Additional Figures

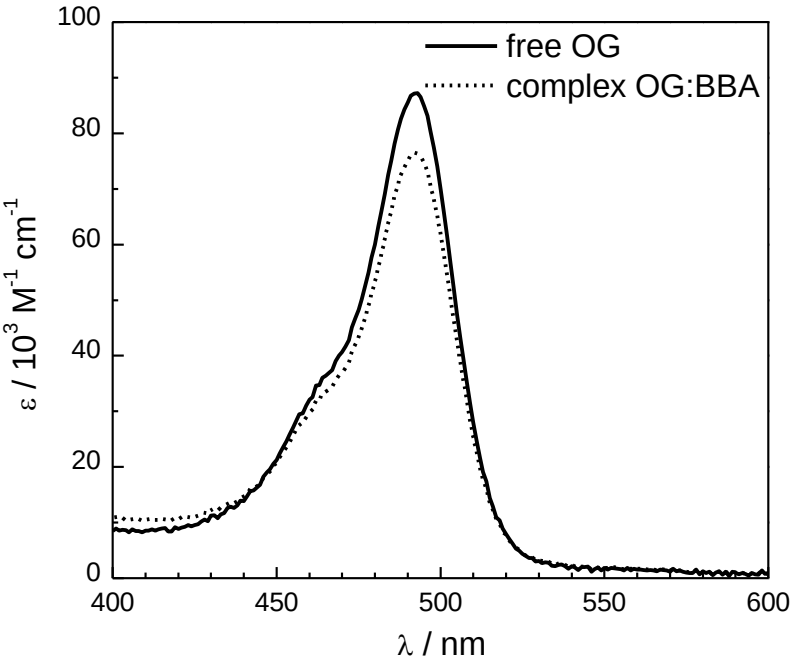


Figure 1S: Absorption spectra of free OG and complex OG:BBA obtained by global fit of function $A(\lambda) = \left(A_{OG}(\lambda) + A_{OG:BBA}(\lambda)\, K\,[BBA]\right) / \left(1 + K\,[\mathrm{BBA}]\right)$ to a series of absorption spectra of OG in the presence of different concentrations of BBA. Non-linear fit parameter: K (free). Linear fit parameters: $A_{\mathrm{OG}}(\lambda)$ and $A_{\mathrm{OG:BBA}}(\lambda)$ for the wavelength range of the absorption spectra (400 – 600 nm). BBA concentration range: 0 – 2.2 mM.

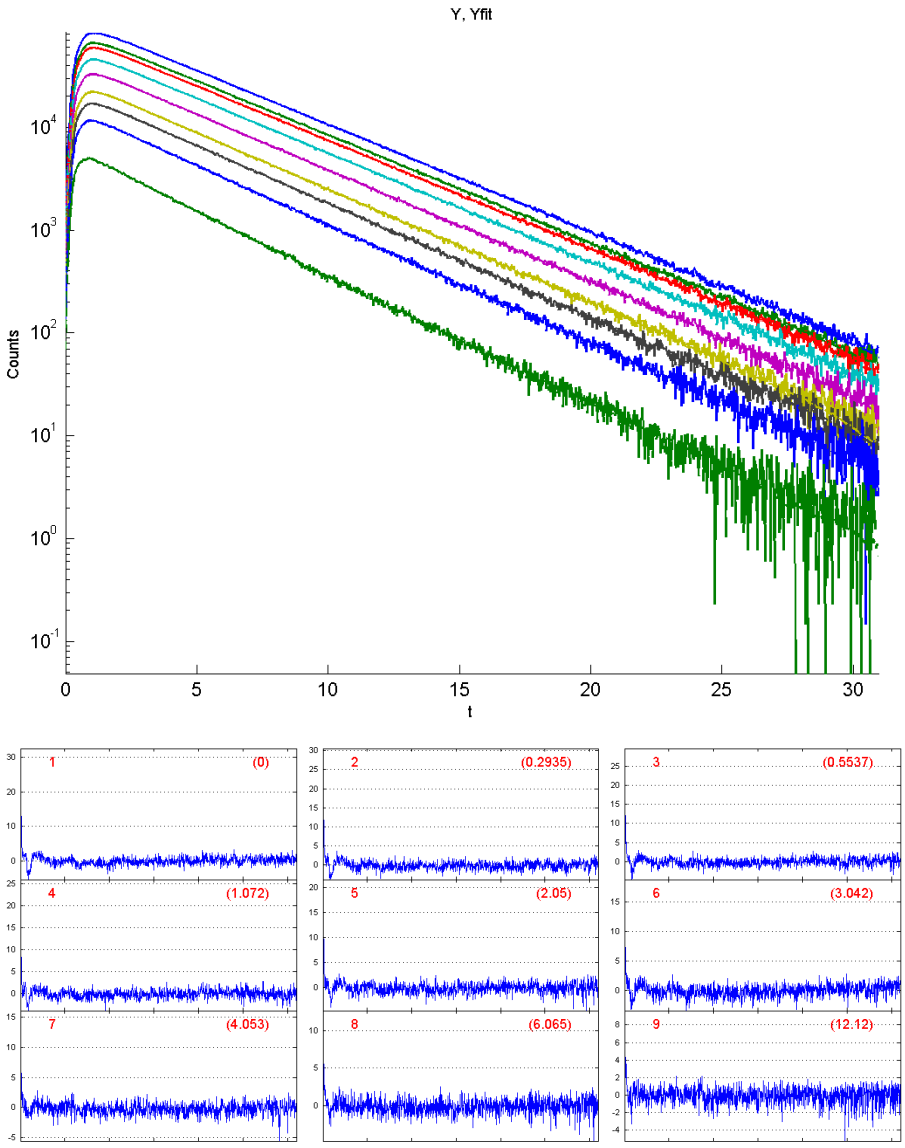


Figure 2S: Global target fit of a triexponential function to a series of fluorescence decays of OG in the presence of different concentrations of BBA, with $\tau_2 = \tau_B$ and $\tau_3 = \tau_A$ given in equation (2). Non-linear fit parameters: $\tau_1 = 0.08$ ns (fixed), τ_2 (free), k_{OG} (free) and k_1 (free). Linear fit parameters: preexponential factors. BBA concentration range: 0 – 12 mM. Upper panel: time resolved fluorescence intensity and fitted decays vs. time in ns. [BBA] increases from top do bottom. Lower panel: residuals for each decay. [BBA]/mM in parenthesis in the upper right corner. Lower scale is time in ns as in the upper panel.

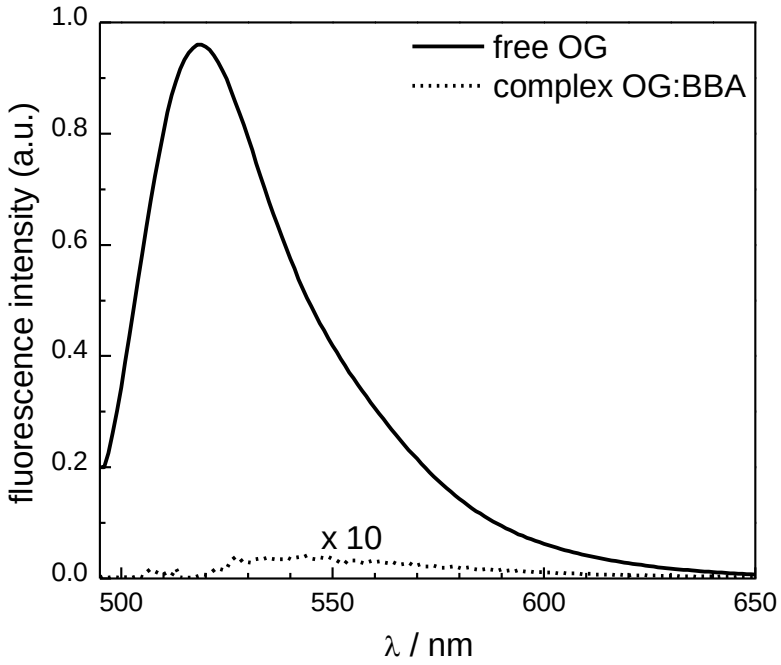


Figure 3S: Emission spectra of free OG and complex OG:BBA obtained by global fit of the function given in equations (1) to a series of fluorescence emission spectra of OG in the presence of different concentrations of BBA. Non-linear fit parameters: K (free), R_1 (fixed to the value determined from fluorescence decays: $R_1 = k_1/k_{OG}$ = 15 mol^{-1} dm^3) and R_ε (fixed to the value determined from absorption spectra: $R_\varepsilon = \varepsilon_{OG}/\ \varepsilon_{OG:BBA}$=1.14). Linear fit parameters: $F_{OG}(\lambda)$ and $F_{OG:BBA}(\lambda)$ for the wavelength range of the emission spectra (495 – 650 nm).

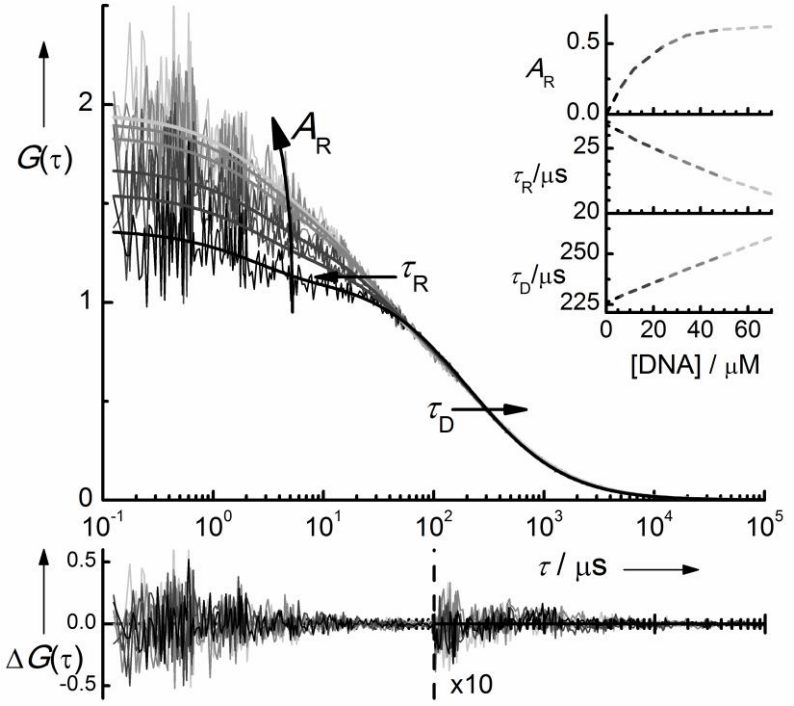


Figure 4S: FCS curves for the titration of BBA-OG-long with AAATTT-hp DNA (experimental data and fitted curves). Insets: variation of the mean diffusion time $\overline{\tau}_D$, the relaxation time τ_R and the relaxation amplitude A_R with $[DNA]_0$ calculated from the fit parameters. The lower panel shows the fit residuals, where the range above 100 µs is amplified in order to facilitate visual examination.

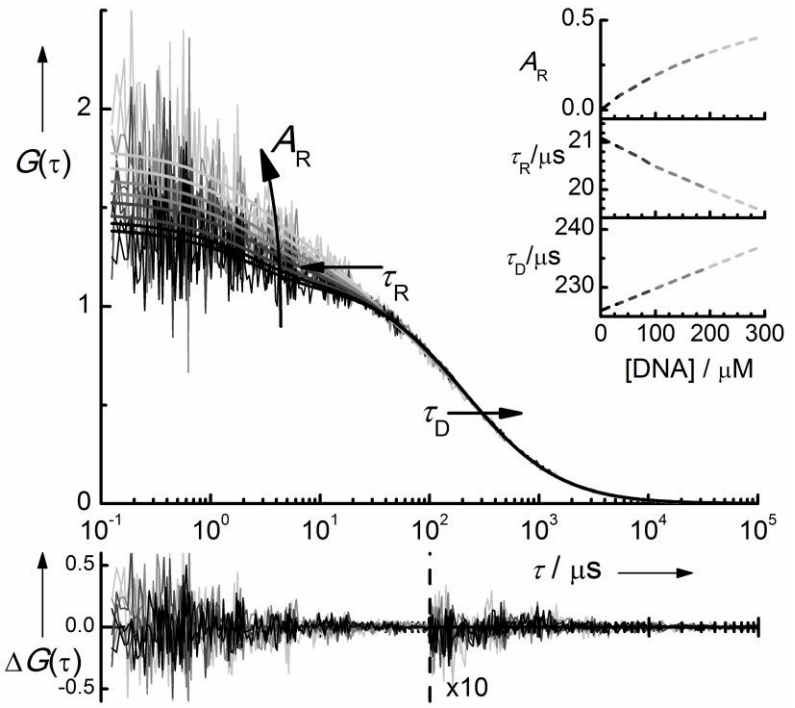
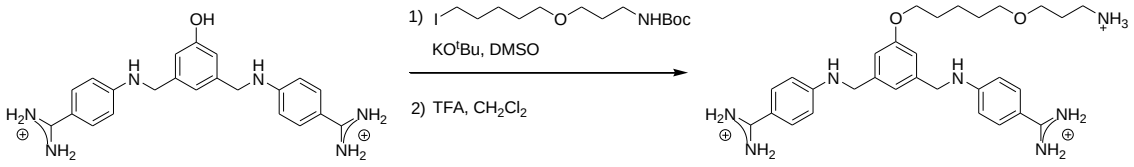


Figure 5S: FCS curves for the titration of BBA-OG-long with GGCCC-hp DNA (experimental data and fitted curves). Insets: variation of the mean diffusion time $\overline{\tau}_D$, the relaxation time τ_R and the relaxation amplitude A_R with $[DNA]_0$ calculated from the fit parameters. The lower panel shows the fit residuals, where the range above 100 µs is amplified in order to facilitate visual examination.

Experimental Section

Synthesis and purification of BBA-OG-long

Synthesis of 4-{[(3-{[5-(3-aminopropoxy)pentyl]oxy}-5-{[(4-carbamimidoylphenyl)-amino]-methyl}phenyl)methyl]amino}benzene-1-carboximidamide (BBA-long)



1 **BBA-long**

Potassium tert-butoxide (29 mg, 0.26 mmol, 4 equiv) was added to a solution of the bis-amino benzamidine **1** (40 mg, 0.065 mmol) in dry DMSO (1.3mL). After 30 min, *tert*-butyl-3-(5-iodopentyloxy)propylcarbamate (28 mg, 0.078 mol, 1.2 equiv) was added in portions. The reaction mixture was stirred under Ar at rt for 2 h. The crude reaction was directly purified by preparative reverse-phase chromatography (Büchi Sepacore) (gradient: 15% B, 5 min; 15% → 95 % B, 30 min.). The combined fractions were concentrated and freeze-dried. The isolated Boc-protected compound was dissolved in CH_2Cl_2 (1 mL) and cooled to 0 ºC. TFA (1 mL) was added dropwise and the resulting solution was stirred at 0 ºC for 1h and at room temperature for other 2 h. The solvent was removed under reduced pressure, and the residual TFA was removed by co-distillation with CH_2Cl_2. The residue was purified by preparative reverse-phase chromatography (Büchi Sepacore) (gradient: 0% B, 5 min; 0% → 50 % B, 30 min.). The freeze-dried solid was identified as the desired product (**BBA-long**) (45.4 mg, 0.052 mmol, 80% overall yield for the 2-step process).

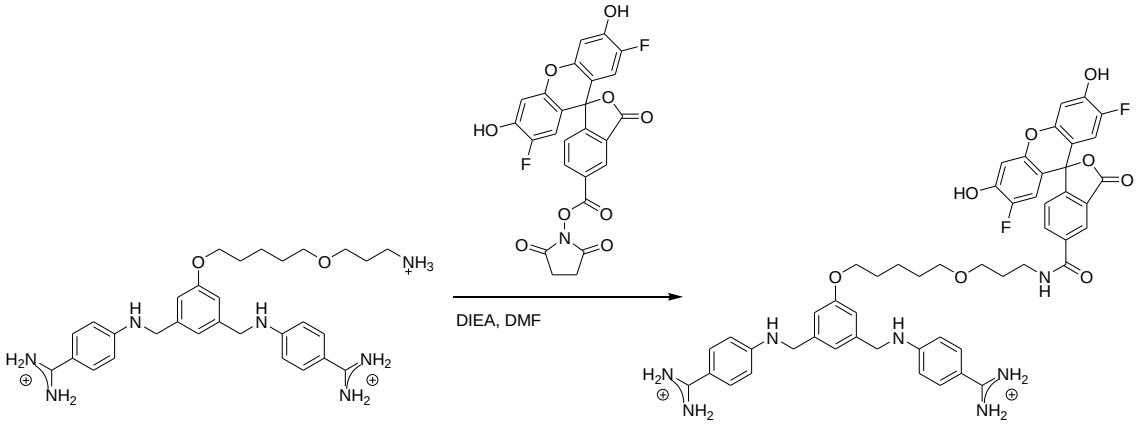
^{1}H RMN (300 MHz, *DMSO-d_6* δ): 1.28 (s, 2H, NH), 1.50 (dd, *J* = 14.9, 8.3 Hz, 2H), 1.63 (td, *J* = 13.4, 6.6 Hz, 2H), 1.68-1.83 (m, 2H), 1.91 (td, *J* = 12.8, 6.4 Hz, 2H), 3.04 (t, *J* = 7.0 Hz, 2H), 3.46 (t, *J* = 6.4 Hz, 2H), 3.55 (t, *J* = 5.8 Hz, 2H), 3.92 (t, *J* = 6.3 Hz, 2H), 4.36 (s, 4H), 6.68 (d, *J* = 8.9 Hz, 4H), 6.78 (s, 2H), 6.93 (s, 1H), 7.57 (d, *J* = 8.9 Hz, 4H).

^{13}C RMN (300 MHz, *DMSO-d_6* δ): 22.4 (CH_2), 27.1 (CH_2), 28.7 (CH_2), 28.9 (CH_2), 37.7 (CH_2), 46.1 (CH_2), 67.4 (CH_2), 67.7 (CH_2), 70.7 (CH_2), 111.4 (CH), 111.7 (CH), 112.8 (C),

117.4 (CH), 129.1 (CH), 140.8 (C), 153.9 (C), 159.8 (C), 165.6 (C).

ESI$^+$-MS: [M+H] calc. for $C_{30}H_{42}N_7O_2$ = 532.3395 found 532.3405; $C_{36}H_{44}F_9N_7O_8$ (M.W. 873.7623).

Synthesis of 5-{[3-({5-[3,5-bis({[(4-carbamimidoylphenyl)amino]-methyl})phenoxy]pentyl}-oxy)propyl]carbamoyl}-2-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (BBA-OG-long)



BBA-long **BBA-OG-long**

A solution of the derivatizated bis-amino benzamidine **(BBA-long)** (3.7 mg, 4.3 µmol, 1.1 equiv) in 250 µL of DMF/DIEA 0.2 M was added to (2.0 mg, 3.9 µmol) Oregon Green 488 succinimidyl ester and stirred for 2 h. The reaction mixture was purified by high pressure reverse-phase chromatography (gradient: 15% B, 5 min; 15%→95 % B, 30 min.). The appropriated fractions were collected, concentrated and freeze-dried to obtain the desired conjugate **BBA-OG-long** (2.6 mg, 2.2 µmol, 57 %).

^{1}H RMN (500 MHz, *MeOD-d_4* δ): 1.56-1.42 (m, 2H), 1.65-1.57 (m, 2H), 1.76-1.66 (m, 2H), 1.92 (p, J = 6.3 Hz, 2H), 3.48 (t, J = 6.3 Hz,2), 3.54 (t, J = 6.8 Hz,2H), 3.57 (t, J = 5.9 Hz, 2H), 3.89 (t, J = 6.3 Hz, 2H), 4.35 (s, 4H), 6.42 (d, J = 10.9 Hz, 2H), 6.69 (d, J = 8.8 Hz, 4H), 6.76 (s, 2H), 6.85 (d, J = 7.4 Hz, 2H), 6.91 (s, 1H), 7.31 (d, J = 8.0 Hz, 1H), 7.57 (d, J = 8.9 Hz, 4H), 8.19 (dd, J = 8.0, 1.6 Hz, 1H), 8.42 (s, 1H).

ESI$^+$-MS: [M+H] calc. for $C_{51}H_{50}F_2N_7O_8$ = 926.3683 found 926.3680; $C_{55}H_{51}F_8N_7O_{12}$ (M.W. 1154.0204).

FCS setup

The confocal epi-illuminated apparatus used for the FCS measurements has been described elsewhere.[1] 100 µL samples were deposited on a glass bottom microplate (Whatman Ltd.). The samples were excited by the continuous linearly polarized light of a 489 nm laser diode (Becker&Hickl, BDL 485 SMC, DE) coupled to a monomode optical fiber (Point-Source, kineFLEX P 1 S 405 0.7, UK). The light output of the fiber was collimated (Schäfter&Kirchhoff, 60FC-4-6,2-01-DI, DE), spectrally cleaned (Semrock, Brightline HC 482/18, US), redirected by a dichroic mirror (Semrock, Brightline BS R488, US) and focused into the sample by a high aperture microscope objective (Olympus, UPLSAPO 60xW/1.20, water immersion) mounted in an inverted microscope (Olympus, IX 71). The fluorescence was collected by the same objective and then refocused through the dichroic mirror onto a pinhole (Thorlabs, ∅=50 µm, US) in the image plane. The light passing the pinhole was collimated, then split into two beams by a nonpolarizing beamsplitter cube (Newport, 05BC17MB.1, US) and each focused onto avalanche photodiodes (MPD50CTC APD, ∅=50 µm, MPD, Italy). Band pass filters (Semrock, Brightline HC 525/45, US) in front of the detectors discriminated fluorescence from scattered laser light. Both output signals were processed and stored by TCSPC modules (SPC 132, Becker & Hickl GmbH, Berlin, Germany). Correlation curves were calculated with the Single Photon Counting software by Becker & Hickl GmbH.

Typically 20 million photons were collected for each correlation curve with count rates in the range of 2.5 to 29 kHz, depending on the sample. All measurements were made at stabilized temperature, 25.0 ± 0.5°C. The excitation power as measured in the focus of the microscope objective by a power meter (Thorlabs, PM30 120, US) was typically 80 µW, corresponding to a mean irradiance of $I_0/2 = P/(\pi\, w_{xy}^2)$ = 9 kW cm-2, assuming a Gaussian intensity distribution along the optical axis. P is the excitation power in the sample.[2] The focal area and the detection volume were calibrated with Rhodamine 123 in aqueous solution at low irradiance yielding a radial $1/e^2$ radius of w_{xy} = 0.53 µm. The value of $D_{R123} = (4.6 \pm 0.4) \times 10^{-10}$ m^2s^{-1} is estimated from PFG-NMR[3] and dual focus FCS[4] data. The diffusion coefficients are given for 25°C.

References

[1] D. Granadero, J. Bordello, M. J. Pérez-Alvite, M. Novo, W. Al-Soufi, *Int. J. Mol. Sci.* **2010**, *11*, 173-188.

[2] C. Eggeling, J. Widengren, R. Rigler, C. A. M. Seidel, *Anal. Chem.* **1998**, *70*, 2651-2659.

[3] P. O. Gendron, F. Avaltroni, K. J. Wilkinson, *J. Fluoresc.* **2008**, *18*, 1093-1101.

[4] C. Muller, A. Loman, V. Pacheco, F. Koberling, D. Willbold, W. Richtering, *Europhys. Lett.* **2008**, *83*, 46001.