

Ratiometric nanoparticle probe based on FRET-amplified phosphorescence for oxygen sensing with minimal phototoxicity

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Experimental section

Materials

All reagents and solvents were obtained from commercial suppliers and used without further purification unless otherwise indicated. PMMA-MA (1.3% methacrylic acid; Mn, ~15,000; Mw, ~34,000), PLGA (poly(lactide-co-glycolic acid), lactide 50 mole%, glycolide 50 mole%, Mn 24,000), platinum octaethyltetraphenylporphyrin (PtOEP) were purchased from Sigma-Aldrich. Qdot™ 525 Streptavidin Conjugate was purchased from ThermoFisher Scientific. Sodium phosphate monobasic (> 99.0%, Sigma-Aldrich) and sodium phosphate dibasic dihydrate (> 99.0%, Sigma-Aldrich) were used to prepare 20 mM phosphate buffers at pH 7.4. Milli-Q water (Millipore) was used in all experiments.

Synthesis and characterization of BlueCy-TPB: Intermediates 3-methyl-2-(methylthio)benzo[d]thiazol-3-ium iodide¹ and 2-methyl-3-octadecylbenzo[d]thiazol-3-ium iodide² were synthesized by following the procedure reported in the literature.

BlueCy: A mixture of 3-methyl-2-(methylthio)benzo[d]thiazol-3-ium iodide (**1**) (1 eq., 48 mg, 0.15 mmol), 2-methyl-3-octadecylbenzo[d]thiazol-3-ium iodide (**2**) (1 eq., 78.6 mg, 0.15 mmol) and triethylamine (2 eq., 41 μ L, 0.30 mmol) in 3 mL of absolute ethanol was refluxed for 5 hours. After cooling, the reaction mixture was added with diethyl ether to precipitate the **BlueCy** dye that was separated by vacuum filtration. The obtained crude product was purified on a silica column (CH₂Cl₂/MeOH 95/5) to give a dark yellow powder of **BlueCy** (68 mg, 68% yield). ¹H NMR (400 MHz, CDCl₃): δ 8.04 (dd, J = 8.0 Hz, 2H; HAr), 7.41–7.34 (m, 2H; HAr), 7.32–7.20 (m, 4H; HAr), 6.88 (s, 1H; -CH=), 4.79 (t, J = 7.2 Hz, 2H; N⁺CH₂), 4.19 (s, 3H; CH₃N⁺), 1.84 (q, J =7.2 Hz, 2H; CH₂CN⁺), 1.48 (q, J =7.2 Hz, 2H; CH₂CCN⁺), 1.28–

1.19 (m, 28H; CH₂), 0.86 ppm (t, $J=6.6$ Hz, 3H; CH₃); ¹³C NMR (100 MHz, CDCl₃): δ 162.21, 161.74, 140.52, 140.28, 128.47, 128.44, 125.56, 125.36, 124.93, 124.86, 123.65, 123.52, 112.98, 112.79, 84.18, 48.35, 36.77, 32.04, 29.81, 29.77, 29.72, 29.67, 29.53, 29.47, 27.61, 27.08, 22.81, 14.24 ppm; HRMS (ESI), calcd for C₃₄H₄₉N₂S₂: 549.333, found: 549.336 [M]⁺.

BlueCy-TPB: **BlueCy** (1 eq., 11.8 mg, 0.017 mmol) and potassium tetrakis(pentafluorophenyl)borate (3 eq., 38.7 mg, 0.052 mmol) were mixed in 0.6 ml of dichloromethane, TLC has shown instant conversion. The crude product was purified by preparative TLC using CH₂Cl₂/MeOH 98/2 as an eluent to obtain pure **BlueCy-TPB** (20.36 mg, 95% yield). ¹H NMR (400 MHz, CDCl₃): δ = 7.86 (t, $J=6.8$ Hz, 2H; HAr), 7.66 (t, $J=7.6$ Hz, 2H; HAr), 7.52–7.48 (m, 4H; HAr), 6.25 (s, 1H; -CH=), 4.30 (t, $J=7.2$ Hz, 2H; N⁺CH₂), 3.84 (s, 3H; CH₃N⁺), 1.90 (q, $J=7.2$ Hz, 2H; CH₂CN⁺), 1.46 (q, $J=7.2$ Hz, 2H; CH₂CCN⁺), 1.36 (q, $J=6.8$ Hz, 2H; CH₂), 1.26–1.23 (m, 26H; CH₂), 0.87 ppm (t, $J=6.0$ Hz, 3H; CH₃); ¹³C NMR (100 MHz, CDCl₃): δ = 162.84, 162.35, 149.60, 147.19, 140.39, 140.13, 139.57, 137.63, 137.12, 135.18, 129.36, 126.22, 126.18, 125.28, 125.04, 123.23, 123.13, 113.30, 113.07, 81.20, 47.46, 33.54, 32.15, 29.92, 29.88, 29.84, 29.79, 29.69, 29.58, 29.45, 27.41, 27.15, 22.90, 14.30 ppm.

Preparation of NPs: PMMA-MA or PLGA solutions at 1 mg mL⁻¹ in acetonitrile/dioxane mixture, containing the desired amount of dyes (1 to 18 wt% relative to the polymer for donor (**BlueCy-TPB**) optimizations and 12 wt% for the donor acceptor system), were added rapidly and under vigorous stirring (shaking, Thermomixer comfort, Eppendorf, 1100 rpm) to a tenfold volume excess aqueous solution (pH 7.4 phosphate buffer) at 21 °C. In case of FRET NPs, **PtOEP** was also added to PMMA-MA and **BlueCy-TPB** solution in acetonitrile before the nanoprecipitation to corresponding concentration; the optimal **PtOEP** concentration in the oxygen nanoprobe was 0.071 wt% (1 mM) with respect to the polymer. The particle solution was then quickly diluted fivefold with phosphate buffer (pH 7.4) for spectroscopic measurements. For single-particle analysis these solutions were diluted 500-fold from the precipitated NP solutions. For cell experiments, the particle solutions were further diluted 30-50 times to get a particle concentration of 60 pM in Opti-MEM.

Characterization of NPs: The determination of size of the polymeric nanoparticles were performed on a Zetasizer Nano ZSP (Malvern Instruments S.A.). The mean value of the diameter of the size distribution per volume was used for analysis. Absorption and emission

spectra were recorded on a Cary 4000 Scan ultraviolet-visible spectrophotometer (Varian) and FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon) equipped with a thermostated cell compartment, respectively. The fluorescence spectra were corrected for detector response and lamp fluctuations. QYs of different loading for **BlueCy-TPB** were calculated using 7-diethylamino coumarin-3-carboxylic acid in dioxane as standard ($\phi = 0.93$)³ following the common protocol. Fluorescence lifetime was measured by using time-correlated single-photon counting (TCSPC) technique using excitation pulses at 405 nm provided by a pulse-picked frequency doubled Ti:sapphire laser (Tsunami, Spectra Physics) pumped by a Millennia X laser (Spectra Physics). Emission was collected through a polarizer set at the magic angle and an 8 nm bandpass monochromator (Jobin-Yvon H10) at 485 nm. The instrumental response function was recorded with a polished aluminium reflector. Fluorescence decays were analyzed by using a standard iterative reconvolution method, assuming a multiexponential decay function. Phosphorescence lifetime was measured by using 405 nm LED excitation using Edinburg FS5 spectrofluorometer by monitoring at 650 nm. Lifetime decays were analyzed by using Edinburgh Instruments' FAST software. To calculate FRET efficiency based on fluorescence spectra, a classical equation was used: $E_{FRET} = (I_D - I_{DA})/I_D \times 100\%$, where I_D is the donor maximum intensity and I_{D-A} is the maximum intensity of the donor in the presence of the acceptor.

The amplification factor of the acceptor emission (antenna effect, AE) was expressed as the ratio of the maximal excitation intensity of the donor to that of the acceptor with correction from the emission of the donor dyes at 650 nm:

$$AE = \frac{I_{D-FRET}^{ex} - I_D^{ex} \times f}{I_{A-FRET}^{ex}}$$

where I_{D-FRET} and I_{A-FRET} are the maximal excitation intensities of donor and acceptor in FRET NPs, respectively, I_D^{ex} is the excitation intensities at the wavelengths of the excitation maximum of donor without acceptors, f is the correction factor calculated as the ratio of the maximum emission intensity of the donor for FRET NPs to that for NPs without acceptor dyes.

Single-Particle Fluorescence Microscopy: For single-particle fluorescence microscopy measurements, the **BlueCy-TPB** and **BlueCy-TPB/PtOEP** NPs and the reference QDs (**QD525**) were immobilized on a LabTek glass chambers by following the previously reported

protocol^{26c} followed by extensive washing with Milli-Q water. The surfaces were left in Milli-Q water during microscopy. Single-particle measurements for the donor particles of different loading were performed in the epi-fluorescence mode using Nikon Ti-E inverted microscope with a 100x objective (Apo TIRF, oil, NA 1.49, Nikon). The excitation was provided by light emitting diodes (SpectraX, Lumencor) at 395 nm. The emission signal was recorded with a Hamamatsu Orca Flash 4 camera. The exposure time was set to 200 ms per image frame. To enable two-color image detection for **BlueCy-TPB/PtOEP** particles, corresponding to cyanine donor and porphyrin acceptor signals, W-VIEW GEMINI image splitting system (Hamamatsu) was used with a dichroic of 640 nm (Semrock FF640-FDi01-25x36) and with filters of 527 ± 35 nm for donor emission. The images were acquired using NIS-elements software. Single-particle analysis was performed using the Fiji software. Particle locations were detected through a Fiji routine applied to a projection (average intensity) of 10-20 frames.

Quantification of Dissolved Oxygen Concentration in Solution: The oxygen concentration in the NP solutions were varied by using sodium sulfite (Na_2SO_3), a commonly used scavenger of molecular oxygen. Different concentrations of sodium sulfite were freshly prepared in milli-Q water and added to NP solution for the measurement of oxygen concentration. The measurements were carried out using a pre calibrated Vernier Optical DO probe by placing the tip of the probe into the sample of interest. The Vernier Optical DO Probe is connected with a portable computer unit with the software LabQuest 2 (version 2.8.5). The measurement has been recorded each time at 21 °C after giving a certain delay (*ca.* 2-3 min) to compensate the temperature and pressure. The oxygen concentration was determined in the units of mg/L or percent saturation (%) for the different samples. The concentration values were taken as an average of three independent measurements.

Phototoxicity measurements: HeLa cells (ATCC® CCL-2™) cells were seeded onto an ibidi® treated glass petridish and incubated for 18 hours. For imaging, the culture medium was removed and the attached cells were washed two times with PBS and one time with Opti-MEM (Gibco-Invitrogen). Then, a freshly prepared solution of **BlueCy-TPB/PtOEP** and PtOEP (1% DMSO) in Opti-MEM was added to the cells and incubated for 3 h. The samples were irradiated using appropriate lasers (395 nm for DA NPs and 550 nm for PtOEP) for 5 min and waited for another 5 min for the microscopic measurements. The measurements were taken using brightfield DIC mode and W-VIEW GEMINI image splitting system as described earlier. The different concentrations of PtOEP (60 nM to 1 μM) were used for optimizing the

brightness and observed that at 500 nM concentration of PtOEP the signal was matching with that of the **BlueCy-TPB/PtOEP** NPs.

Cellular Imaging of Oxygen Gradients in HeLa Cells: HeLa cells (ATCC® CCL-2™) were grown in Dulbecco's modified Eagle medium (without phenol red, Gibco-Invitrogen), supplemented with 10% FBS (Lonza), L-glutamine, and 1% antibiotic solution (penicillin-streptomycin, Gibco-Invitrogen) at 37 °C in humidified atmosphere containing 5% CO₂. Further the cells were seeded onto the Ibidi® treated channels in the μ-slide (see above). After letting the cells adhere for 18 h, the cell culture medium was removed, and the cells were rinsed twice with opti-MEM, followed by incubation with a freshly prepared solution of the donor alone (control) and donor-acceptor NPs. After 3 h of incubation, the NP suspension was removed, and the cells were rinsed with Opti-MEM, and finally the measurements were taken in the same. Oxygen sensing experiments were performed by placing the chambered microscope μ-slides on a Nikon Ti-E inverted microscope (see above) using a 20X air objective (Nikon CFI Plan Apo, NA = 0.75). One outlet of the μ-slide was closed after adding sodium sulfite (oxygen restricted end) and the other outlet was exposed to air (oxygen abundant end). The different channels were recorded as follows by using the excitation at 395 nm, and emission recorded from 490 to 560 nm (for donor emission) and from 640 to 670 nm (for acceptor emission). W-VIEW GEMINI image splitting system (Hamamatsu) was used with dichroic 640 nm (Semrock FF640-FDi01-25x36) to image both donor and acceptor channels (see above). The stepwise scan from one end to the other end of the μ-slide was performed by setting up each scan in 1 mm intervals, which was required to image the hypoxic or normoxic regions in HeLa cell lines. The large scale panoramic image was obtained using the same setup with 10X air objective (Nikon CFI Plan Apo, NA = 0.45) and an automated function of NIS-elements software. Image analysis was performed using the Fiji software.

Transmission Electron Microscopy

Carbon-coated copper-rhodium electron microscopy grids with a 300 mesh (Euromedex, France) were surface treated with a glow discharge in amylamine atmosphere (0.45 mbar, 4 – 4.5 mA, 22 s) in an Elmo glow discharge system (Cordouan Technologies, France). Then, 5 μL of the particle solution (**BlueCy-TPB** and **BlueCy-TPB/PtOEP** NPs) were deposited onto the grids and left for 2 min. The grids were further stained for 1 min with a 2% uranyl acetate solution. Further the grids were observed through a Philips CM120 transmission electron

microscope equipped with a LaB6 filament and operating at 100 kV. Areas covered with nanoparticles of interest were recorded at different magnifications on a Peltier cooled CCD camera (Model 794, Gatan, Pleasanton, CA). Image analysis was performed using the Fiji software.

Theoretical calculation of FRET efficiency. FRET efficiency inside NPs was calculated assuming (i) a homogenous distribution of fluorophores and (ii) absence of energy migration within the donor dyes. To this end, the model of energy transfer to multiple acceptors in a homogeneous solution of donors and acceptors, without D–A diffusion, was used.¹ In this case, the steady-state intensity of the donor is given by

$$\frac{F_{DA}}{F_D} = 1 - \sqrt{\pi} * \gamma * \exp(\gamma^2) [1 - \text{erf}(\gamma)].$$

In this expression $\gamma = \frac{A}{A_0}$, where A is the acceptor concentration. A_0 is the critical concentration representing the acceptor concentration required for 76% energy transfer. It can be calculated by $A_0 = \frac{447}{R_0^3}$, where R_0 is the Förster radius in Å. The error function $\text{erf}(\gamma)$ is given by:

$$\text{erf}(\gamma) = \frac{2}{\sqrt{\pi}} \int_0^{\gamma} \exp(-x^2) dx$$

The theoretical FRET efficiency could be calculated as:

$$E_{FRET} = 1 - \frac{F_{DA}}{F_D} = \sqrt{\pi} * \gamma * \exp(\gamma^2) [1 - \text{erf}(\gamma)]$$

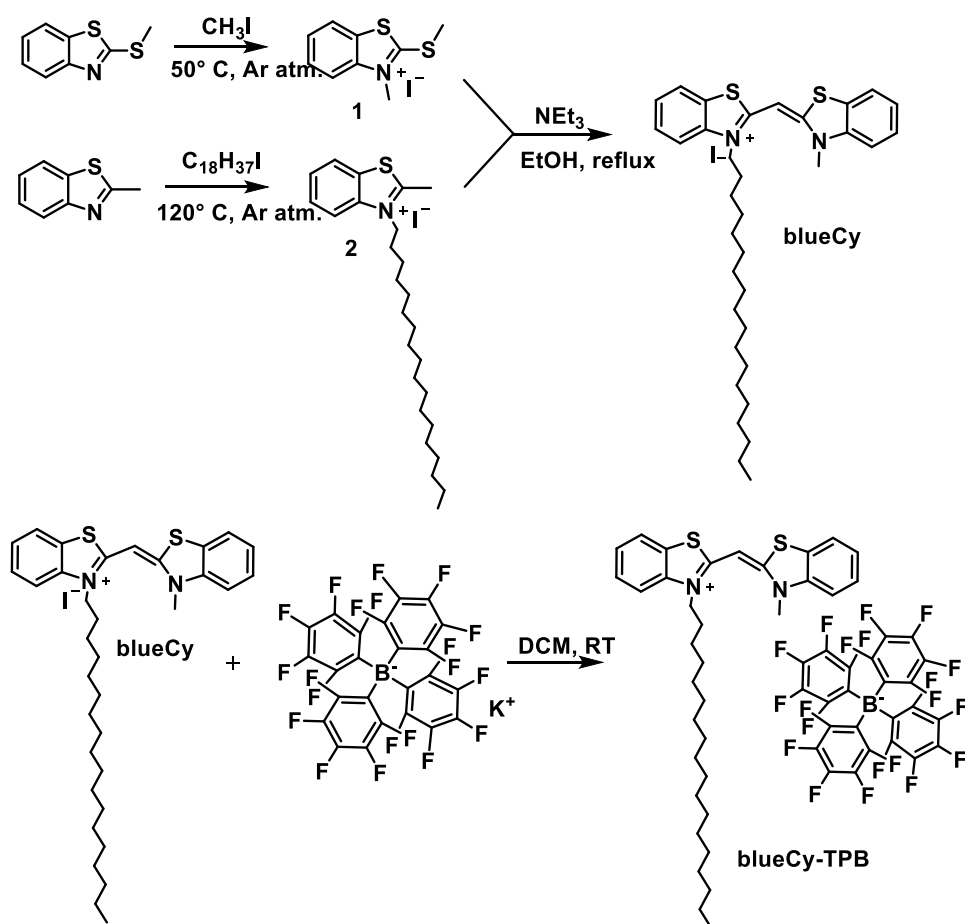
The Förster radius was calculated by: $R_0 = 0.211 \left[\frac{k^2 Q_D J(\lambda)}{n^4} \right]^{1/6}$.

The overlap integral $J(\lambda)$ was calculated using the FluorTools software.⁵ As emission spectrum of the donor dyes inside NPs depends on their loading, calculation of the Förster was done using emission spectrum of NPs loaded with **BlueCy-TPB** at 12 wt%.

Setting up oxygen gradients in a microfluidics slide

The micro slides for gradient were purchased from Clini Sciences (ibidi® μ -Slide I; 0.6/0.8 Luer ibidiTreat: 1.5 polymer coverslip, tissue culture treated, sterilized). The gradient

generation and stability was tested by adding the aqueous solution of Nile Red (as a colorimetric indicator for gradient) to the 50 mm long and 5 mm width channel and introduced a reducing agent sodium dithionate to one outlet, which was then capped immediately after the addition with parafilm. The second outlet was left open and exposed to air to make sure the presence of oxygen. During the flow of sodium dithionate we could observe the disappearance of the color of Nile Red with a clear formation of gradient. Then, the experiment was repeated with **BlueCy-TPB/PtOEP** NPs using sodium sulfite as an oxygen scavenger and the measurements was done under wide-field fluorescence microscopy as described earlier.



Scheme S1. Synthetic route to **BlueCy-TPB**.

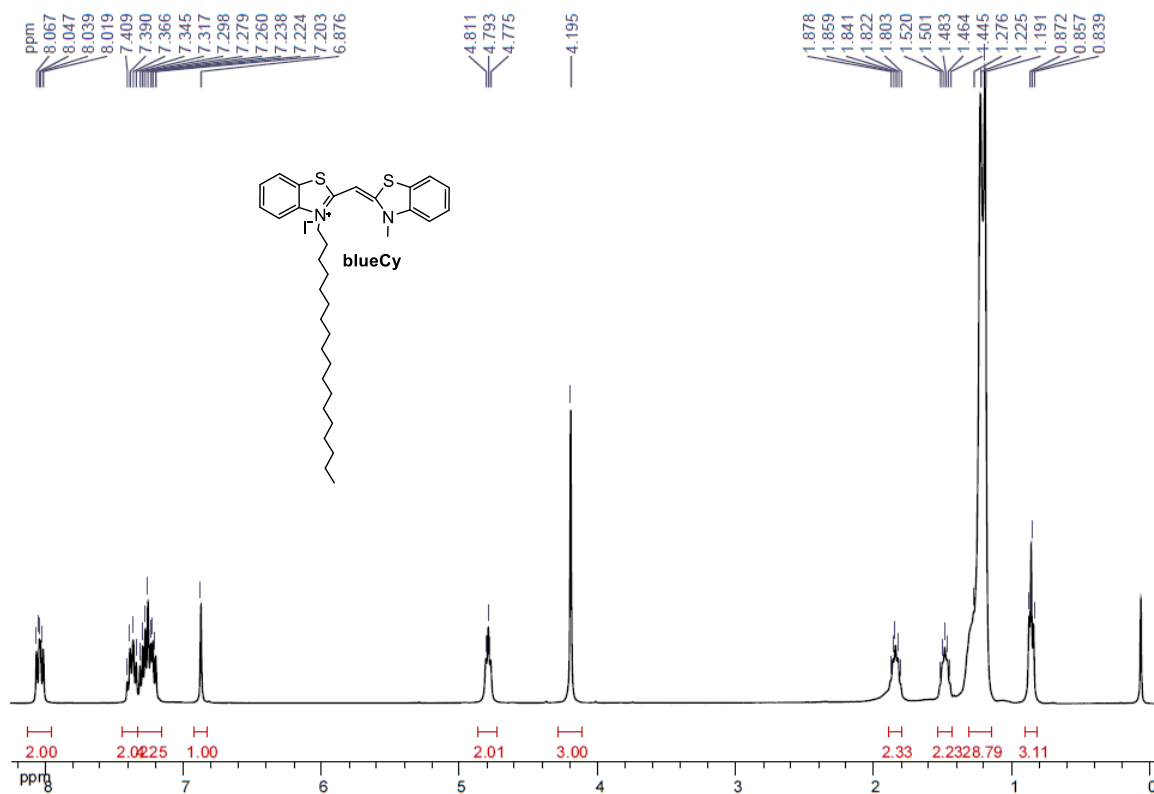


Figure S1. ¹H NMR spectrum of **BlueCy** in CDCl₃.

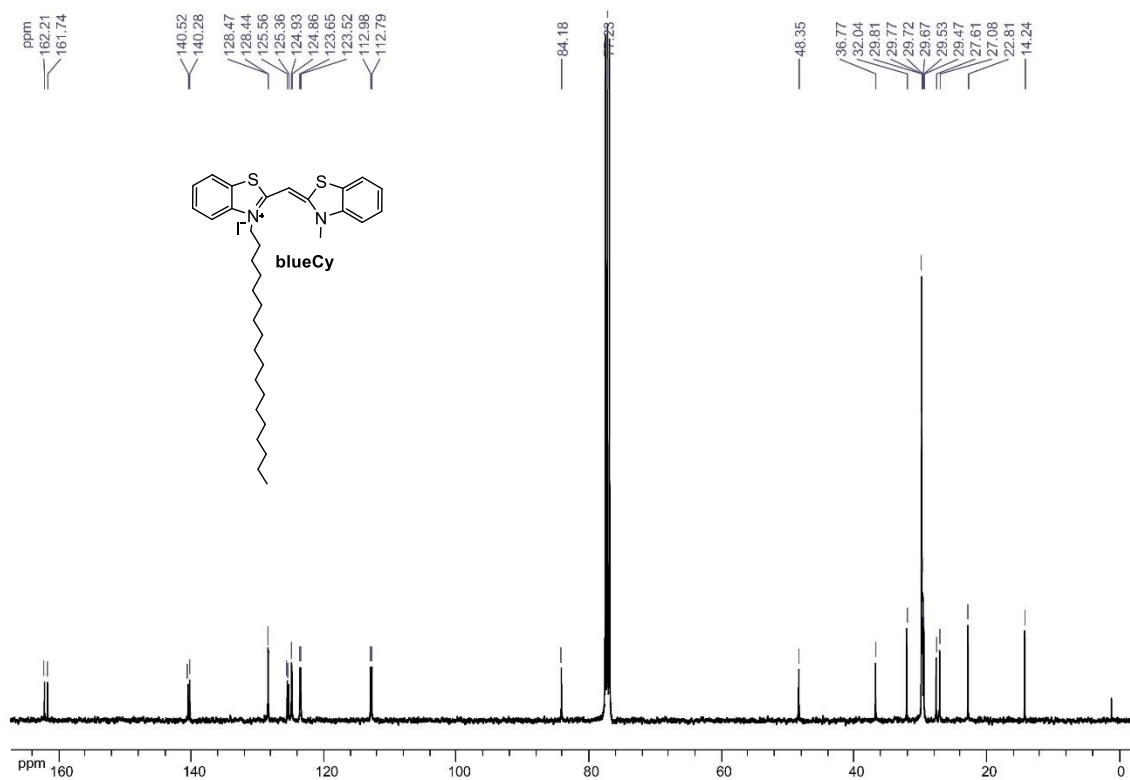


Figure S2. ^{13}C NMR spectrum of **BlueCy** in CDCl_3 .

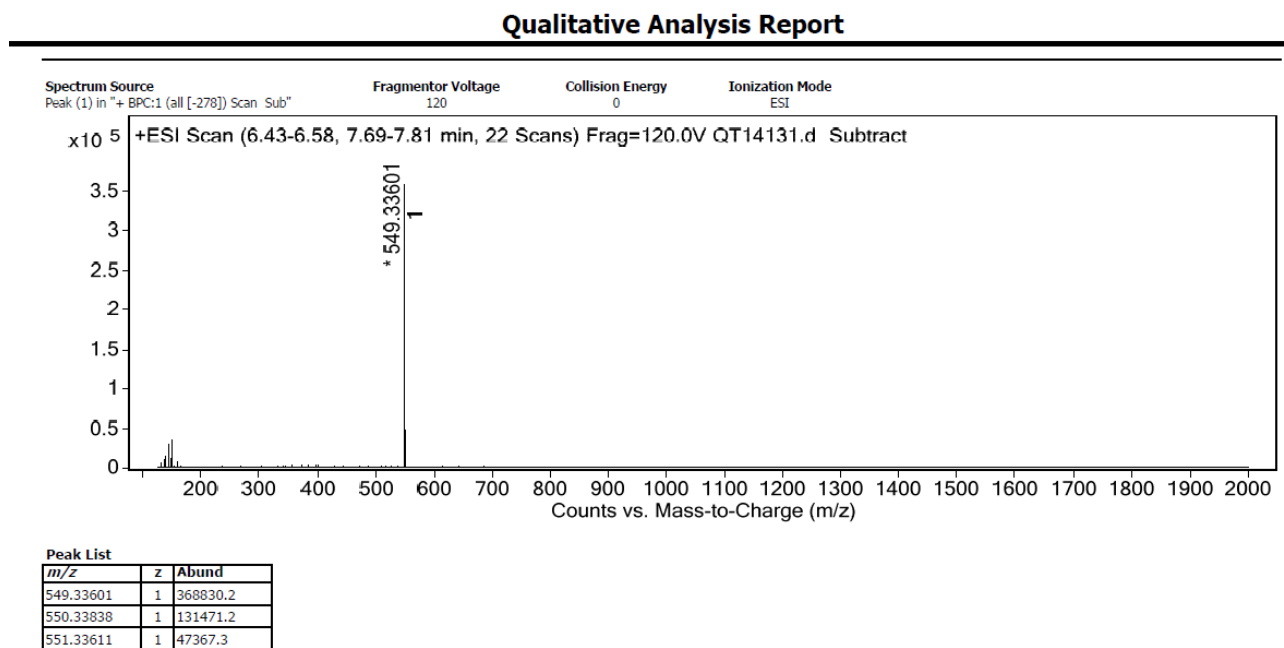


Figure S3. HRMS spectra of **BlueCy**.

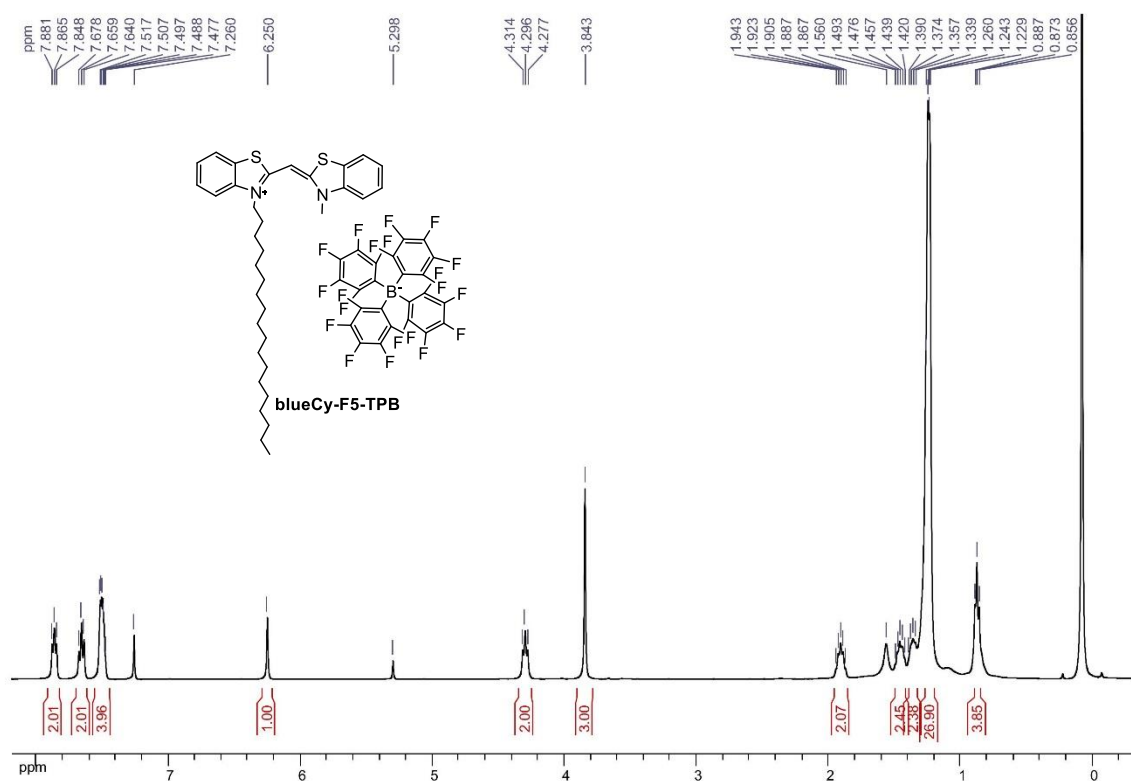


Figure S4. ^1H NMR spectrum of **BlueCy-TPB** in CDCl_3 .

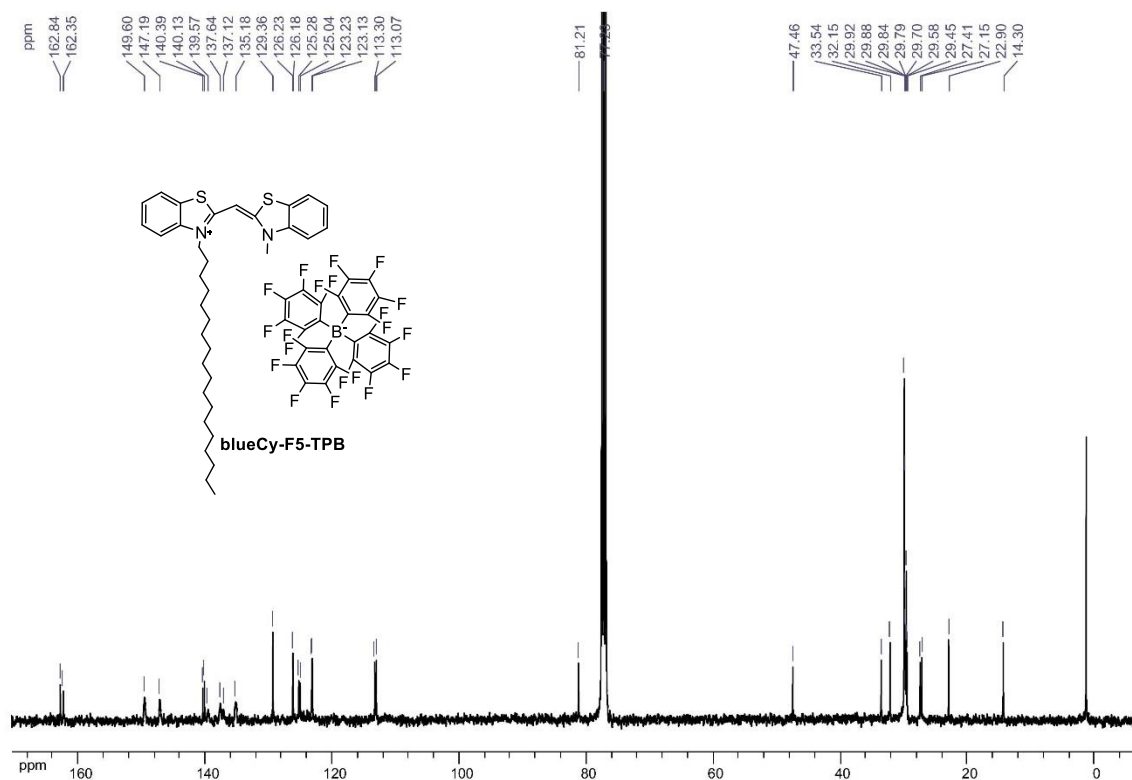


Figure S5. ^{13}C NMR spectrum of **BlueCy-TPB** in CDCl_3 .

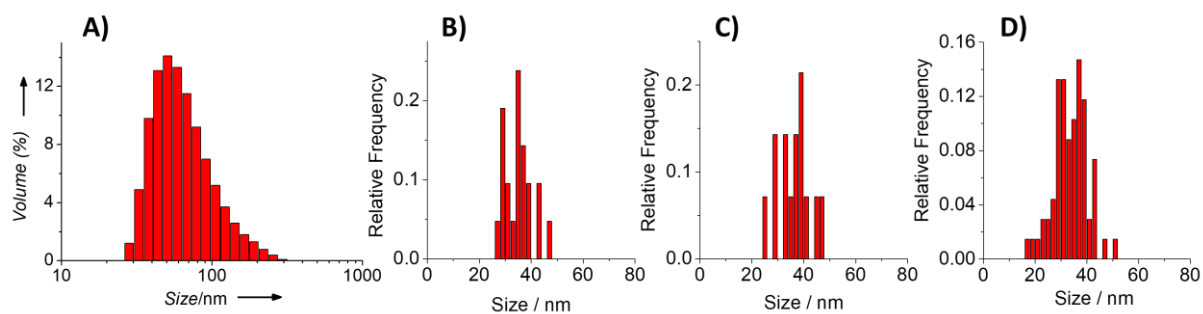


Figure S6. Particle size analysis by DLS and TEM. (A) DLS size distribution of **BlueCy-TPB/PtOEP PMMA-MA NPs** at 12 wt% donor loading (donor : acceptor molar ratio 100:1). (B) Size distribution according to TEM of different PMMA-MA NPs: PMMA-MA NPs, BlueCy-TPB (12 wt%) NPs and BlueCy-TPB/PtOEP NPs.

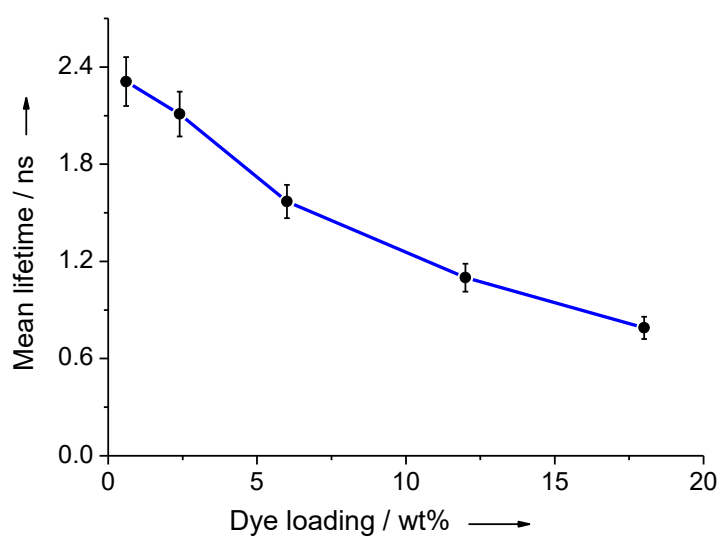


Figure S7. Mean fluorescence lifetime of **BlueCy-TPB** loaded PMMA-MA NPs at various dye loading percentages. The analysis of multiple decay components is given in Table S3.

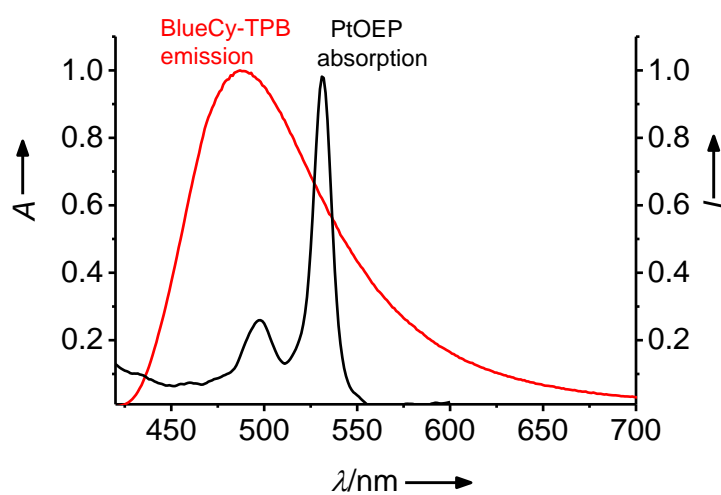


Figure S8. Absorption spectra of the **PtOEP** acceptor and emission spectra of the **BlueCy-TPB** donor in acetonitrile showing the effective overlap for the efficient FRET.

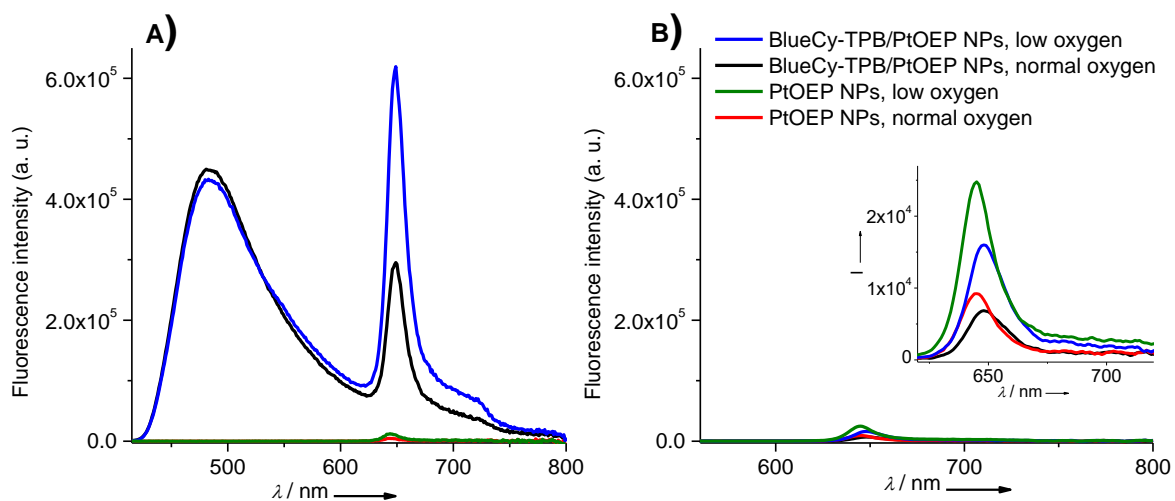


Figure S9. Emission spectra of NPs loaded with **BlueCy-TPB** (12 wt%, 100 mM) and **PtOEP** (0.071 wt%, 1 mM) and **PtOEP** (1 mM loading) alone in normal oxygen concentration or in the presence of 1.4 mM sodium sulfite (low oxygen) in solution obtained by exciting at A) 405 nm and B) 537 nm. Inset of B) shows the expanded region for better visualization.

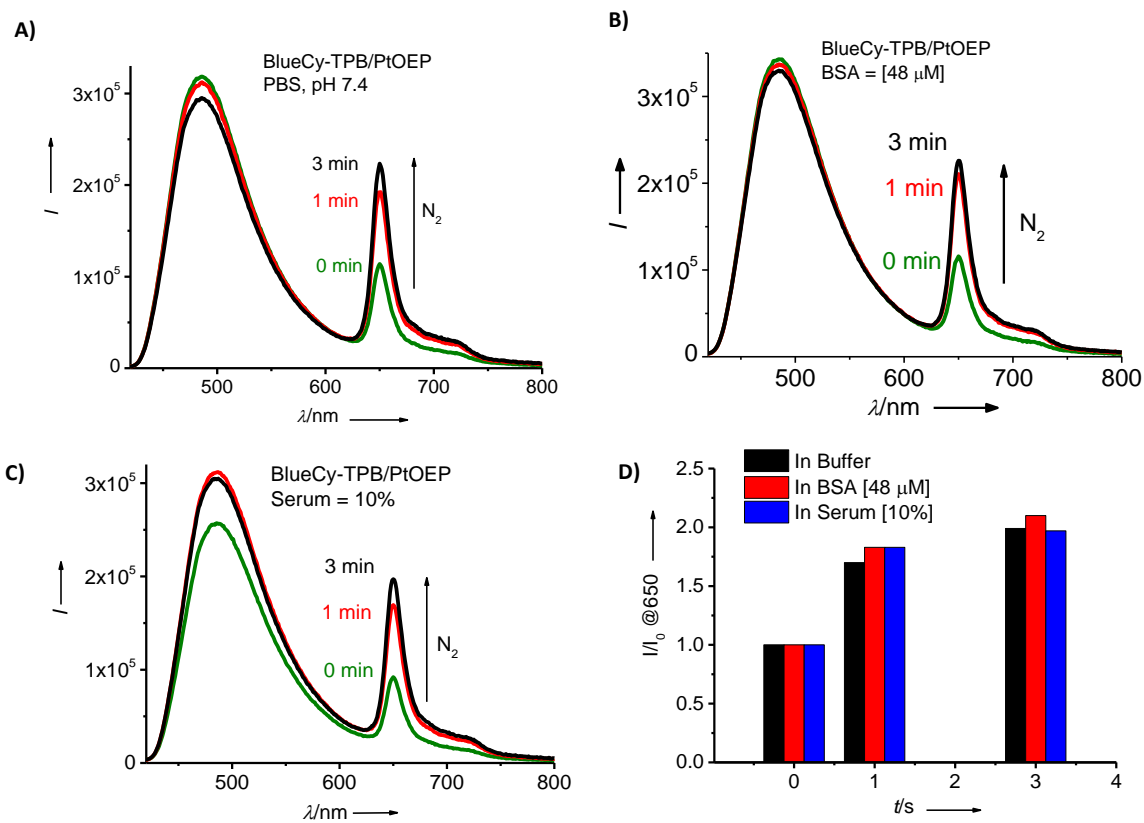


Figure S10. Sensitivity of the probe towards the purging of nitrogen (deoxygenation) in different biologically relevant media such as A) PBS, pH 7.4; B) BSA (48 μM); and C) Serum (10%) under similar conditions. D) The bar diagram showing the comparison between these three conditions by plotting the intensity at 650 nm vs time of purging.

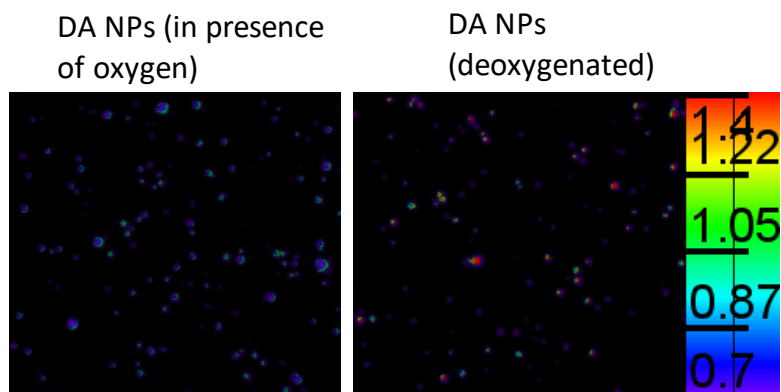


Figure S11. Ratiometric images of **BlueCy-TPB/PtOEP** NPs in the presence and absence of oxygen. The color scale of the right represents acceptor to donor intensity ratio.

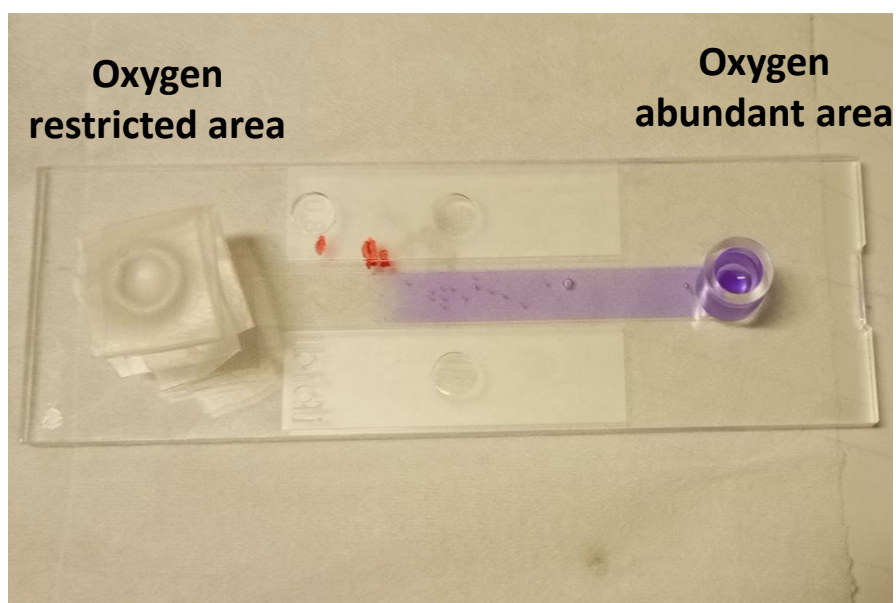


Figure S12. Photo of a microfluidic chamber during optimization of stable oxygen gradient generation in the microfluidic slide using sodium dithionite reducing agent and Nile Red (reduction-sensitive dye).

Table S1. Spectroscopic properties of **BlueCy-TPB** in various solvents.

Solvent	λ_{abs} (nm)	λ_{fl} (nm)	QY (%) ^a
Dioxane	427	479	0.33
Methanol	424	469	0.10
Ethanol	425	478	0.08
Phosphate Buffer	431	492	6.1
Glycerol	428	474	21

λ_{abs} , absorption maximum; λ_{fl} , fluorescence maximum; QY, Quantum yield of fluorescence;
^a QYs for **BlueCy-TPB** were calculated using 7-diethylamino coumarin-3-carboxylic acid in dioxane as standard ($\Phi = 0.93$).

Table S2. Size, polydispersity (PDI) and fluorescence quantum (QY) of **BlueCy-I** and **BlueCy-TPB** in PMMA-MA NPs at different dye loading.

Dye Loading (mM)	BlueCy-I (in PMMA-MA)			BlueCy-TPB (in PMMA-MA)		
	Size (nm)	PDI	QY (%)	Size (nm)	PDI	QY (%)
5	110±10	0.17	31±2	47±3	0.11	36±2
10	190±10	0.25	27±2	47±4	0.10	34±2
50	460±20	0.27	17±1	47±3	0.10	24±2
100	800±40	0.73	11±1	48±4	0.12	17±1
150	800±40	0.67	8.8±0.5	49±3	0.13	11±1

Table S3. Time-resolved fluorescence spectroscopy data for PMMA-MA NPs at different **BlueCy-TPB** loading and for FRET NPs.^a

Donor Loading (%)	τ_1 (nm)	α_1 (%)	τ_2 (nm)	α_2 (%)	τ_3 (nm)	α_3 (%)	$\langle\tau\rangle$ (nm)
0.6	-	-	0.71	12.1	2.53	87.9	2.31±0.15
2.4	0.16	2.92	1.01	18.8	2.45	78.3	2.11±0.14
6	0.17	6.45	1.00	40.4	2.17	53.1	1.57±0.10
12	0.18	12.7	0.84	43.4	1.63	43.9	1.10±0.09
18	0.16	15.9	0.61	47.4	1.31	36.6	0.79±0.07
FRET NPs	0.17	15.0	0.63	45.6	1.42	39.5	0.87±0.07

^a $\tau_1 - \tau_3$ are fluorescence decay times of **BlueCy-TPB** and $\alpha_1 - \alpha_3$ are corresponding amplitudes. $\langle\tau\rangle$ is average fluorescence lifetime **BlueCy-TPB**. FRET NPs correspond to PMMA-MA NPs containing 12% (100 mM) of **BlueCy-TPB** and 0.071 wt% (1 mM) of **PtOEP**.

Table S4. Brightness calculated for **BlueCy-TPB** in PMMA-MA at different loading.

Dye Loading (wt%)	Dye Loading (mM)	Brightness ^a	
		Theoretical ($M^{-1}cm^{-1}$)	Experimental ($M^{-1}cm^{-1}$)
0.6	5	2.3×10^6	2.2×10^6
2.4	20	8.8×10^6	9.9×10^6
6	50	1.6×10^7	1.8×10^7
12	100	2.3×10^7	2.6×10^7
18	150	2.2×10^7	2.8×10^7

^a Theoretical brightness was measured by using the equation $N(\text{number of dyes}) \times \epsilon(\text{molar absorptivity}) \times \phi(\text{quantum yield})$ and experimental values obtained through single particle fluorescence images in comparison with QD525.

Table S5. Phosphorescence lifetime of **BlueCy-TPB/PtOEP** NPs by the successive variation of oxygen concentration in the solution.

Oxygen Conc (mg/mL)	Phosphorescence lifetime (μ s)
7.5	60.6
5.3	83.2
4.1	108
2.4	122
0.03	138

References

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