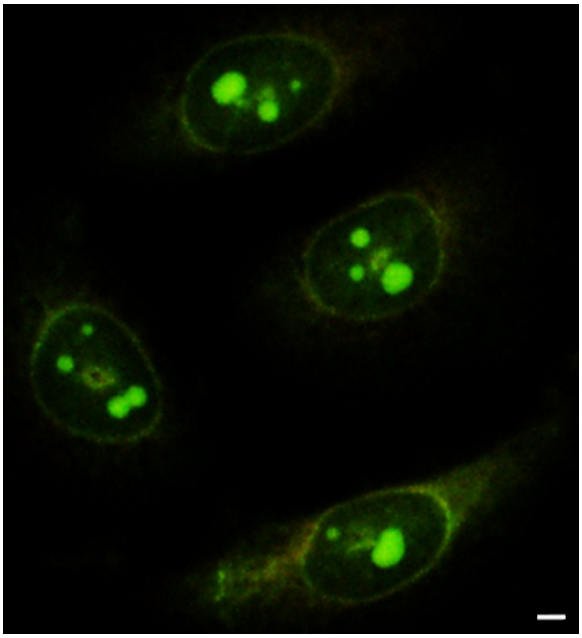


Fig. S10A. Fluorescence microscopic images of living PC3 cells incubated with **Styryl-TO** (5 μM) for 15 min. 1000× magnification was utilized in the imaging. Scale bar is 10 μm.

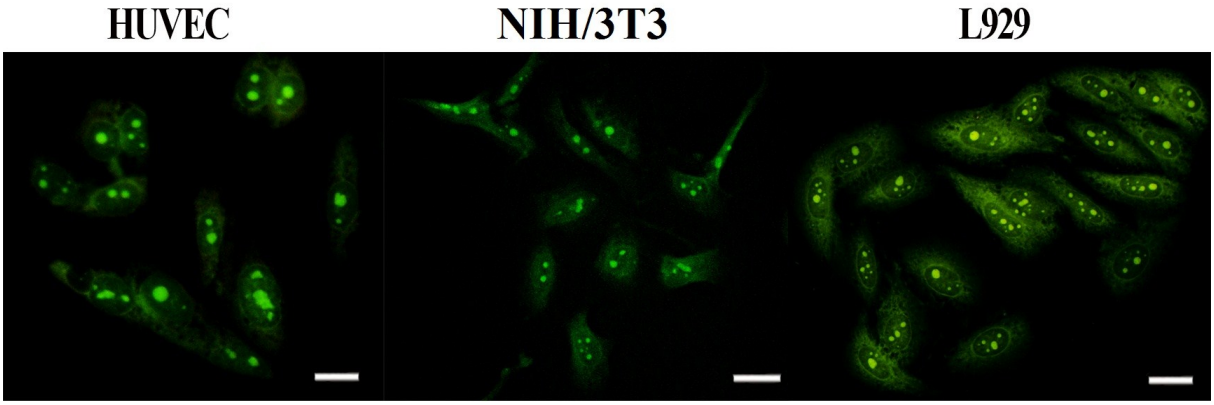


Fig. S10B. Fluorescence microscopic images of living HUVEC, NIH-3T3, L929 cells incubated with **Styryl-TO** (5 μM) for 15 min. 1000× magnification was utilized in the imaging. Scale bar is 10 μm.

6. DNase and RNase digestion tests

Cells were first fixed by pure methanol for 1 min at ambient temperature. The cell membrane was then permeabilized by immersing the cell in 1 % Triton X-100 for 2 min. After rinsing with PBS twice, 1 ml 5 μM dye PBS solution was added into three adjacent wells. Cells were then incubated in this dye PBS solution for 15 min in ambient temperature before being rinsed

by clean PBS twice. A total of 100 ml clean PBS (as control experiment), 50 mg/ml DNase (Sigma), or 75 mg/ml DNase-Free RNase (GE) was added into the three adjacent wells and incubated at 37 ^{o}C in 5% CO_2 for 3 hr. Cells were rinsed by clean PBS twice more before imaging. For each dye test, the fluorescent imaging pictures were obtained by using an equal exposure time for control, DNase, and RNase experiments. **Styryl-TO**, SYTO RNAselect and **E36** were made in 5 μM PBS solution for the test. The cells stained with Styryl-TO, SYTO RNAselect and E36 were imaged with a Leica DMRB fluorescence microscope, equipped with a FITC filter (420-495 nm) for observation of Styryl-TO signal. 1000× magnification was utilized in the imaging. Duplicate experiments have been tested. The brightness and contrast of all images were adjusted to improve picture quality.

7. Photo-stability

PC3 cells were incubated in 1mL culture solution consist of 5 μM **Styryl-TO** or 5 μM RNASelect for 15min and imaged by Inverted Fluorescence Microscope. RNASelect and **Styryl-TO** stained Cells were imaged at FITC channel. The initial intensity referred to the first scan of each dye. Photo-stability is an important factor for Evaluation applicability of new fluorescent probes in live cell imaging. **Styryl-TO** exhibited good cell tolerability under the cell imaging concentration after of incubation 24 h. The photo-stability of **Styryl-TO** and SYTO RNASelect in cell imaging were also examined. The laser powers 490 nm, 497 nm and 475 nm were used to irradiate the RNASelect, **E36** and **Styryl-TO** RNA buffer solution respectively and the fluorescence were collected at 530 nm for RNASelect, 548 for **E36** and 535 nm for **Styryl-TO** under continuous irradiation for 600 scans in 100 min by Fluorescence spectrometer LS245 (Perkin Elmer). Fluorescence spectra of 5 μM **Styryl-TO** and RNASelect in the presence of 100 mg/mL RNA in 10 mM Tris- HCl buffer containing 60 mM KCl. The MTT results showed that 86.7% of cells were alive after incubation 24 h.

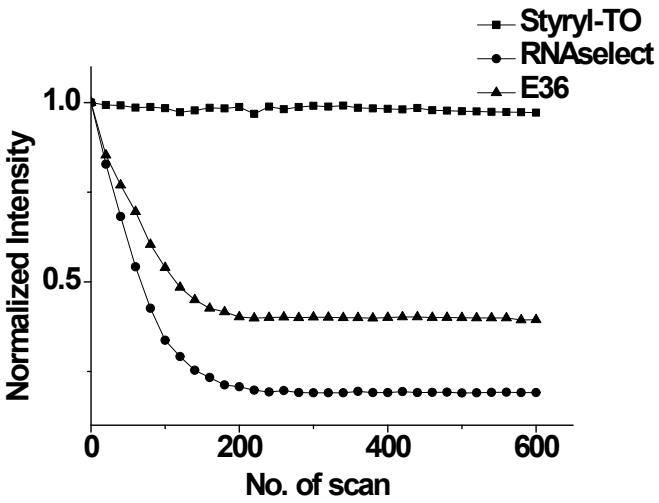


Fig. S11. Photo-stability of **Styryl-TO** (5 μM) **E36** (5 μM) and SYTO RNASelect (5 μM) with increasing number of scans

References

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[2] F. H. Stootman, D. M. Fisher, A. Rodger, J. R. Aldrich-Wright, *Analyst*, 2006, **131**, 1145.

[3] X. Xie, B. Choi, E. Largy, R. Guillot, A. Granzhan, M. P. Teulade Fichou, *Chem. Eur. J.,* 2013, **19**, 1214.