Supporting information

for

A Bright Red Fluorescent Cyanine Dye for Live-Cell Nucleic Acid Imaging, with High Photostability and Large Stokes Shift


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Protocols for dye syntheses

Known compound TO3 was synthesized by a published method\cite{S1}.

![Chemical structure of TO3-CN]

(a) \(\text{CH}_3\text{C}_6\text{H}_4\text{SO}_3\text{CH}_3\), 120 °C, 1h; (b) CNCH\(_2\)COOH, Pyridine, Et\(_3\)N, rt., overnight; (c) DMF, POCl\(_3\), 90 °C, 2h; (d) 210 °C, 30min; (e) CH\(_3\)CH\(_2\)I, toluene, reflux, 12h

**Scheme S1.** Synthetic route of dyes TO3-CN.

**Synthesis of 3-methyl-2-(methylthio)-benzothiazolium tosylate (1)** To 2.0 g (11 mmol) 2-(methylthio)-benzothiazole was added 3.0 g (16 mmol) methyl p-toluenesulfonate and heated to 130 °C for 1 h. After cooling to 70 °C, acetone was added until a white precipitate appeared. Reflux was maintained for another 30min before cooling to room temperature. The precipitate was collected by filtration and dried under vacuum to yield 1 (3.02 g, 74.5%). \(^1\)H NMR (400 MHz, CD\(_3\)OD) \(\delta\) (ppm): 8.22 (1 H, d, \(J = 8.2\)), 8.08 (d, \(J = 8.5, 1\) H), 7.85 (t, \(J = 7.8, 1\) H), 7.72 (t, \(J = 14.1, 6.3, 1\) H), 7.68 (d, \(J = 8.1, 2\) H), 7.21 (d, \(J = 8.1, 2\) H), 4.15 (s, 1 H), 3.13 (s, 1 H), 2.35 (s, 1 H). HR -TOF-MS Exact mass calculated for C\(_9\)H\(_{10}\)NS\(_2\) requires 196.0255. Found m/z 196.0261. HR -TOF-MS Exact mass calculated for C\(_7\)H\(_7\)O\(_3\)S\(^-\) requires 171.0116. Found m/z 171.0116.

**2-(3-methyl-2(3H)-benzothiazolylidene)acetonitrile (2)** To a solution of 7.35 g (20 mmol) 3-methyl-2-(methylthio)-benzothiazolium tosylate (1) in 50 mL pyridine, 2.04 g (24 mmol) cyanoacetic acid was added, followed by 2.43 g (24 mmol) of triethylamine. After overnight stirring of the resulting reddish material under a nitrogen atmosphere, the mixture was concentrated under vacuum. Roughly 3 volumes of water were slowly added to the reaction flask with stirring during which time a homogeneous solution was obtained followed by the precipitation of the product. The solids were collected by filtration and washed thoroughly with water to yield 2 (2.57 g, 68.5%) \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) (ppm): 7.39 (d, \(J = 7.7, 1\) H),
7.27 (t, 1H, J = 7.8 Hz), 7.08 (t, J = 7.6, 1H), 6.93 (d, J = 8.1, 1H), 4.22 (s, 1H), 3.32 (s, 3H).

**2-Formyl-2-(3-methyl-2(3H)-benzothiazolylidene)acetonitrile (3)** A 2.3 g (15 mmol) amount of phosphorus oxychloride was added dropwise to 10 mL dimethylformamide (DMF) in an ice bath. After the addition was complete, the solution was allowed to warm to room temperature. A solution of 2.27 g (12 mmol) 2-(3-methyl-2(3H)-benzothiazolylidene)acetonitrile (2) in 15 mL DMF was added. The mixture was then stirred at 90°C for 2 h under a nitrogen atmosphere. Then the mixture was cooled to room temperature and added to ice-water mixture. 1 M NaOH was slowly added to the reaction flask with stirring during which time a homogeneous solution was obtained followed by the precipitation of the product. The solids were collected by filtration and washed thoroughly with water to yield 3 (0.85g, 33%). 1H NMR (400 MHz, CDCl₃) δ (ppm): 9.52 (s, 1H), 7.76 (d, J = 8.0, 1H), 7.57 (t, J = 7.8, 1H), 7.49 – 7.36 (m, 2H), 4.23 (s, 3H).

**1-Ethyl-4-methylquinolinium iodide (4)** 1-Ethyl-4-methylquinolinium iodide 4a was synthesized by mixing lepidine (2.86 g, 20.0 mmol) with ethyl iodide (3.34 g, 24.0 mmol) and heating to reflux for 8 h in 20 mL toluene. After cooling, the solid was crushed, washed several times with ethyl ether and dried under vacuum to give crude product 4 (5.44 g, 91%). HR-TOF-MS Exact mass calculated for C₁₂H₁₄N (M⁺) requires 172.1126. Found m/z 172.1132.

**TO3-CN** 1-Ethyl-4-methylquinolinium iodide 4a (0.60g, 2 mmol) and 2-Formyl-2-(3-methyl-2(3H)-benzothiazolylidene)acetonitrile 3 (0.22g, 1 mmol) were heated to 210 °C for 30 min and then allowed to cool. The mixture was purified by silica flash column chromatography (DCM/methanol=50:1) to yield the product TO3-CN (0.19 g, 38%). The HPLC purity of TO3-CN was determined to be 98.44%. 1H NMR (400 MHz, DMSO) δ (ppm): 8.99 (d, J = 6.8, 1 H), 8.55 (d, J = 8.0, 1 H), 8.39 (d, J = 8.7, 1 H), 8.29 (d, J = 6.8, 1 H), 8.21 – 8.11 (2 H, m), 8.01 (d, J = 7.1, 1 H), 7.98 – 7.89 (1 H, m), 7.77 (d, J = 8.2, 1 H), 7.60 (t, J = 7.9, 1 H), 7.44 (t, J = 8.0, 1 H), 7.19 (d, J = 14.4, 1 H), 4.86 (q, J = 7.3, 2 H), 4.12 (2 H, s), 1.54 (t, J = 7.1, 3 H). 13C NMR (101 MHz, DMSO) δ (ppm): 165.6, 145.5, 143.3, 141.9, 138.1, 137.0,
135.0, 129.0, 128.5, 125.9, 125.6, 124.9, 123.0, 119.3, 119.2, 118.1, 114.1, 114.1, 110.0, 75.9, 51.4, 36.7, 15.5. HR-TOF-MS Exact mass calculated for $\text{C}_{23}\text{H}_{20}\text{N}_{3}\text{S}$ requires 370.1378. Found m/z 370.1365.

Figure S1. The absorption and fluorescent spectra of $\text{TO3-CN}$ (left) and $\text{TO3}$ (right) in different solvents.

The absorption blue shift is $\Delta S = \Delta S_0 - \Delta S_1$

Figure S2. The calculated dipole moments of the ground state (9.7727) and the excited state (9.5190) of $\text{TO3-CN}$. 
**Figure S3.** The calculated dipole moments of the ground state (3.9069) and the excited state (5.0688) of TO3.

**Figure S4.** Absorbance and emission spectra of TO3 in the absence and presence of CT DNA (left) or RNA (right) in buffer.

**Figure S5.** Absorbance and emission spectra of TO3-CN in the absence and presence of CT DNA (left) or RNA (right) in buffer.
**Figure S6.** Fluorescence response of TO3 (1 μM) to CT DNA (red line), tRNA (black line), and BSA (blue line) at different concentrations in Tric-HCl buffer (10 mM, pH = 7.4).

**Figure S7.** CD spectra during the titration of a 10.5 μM solution of TO3 with CT DNA at 20 °C in buffer. The [base pair]/[TO3] molar ratios are 0.33, 0.67 in (a), 1, 1.33, 1.67, 2, 4 in (b) and 8, 16, 20, 30 and 40 in (c). The black dashed line refers to TO3 without CT DNA. The arrows indicate how the CD bands respond to the increases in the CT DNA concentration.

**Figure S8.** CD spectra during the titration of a 21 μM solution of TO3-CN with CT
DNA at 20 °C in buffer. The [base pair]/[TO3-CN] molar ratios are 0.33, 0.67 in (a) and 2, 4, 8, 20 and 40 in (b). The black dashed line refers to TO3-CN without CT DNA. The arrows indicate how the CD bands respond to the increases in the CT DNA concentration.

**Figure S9.** Live-cell staining and DNase and RNase digest experiments with TO3-CN in HeLa cells. TO3-CN (excited at 559 nm and collected at 575 nm to 620 nm) was cultured at 2 μM concentration for 45 min. The scale bar represents 20 μm.

**Figure S10.** Colocalization imaging of HeLa cells stained with 3.0 μM SYTO 9 and
2.0 μM TO3-CN for 45 min at 37 °C. (a) Confocal image from SYTO 9 on channel 1 (495—535 nm, λex = 488 nm). (b) Confocal image from TO3-CN on channel 2 (575—620 nm, λex = 559 nm). (c) Merged image of channels 1 and 2. (d) Intensity profile of ROIs across MCF-7 cells. (e) Correlation plot of SYTO 9 and TO3-CN intensities.

**Figure S11.** Comparisons of the cytotoxicity of TO3 and TO3-CN at various concentrations (1, 3, and 5 μM) in living COS7 or HeLa cells for 6 h.
Figure S12. The HPLC purity of TO3-CN

\[ \text{Processed Channel Descr.: PDA 550.0 nm} \]

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\[ ^1\text{H and } ^{13}\text{C NMR spectra} \]
$^{1}H$ NMR spectrum of TO3-CN

$^{13}C$ NMR spectrum of TO3-CN