Cyanine fluorophore derivatives with enhanced photostability


<table>
<thead>
<tr>
<th>Supplementary Figure 1</th>
<th>General schematic representation of TSQ conjugation to Cy5.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Figure 2</td>
<td>Spectral properties of Cy5-TSQ conjugates.</td>
</tr>
<tr>
<td>Supplementary Figure 3</td>
<td>Effect of TSQs on Cy5 fluorescence emission intensity.</td>
</tr>
<tr>
<td>Supplementary Figure 4</td>
<td>Dependence of the photon emission and photobleaching rates on illumination intensity.</td>
</tr>
<tr>
<td>Supplementary Figure 5</td>
<td>Blinking behavior measured at high time resolution.</td>
</tr>
<tr>
<td>Supplementary Figure 6</td>
<td>Relative rate of singlet oxygen generation of Cy5-TSQ conjugates compared with Cy5.</td>
</tr>
<tr>
<td>Supplementary Figure 7</td>
<td>Comparison of TSQs in solution and attached proximal to Cy5.</td>
</tr>
<tr>
<td>Supplementary Figure 8</td>
<td>Enhanced photostability of Cy5-TSQ conjugates in an oxygenated environment.</td>
</tr>
<tr>
<td>Supplementary Table 1</td>
<td>Photobleaching time constants.</td>
</tr>
<tr>
<td>Supplementary Note</td>
<td>Chemical synthesis of TSQ-NHS and TSQ-NH2 derivatives and Cy5-TSQ conjugates.</td>
</tr>
</tbody>
</table>

Note: Supplementary videos 1 and 2 are available on the Nature Methods website.
Supplementary Figure 1. General schematic representation of TSQ conjugation to Cy5. COT, NBA or TX is chemically modified to contain an amine moiety (Supplementary Note) that can be covalently attached to bis-NHS reactive Cy5. After purification of the monoreacted species, the product is competent for coupling to a biomolecule of interest.
Supplementary Figure 2. Spectral properties of Cy5-TSQ conjugates. (a) Normalized absorption and (b) emission spectra (excited at 629 nm) for Cy5 (grey), Cy5-COT (olive), Cy5-NBA (red) and Cy5-TX (blue), corrected to same absorbance to ensure all dyes absorb equal amount of photons. The difference in fluorescence intensity reports on variability in quantum yield. (c) The fluorescence lifetime measurements, fit by double exponential functions (black lines), indicate modest variation in the weighted average of fluorescence lifetime: Cy5 (1.122 ns); Cy5-COT (1.271 ns); Cy5-NBA (1.154 ns); and Cy5-TX (0.9565 ns). All measurements were taken using free dyes in 100% methanol (Online Methods).
Supplementary Figure 3. Effect of TSQs on Cy5 fluorescence emission intensity. (a) The mean fluorescence intensities (in photons detected per 100 ms frame) under conditions of TSQs in solution (TSQ sln) and directly conjugated to the dye (Cy5-TSQ). (b) Average number of photons detected (µ) for Cy5 and Cy5-TSQ derivatives prior to photobleaching (τon x average number of photon emitted per 100 ms frame). Error bars are the s.d. across n=6 movies from at least two independent experiments.
Supplementary Figure 4. Dependence of the photon emission and photobleaching rates on illumination intensity. Single-molecule fluorescence traces were obtained for fluorescently labeled DNA duplexes at 100 ms time resolution at various laser powers reflecting illumination intensities typical for single-molecule imaging. (a) Mean fluorescence intensities (average number of photons detected per 100 ms frame). (b) the average total time in fluorescent states (Total $\tau_{\text{on}}$) prior to photobleaching. Total $\tau_{\text{on}}$ is the sum of all fluorescence dwells observed prior to photobleaching. Cy5 (gray squares), Cy5-COT (green circles), Cy5-NBA (red triangles), and Cy5-TX (blue inverted triangles).
Supplementary Figure 5. Blinking behavior measured at high time resolution. Single-molecule fluorescence traces of Cy5-labeled DNA duplexes performed at 75 mW laser power and 10 ms time resolution to investigate high-frequency blinking behavior potentially undetected at 100 ms (Fig. 1). (a) Representative traces of Cy5 fluorescence under direct excitation in the absence of TSQ (Cy5) and with COT, NBA, or TX directly coupled to the dye. (b) Bar graphs showing average dwell times in the fluorescent state ($\tau_{on}$) with TSQs directly conjugated to Cy5 (Cy5-TSQ). (c) Comparative intensities of Cy5 parent and Cy5-TSQ conjugates. Error bars are the s.d. across n=6 movies from at least two independent experiments.
Supplementary Figure 6. Relative rate of singlet oxygen generation of Cy5-TSQ conjugates compared with Cy5. Singlet oxygen generation of Cy5 and the Cy5-TSQ derivatives was monitored as described in Online Methods. A control experiment with PCD demonstrated that molecular oxygen in solution is required for singlet oxygen generation.
Supplementary Figure 7. Comparison of TSQs in solution and attached proximal to Cy5.

The average $\tau_{on}$ is shown from experiments in which TSQs are present at 1 mM in solution (TSQ sln) or proximally linked at the second base position from the 3’ end of the complementary DNA strand (TSQ-2). Error bars are the s.d. from n≥3.
Supplementary Figure 8. Enhanced photostability of Cy5-TSQ conjugates in an oxygenated environment. Single-molecule fluorescence traces of fluorescein-labeled DNA duplexes were collected at 60 mW laser power and 20 ms time resolution. (a) Representative traces of Cy5 fluorescence under direct excitation in the absence of TSQ and with COT, NBA, or TX directly coupled to the dye. (b) Bar graphs showing average dwell times in the fluorescent state ($\tau_{on}$). (c) Comparative intensities of Cy5 parent and Cy5-TSQ conjugates. Error bars are the s.d. across n=6 movies from at least two independent experiments.
Supplementary Table 1. Photobleaching time constants. The loss of fluorescence at the surface due to photobleaching (Fig. 3a,b) was fit to an exponential function. The time constants (seconds) are shown with oxygen scavenging (+PCD) and without oxygen scavenging (-PCD).

<table>
<thead>
<tr>
<th>Sample</th>
<th>+PCD (s)</th>
<th>-PCD (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy5</td>
<td>3.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Cy5-COT</td>
<td>47.6</td>
<td>3.4</td>
</tr>
<tr>
<td>Cy5-NBA</td>
<td>43.7</td>
<td>3.4</td>
</tr>
<tr>
<td>Cy5-TX</td>
<td>145.0</td>
<td>1.1</td>
</tr>
</tbody>
</table>
Supplementary Note

Chemical synthesis of TSQ-NHS and TSQ-NH₂

**COT-NHS, COT-NH₂**

To a stirred solution of cyclooctatetraene (3.0 g, 28.8 mmol) in DCM (30 ml) was slowly added a solution of Br₂ (4.6 g, 28.8 mmol) in DCM (20 ml) at -70 °C. The resulting solution was stirred at -70 °C for 1 h, at which point a solution of potassium tert-butoxide (4.5 g, 40 mmol) in THF (20 ml) was added dropwise. The reaction mixture was stirred at -60 °C for 4 h, warmed to -10 °C, and poured into ice water. Using a small amount of MgSO₄ to break up the emulsion, the organic layer was removed and the aqueous layer extracted with diethyl ether (3 x 20 mL). The combined extracts were dried over MgSO₄, filtered, and concentrated to give COT-Br as light yellow oil (5.1 g, 97%) which was used without further purification. ¹H NMR (CDCl₃): δ 6.22 (s, 1H), 5.74 – 5.98 (m, 5H), 5.62 (s, 1H); ¹³C NMR (CDCl₃): δ 133.2, 133.1, 132.8, 132.4, 132.0, 130.9, 121.4.

To a stirred 0 °C solution of allyloxy-tert-butylsilane (1.0 g, 5.8 mmol) in THF (5 ml) was added 9-borabicyclo[3.3.0]nonane (0.5 M in THF, 13 mL, 6.5 mmol). The reaction solution was slowly warmed to rt and stirred for 3 h. At that point COT-Br (1.27 g, 6.9 mmol), NaOH (3 M solution, 5.7 ml, 17.1 mmol), and tetrakis(triphenylphosphine)palladium(0)(100 mg, 0.88 mmol) were added, and the mixture was heated at reflux overnight. At that point, the reaction mixture was cooled, diluted with 1:1 hex/EtOAc, washed with water and brine, then dried over MgSO₄, filtered, and concentrated. The residue was purified by silica gel chromatography (1:20 EtOAc/hex) to provide the desired product as a light brown liquid (1.0 g, 3.62 mmol, 62%). ¹H NMR (CDCl₃): δ 5.85-5.72 (m, 6H), 5.61 (s, 1H), 3.72 (t, J = 6.2 Hz, 2H), 2.15 (s, 2H), 1.73-1.61 (m, 2H), 0.90 (s, 9H), 0.06 (s, 6H); ¹³C NMR (CDCl₃): δ 143.9, 134.1, 132.2, 132.1, 131.8, 131.5, 131.0, 126.8, 62.4, 32.1, 31.3.

To a stirred rt solution of COT-OTBS (700 mg, 2.5 mmol) in THF (2 ml) was added tetrabutylammonium fluoride (1M in THF, 5 mL, 5 mmol). The resulting solution was stirred for 2 h, at which point it was diluted with EtOAc (20 ml), washed water and brine, dried over MgSO₄, filtered, and concentrated. The residue was purified by silica gel...
chromatography (1:3 EtOAc/hex) to afford the target compound as a light yellow oil (300 mg, 73%). $^1$H NMR (CDCl$_3$): $\delta$ 5.96-5.67 (m, 6H), 5.62 (s, 1H), 3.72 (t, $J = 6.2$ Hz, 2H), 2.22 (s, 2H), 1.69 (t, $J = 6.2$ Hz, 2H); $^{13}$C NMR (CDCl$_3$): $\delta$ 143.9, 134.1, 132.2, 132.1, 131.8, 131.5, 131.0, 126.8, 62.4, 34.1, 31.3.

To a stirred 0 °C solution of COT-OH (140 mg, 0.86 mmol) in acetone (3 ml) was added freshly made Jones reagent (3 M, 576 µL, 1.7 mmol). The reaction was stirred at 0 °C for 1 h, and then quenched by the addition of MeOH. The solvent was removed in vacuo and the resulting residue taken up in EtOAc and water, then extracted with EtOAc (3 x 20 ml). The combined organic layers were dried over Na$_2$SO$_4$ and concentrated. The resulting residue was passed through a short plug of silica gel using 2:1 EtOAc/hex to provide 40 mg of the crude acid (estimated yield 26%), which was taken on without further purification.

To a stirred 0 °C solution of COT-COOH (40 mg, 0.23 mmol) and NHS-OH (31 mg, 0.25 mmol) in THF (5 ml) was added a solution of dicyclohexylcarbodiimide (56 mg, 0.25 mmol) in THF (3 ml) dropwise. The reaction mixture was slowly warmed to room temperature and stirred overnight. At that point, the resulting slurry was filtered and washed with THF. The combined filtrate and wash was then dried over Na$_2$SO$_4$ and concentrated. The resulting residue was purified by silica gel chromatography (1:5 acetone/DCM) to provide COT-NHS as a yellow oil (31 mg, 48%). $^1$H NMR (CDCl$_3$): $\delta$ 5.96-5.72 (m, 7H), 5.68 (s, 1H), 2.87 (s, 4H), 2.77 (t, $J = 6.2$ Hz, 2H), 2.48 (brs, 2H); $^{13}$C NMR (CDCl$_3$): $\delta$ 169.0, 168.0, 141.3, 132.7, 132.0, 131.7, 131.5, 131.4, 128.0, 32.1, 39.5, 25.6.

To a stirred 0 °C solution of ethylenediamine (60 mg, 1 mmol) in DCM (3 ml) was slowly added a solution of COT-NHS (27mg,0.1mmol) in DCM (2 ml).The solution was warmed to rt and stirred for 1 h, then diluted with DCM (15 ml), washed with saturated aq. Na$_2$CO$_3$ solution and brine, then dried over Na$_2$SO$_4$, filtered, and finally concentrated to give 15 mg of a yellow oil (approximately 68%), which was carried on without further purification. ESI-MS: m/z calculated for C$_{13}$H$_8$N$_2$O [M+H]$^+$ 219.1, found: 219.1.
NBA-NHS and NBA-NH₂

\[
\text{O}_2\text{N} - \text{O} - \text{N} - \text{O} - \text{O} - \text{N} - \text{O}
\]

Synthesized following a previously published procedure\(^1\).

Trolox-NHS and Trolox-NH₂

\[
\text{O}_2\text{N} - \text{N} - \text{NH}_2
\]

Synthesized following a previously published procedure\(^2\).

\[
\begin{align*}
\text{HO} & - \text{O} - \text{O} - \text{N} - \text{O} - \text{O} - \text{N} - \text{O} - \text{N} - \text{NH}_2 \\
\text{DCM, 0 °C} & \rightarrow \\
\text{HO} & - \text{O} - \text{O} - \text{N} - \text{O} - \text{O} - \text{N} - \text{O} - \text{N} - \text{NH}_2
\end{align*}
\]

To a 0 °C solution of ethylenediamine (160 mg, 2.7 mmol) in DCM (10 ml) was slowly added Trolox-NHS (89 mg, 0.26 mmol) as a solution in DCM (5 ml). The resulting solution was warmed to rt and stirred for 1 h. At that point, the reaction was diluted with DCM (30 ml), washed with saturated aq. Na₂CO₃ solution and brine, then dried over Na₂SO₄, filtered, and concentrated to furnish the crude Trolox-NH₂ (32 mg, approx. 43%) as white solid which was carried forward without further purification. ESI-MS: \(m/z\) calculated for \(C_{16}H_{24}N_2O_3\) [M+H]\(^+\) 293.2, found: 293.2.

Chemical Synthesis of Cy5-TSQ conjugates

One equivalent of TSQ-NH₂ in 20 µL DMSO was mixed in 150 µL of 50 mM potassium borate buffer pH = 8.1 followed by addition of 40 µL of Cy5 bis-NHS ester (1 mg, 1.1 µmol) in DMSO. The mixture was again vortexed and then incubated in the dark at room temperature. The reaction was monitored by LCMS every 5 min until completion with total reaction time typically about 25 min. Once complete, the reaction was diluted to 3 mL with distilled, deionized water (ddH₂O). Monoreacted fluorophore was purified from unreacted and bis-reacted fluorophores using a semipreparative HPLC C18 T3 column (Waters) with a 0.1% formic acid mobile phase in a gradient from 25-65% acetonitrile.
After evaporation of acetonitrile, the product was concentrated and buffer exchanged over a Sep-Pak C18 column and eluted in methanol followed by evaporation in a speed vac.

Unless otherwise stated, all commercially available materials were purchased from Aldrich, TCI, or Alfa Aesar and were used without any further purification. When necessary, solvents and reagents were dried prior to use, using standard protocols. All non-aqueous reactions were carried out in oven-dried glassware under an atmosphere of Argon. Analytical thin layer chromatography (TLC) was performed on silica gel 60, F254 plates (0.25 mm thickness) from SiliCycle. Visualization was accomplished by either irradiation under a 254 nm UV lamp or by staining with an aqueous solution of ceric ammonium molybdate (CAM). Flash chromatography was performed on silica gel 60 (230- 400 mesh). All LC-based separations involved a mobile phase of 0.1% formic acid (v/v) in water (solvent A) and pure acetonitrile (solvent B). HPLC separations were performed using a Varian PrepStar SD-1 solvent delivery system equipped with a Varian ProStar 335 diode array detector and a Waters Atlantis®Prep T3 column (5 µm, 19 x 150 mm), with a similarly packed guard column. LCMS separations were performed using a Waters ACQUITY UPLC system equipped with ACQUITY PDA (diode array) and FLR (fluorescence) detectors, a Waters Micromass SQD 2000 spectrometer, and a Waters ACQUITY HSS T3 column (1.8 µm, 2.1 x 100 mm). $^1$H and $^{13}$C NMR spectra were acquired on a Bruker DRX-500 spectrometer at 500 MHz for $^1$H and 125 MHz for $^{13}$C. Chemical shifts are expressed in parts per million downfield from tetramethylsilane (TMS), using either TMS or the solvent resonance as an internal standard (TMS, $^1$H: 0 ppm; chloroform, $^{13}$C: 77.0 ppm). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constant, and integration.
Chemical structures of TSQ Conjugated Cy5 NHS ester

**COT-Cy5-NHS:**

LCMS: 25-65% B over 2.5 min, rt = 1.73 min
HPLC: 25-65% B over 25 min, rt = 13.01 min
ESI-MS: m/z calculated for $C_{54}H_{65}N_5O_{12}S_2$  
\[
[M+H]^+ \text{ 1040.4, found 1040.7}
\]

**NBA-Cy5-NHS:**

LCMS: 25-65% B over 2.5 min, rt = 1.28 min
HPLC: 25-65% B over 25 min, rt = 7.33 min
ESI-MS: m/z calculated for $C_{50}H_{60}N_6O_{13}S_2$  
\[
[M+H]^+ \text{ 1017.4, found: 1017.7}
\]

**TX-Cy5-NHS:**

LCMS: 25-65% B over 2.5 min, rt = 1.80 min
HPLC: 25-65% B over 25 min, rt = 13.65 min
ESI-MS: m/z calculated for $C_{57}H_{71}N_5O_{14}S_2$  
\[
[M+H]^+ \text{ 1114.5, found: 1114.8}
\]
Final products of NHS activated Cy5 derivatives characterized by LC/MS

References:

