A photoswitchable solvatochromic dye for probing membrane ordering by RESOLFT super-resolution microscopy


[a] Dr. A. T. Frawley, K. G. Leslie, Dr. V. Wycisk, Prof. H. L. Anderson
Department of Chemistry, University of Oxford
Chemistry Research Laboratory, Mansfield Road, Oxford, OX1 3TA, UK 1
E-mail: andrew.frawley@chem.ox.ac.uk; harry.anderson@chem.ox.ac.uk

[b] Dr. S. Galiani, Dr. D. Shrestha, Prof. C. Eggeling
MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine
University of Oxford
Oxford, OX3 9DS, UK

[c] Prof. C. Eggeling
Institute of Applied Optics and Biophysics
Friedrich-Schiller-University
Jena, Max-Wien Platz 4, 07743 Jena, Germany

[d] Prof. C. Eggeling
Leibniz Institute of Photonic Technology e.V.,
Albert-Einstein-Strasse 9, 07745 Jena, Germany

[e] Prof. C. Eggeling
Jena Center for Soft Matter (JCSM),
Philosophenweg 7, 07743 Jena.
Email: christian.eggeling@uni-jena.de

‡These authors contributed equally
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1. General Methods

All reagents were purchased from commercial sources and used as received. Normal phase flash column chromatography was carried out using SiO$_2$ (60 Å pore size, 40–63 μm particle size, Aldrich, UK) as the stationary phase.

NMR spectra were acquired at 298 K on a Bruker Ascend 600 instrument, equipped with a 5 mm BB-F/1H cryoprobe. NMR chemical shifts are reported in ppm relative to SiMe$_4$ (δ = 0) and were referenced internally with respect to residual solvent protons. Coupling constants are reported in Hz.

Electrospray mass spectrometry was carried out on a Waters Micromass LCT Premier XE spectrometer using 90:10 MeOH:H$_2$O (+0.1% formic acid) as the mobile phase. High-resolution mass spectra (HRMS) were obtained on Waters BioAccord system.

HPLC analysis

Reverse phase HPLC was performed at 298 K using an Agilent 1100 Series system comprising an autosampler (G1313A), a vacuum degassing unit (G1379A), a quaternary pump (G1311A), a column oven (G1316A), a diode array detector (G1315B), and a fraction collector (G1364C). The instrument was operated using ChemStation software. For analytical HPLC an Agilent Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm particle size) was used with a flow rate of 1.0 mL/min.

Method A

<table>
<thead>
<tr>
<th>Time / min</th>
<th>%H$_2$O</th>
<th>%CH$_3$OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>33</td>
<td>95</td>
<td>5</td>
</tr>
</tbody>
</table>

Optical analysis

All spectroscopic measurements were conducted in HPLC grade solvents using quartz cuvettes (10 mm path length, Starna Scientific Ltd, UK). UV-vis absorption spectra were acquired on a Perkin Elmer Lambda 20 spectrometer. Unless otherwise stated, all absorption spectra were recorded at 298 K, with temperature control by a PTP-1 Peltier unit from Perkin Elmer. Fluorescence spectra were acquired at 298 K using an Edinburgh Instruments FS5 spectrofluorometer operating Fluoracle® software, and equipped with a xenon arc lamp (providing 230–1000 nm excitation range), a thermostatic sample holder (SC-20) and both an R13456 PMT detector (200–950 nm spectral coverage, Hamamatsu) and an InGaAs analogue NIR detector (850–1650 nm spectral coverage). Quantum yields were measured by an absolute method using an integrating sphere (SC-30). Lifetimes were measured in time-correlated single photon counting (TCSPC) mode using a picosecond pulsed diode laser (EPL-475) as the excitation source.
2. Synthetic Procedures

Compounds s1 and 2 were synthesized according to literature procedures.[1–3]

![Chemical structure](image)

**Scheme S1.** Preparation of NR-dyad using ethylene diamine linked Nile Red 1 and the NHS ester of SO 2.

**Nile Red derivative 1.** 4-(Ethyl(5-oxo-5H-benzo[a]phenoxazin-9-ylamino)butanoic acid 1 (18 mg, 48 µmol) and HBTU (31 mg, 81 µmol) were dissolved in dry DMF (2 mL). DIPEA (22 mg, 0.17 mmol, 30 µL) was added and the reaction mixture was stirred at room temperature for 45 min. To the solution was added tert-butyl (2- aminoethyl) carbamate (11 mg, 72 µmol) and DIPEA (12 mg, 0.09 mmol, 16 µL). The reaction mixture was stirred at room temperature for 2 h. After the reaction was completed the solvent was removed under reduced pressure. The crude product was dissolved in CH₂Cl₂ and washed with water. The organic layers were dried over Na₂SO₄, filtered and concentrated. The crude product was purified by column chromatography (SiO₂, CH₂Cl₂/ethyl acetate 4:1, 0-4% methanol calculated from the total volume) and Nile Red 1 was obtained as a red solid (17.5 mg, 71%); Rf: 0.44 (SiO₂, ethyl acetate); tᵣ (method A): 18.6 min; ¹H NMR (600 MHz, CDCl₃) δ 8.65 – 8.62 (m, 1H, H-2), 8.29 (dd, J = 7.8, 1.4 Hz, 1H, H-5), 7.71 (ddd, J = 8.3, 7.4, 1.4 Hz, 1H, H-3), 7.64 (td, J = 7.5, 1.3 Hz, 1H, H-4), 7.57 (d, J = 9.0 Hz, 1H, H-12), 6.68 (dd, J = 9.1, 2.7 Hz, 1H, H-13), 6.48 (d, J = 2.7 Hz, 1H, H-15), 6.46 (d, J = 6.2 Hz, 1H, H-23), 6.36 (s, 1H, H-8), 4.97 (s, 1H, H-26), 3.50 – 3.37 (m, 6H, H-18, H-19, H-24), 3.33 – 3.27 (m, 2H, H-25), 2.28 (t, J = 7.0 Hz, 2H, H-21), 1.99 (p, J = 7.2 Hz, 2H, H-20), 1.41 (s, 9H, H-29), 1.27 – 1.20 (m, 6H, H-17); ¹³C NMR (151 MHz, CDCl₃) δ 183.94 (C-7), 172.58 (C-22), 157.33 (C-27), 152.27 (C-9), 151.09 (C-14), 146.81 (C-16), 140.22 (C-10), 132.20 (C-1), 131.86 (C-6), 131.49 (C-3), 131.28 (C-12), 130.10 (C-4), 125.80 (C-5), 125.20 (C-11), 123.97 (C-2), 110.06 (C-13), 105.85 (C-8), 96.67 (C-15), 80.02 (C-28), 50.14 (C-19), 45.63 (C-18), 41.37 (C-24), 40.35 (C-25), 33.20 (C-21), 28.33 (C-29), 23.23 (C-20), 12.49 (C-17); HRMS (ESI⁺) m/z calcd for C₂₉H₃₃N₄O₅⁺: 519.2602 [M+H⁺]; found: 519.2602; UV-Vis
(CH₂Cl₂): \( \lambda_{\text{max}} (\epsilon) = 539 \text{ nm (70,200 mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}) \); fluorescence (CH₂Cl₂): \( \lambda_{\text{exc}} = 500 \text{ nm, } \lambda_{\text{em}} = 605 \text{ nm; } \Phi_{\text{f}} = 64\%; \tau_{\text{f}} = 4.4 \text{ ns.} \\

**NR-dyad.** Nile Red 1 (8.00 mg, 15.4 \( \mu \text{mol} \)) was dissolved in dichloromethane (0.90 mL) and TFA (0.10 mL) was added. The reaction mixture was stirred at 20 °C for 1 h and followed by TLC. The solvent was removed in vacuo and the crude product was co-evaporated with dichloromethane (6 times) to remove the acid. The deprotected product was confirmed by MS ([(ESI\(^+\)] m/z 419.180 [M+H\(^+\)], calc. 419.208 [M+H\(^+\)]) used without further purification. Deprotected Nile Red 2 (8.00 mg, 15.0 \( \mu \text{mol} \)) and spironaphthoxazine NHS ester 2 (17.6 mg, 30.0 \( \mu \text{mol} \)) were dissolved in dry DMF (3.00 mL) and stirred at 20 °C for 20 h. The solvent was removed in vacuo and the crude product was purified by column chromatography (SiO₂, CH₂Cl₂ 100% to CH₂Cl₂/ethyl acetate 80:20, addition of 5% methanol, calculated from total volume) to give dyad **NR-dyad** as a red solid (8.9 mg, 67%); \( R_{\text{f}} \): 0.19 (SiO₂, ethyl acetate); \( t_{\text{f}} \) (method A): 22.0 min; \(^1\)H NMR (600 MHz, CDCl₃) \( \delta \) 8.60 (dd, \( J = 8.3, 8.3 \text{ Hz, 2H, overlapped H-41 and H-2} \)), 8.27 (dd, \( J = 7.8, 1.4 \text{ Hz, 1H, H-5} \)), 7.96 (d, \( J = 8.4 \text{ Hz, 1H, H-44} \)), 7.68 (td, \( J = 7.6, 1.5 \text{ Hz, 1H, H-3} \)), 7.65 – 7.62 (m, 3H, overlapped H-4, H-29 and H-38), 7.61 – 7.57 (m, 1H, H-42), 7.56 (d, \( J = 1.8 \text{ Hz, 1H, H-33} \)), 7.53 (d, \( J = 9.0 \text{ Hz, 1H, H-12} \)), 7.35 (ddd, \( J = 8.4, 6.7, 1.3 \text{ Hz, 1H, H-43} \)), 7.18 (d, \( J = 7.3 \text{ Hz, 1H, H-52} \)), 6.95 – 6.93 (m, 1H, H-53), 6.91 (t, \( J = 7.8 \text{ Hz, 1H, H-26} \)), 6.89 (s, 1H, H-47), 6.81 (t, \( J = 5.4 \text{ Hz, 1H, H-23} \)), 6.73 (t, \( J = 7.3 \text{ Hz, 1H, H-54} \)), 6.64 (d, \( J = 9.1, 2.7 \text{ Hz, 1H, H-13} \)), 6.49 – 6.43 (m, 2H, overlapped H-15 and H-30), 6.34 (s, 1H, H-8), 6.29 (d, \( J = 7.9 \text{ Hz, 1H, H-55} \)), 3.90 (s, 2H, H-49), 3.65 – 3.60 (m, 2H, H-25), 3.54 (q, \( J = 5.3 \text{ Hz, 2H, H-24} \)), 3.44 – 3.35 (m, 4H, overlapped H-18 and H-19), 3.29 – 3.04 (m, 2H, H-50), 2.77 (s, 3H, H-36), 2.30 (t, \( J = 7.0 \text{ Hz, 2H, H-21} \)), 1.98 (p, \( J = 7.1 \text{ Hz, 2H, H-20} \)), 1.36 (s, 3H, H-35 or H-35'), 1.33 (s, 3H, H-35 or H-35') 1.18 (t, \( J = 7.1 \text{ Hz, 3H, H-17} \)); \(^{13}\)C NMR (151 MHz, CDCl₃) \( \delta \) 183.91 (C-7), 173.35 (C-22), 168.84 (C-27), 152.24 (C-9), 151.07 (C-14), 150.72 (C-31), 149.98 (C-56), 148.47 (C-38), 146.76 (C-16), 144.72 (C-46), 144.31 (C-48), 140.12 (C-10), 135.48 (C-32), 132.17 (C-40), 131.85 (C-6), 131.82 (C-1), 131.49 (C-3), 131.26 (C-12), 130.12 (C-51), 130.09 (C-4), 128.03 (C-29), 127.75 (C-42), 127.03 (C-53), 125.78 (C-5), 125.60 (C-45), 125.23 (C-11), 125.18 (C-28), 124.89 (C-52), 124.75 (C-44), 123.94 (C-2), 122.03 (C-41), 121.18 (C-33), 120.68 (C-39), 119.32 (C-54), 110.06 (C-13), 109.97 (C-55), 108.61 (C-47), 106.38 (C-30), 105.79 (C-8), 98.79 (C-37), 96.67 (C-15), 55.34 (C-49), 51.55 (C-34), 50.09 (C-19), 45.59 (C-18), 41.08 (C-24), 40.87 (C-25), 33.22 (C-21), 29.64 (C-36), 29.05 (C-50), 25.47 (C-35 or C-35'), 23.26 (C-20), 20.95 (C-35 or C-35'), 12.46 (C-17); HRMS (ESI\(^+\)) \( m/z \) calcld for C₅₅H₅₂N₂O₄\(^{+}\): 890.4024 [M+H\(^+\)]; found: 890.4037. UV-Vis (CH₂Cl₂): \( \lambda_{\text{max}} (\epsilon) = 537 \text{ nm (111,000 mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}) \); fluorescence (CH₂Cl₂): \( \lambda_{\text{exc}} = 500 \text{ nm, } \lambda_{\text{em}} = 604 \text{ nm; } \Phi_{\text{f}} = 34\%; \tau_{\text{f}} = 3.7 \text{ ns.} \)
3. Photophysical Properties

Figure S1. Normalized absorption spectra of 1 in solvents of varying polarity (298 K, 1 µM).

Figure S2. Normalized emission spectra of 1 in solvents of varying polarity (λ_{exc} 480 nm, 298 K, 1 µM).
Figure S3. Normalized absorption spectra of NR-dyad in solvents of varying polarity (298 K, 1 µM).

Figure S4. Normalized emission spectra of NR-dyad in solvents of varying polarity (λexc 480 nm, 298 K, 1 µM).
Table S1. Photophysical properties of Nile Red derivative 1 in various solvents (298 K, 1 µM).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$E_N^t$ Reichardt polarity parameter$^{[4]}$</th>
<th>$\lambda_{\text{max}}$ (abs) / nm</th>
<th>$\varepsilon(\lambda_{\text{max}})$ / mol$^{-1}$ dm$^3$ cm$^{-1}$</th>
<th>$\lambda_{\text{max}}$ (em) / nm</th>
<th>$\Phi_f$</th>
<th>$\tau$ / ns</th>
</tr>
</thead>
<tbody>
<tr>
<td>THF</td>
<td>0.207</td>
<td>533</td>
<td>72300</td>
<td>596</td>
<td>0.85</td>
<td>4.3</td>
</tr>
<tr>
<td>DCM</td>
<td>0.309</td>
<td>540</td>
<td>70200</td>
<td>605</td>
<td>0.64</td>
<td>4.4</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.355</td>
<td>534</td>
<td>65400</td>
<td>611</td>
<td>0.55</td>
<td>4.6</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.444</td>
<td>552</td>
<td>69600</td>
<td>635</td>
<td>0.48</td>
<td>4.2</td>
</tr>
<tr>
<td>MeOH</td>
<td>0.762</td>
<td>549</td>
<td>65500</td>
<td>641</td>
<td>0.35</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Table S2. Photophysical properties of NR-dyad in various solvents (298 K, 1 µM).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$E_N^t$ Reichardt polarity parameter$^{[4]}$</th>
<th>$\lambda_{\text{max}}$ (abs) / nm</th>
<th>$\varepsilon(\lambda_{\text{max}})$ / mol$^{-1}$ dm$^3$ cm$^{-1}$</th>
<th>$\lambda_{\text{max}}$ (em) / nm</th>
<th>$\Phi_f$</th>
<th>$\tau$ / ns</th>
</tr>
</thead>
<tbody>
<tr>
<td>THF</td>
<td>0.207</td>
<td>531</td>
<td>109000</td>
<td>596</td>
<td>0.59</td>
<td>3.2</td>
</tr>
<tr>
<td>DCM</td>
<td>0.309</td>
<td>536</td>
<td>111000</td>
<td>604</td>
<td>0.34</td>
<td>3.7</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.355</td>
<td>534</td>
<td>120000</td>
<td>612</td>
<td>0.36</td>
<td>3.9</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.444</td>
<td>552</td>
<td>92000</td>
<td>634</td>
<td>0.54</td>
<td>3.6</td>
</tr>
<tr>
<td>MeOH</td>
<td>0.762</td>
<td>554</td>
<td>162000</td>
<td>639</td>
<td>0.15</td>
<td>1.9</td>
</tr>
</tbody>
</table>

4. Calculation of FRET efficiency

Equation S1 was used to calculate Förster radii, $R_0$, for the NR-dyad (see Table S2)$^{[5]}$:

$$R_0 = 0.2108 \left[ \frac{\kappa^2 \Phi_D J(\lambda)}{n^4} \right]^{1/6} \quad \text{(Eq. S1)}$$

where $\kappa^2$ is the dipole orientation factor (assumed to be 2/3 given free rotation between the donor and the acceptor), $\Phi_D$ is the fluorescence quantum yield of the donor in the absence of the acceptor (in this case, compound 1), $n$ is the refractive index of the solvent (1.424 for CH$_2$Cl$_2$), and $J(\lambda)$ is the spectral overlap integral, which is given by Equation S2.$^{[5]}$

$$J(\lambda) = \int f_D \varepsilon_A(\lambda) \lambda^4 \, d\lambda \quad \text{(Eq. S2)}$$

where $f_D$ is the normalized donor emission spectrum, $\varepsilon_A(\lambda)$ is the acceptor molar extinction coefficient at wavelength $\lambda$. The efficiency of the FRET process, $E$, can be calculated from the Förster radius, $R_0$, and the distance between the donor and acceptor, $r$, according to Equation S3. We have used distances measured from molecular mechanics structural optimizations of the dyads.
We have acquired emission data for Nile Red derivative 1 in toluene, dichloromethane (DCM) and acetonitrile (MeCN) and taken the absorption spectra of the open form of the spironaphthoxazine in the same solvents from previous work. The calculated FRET distances and efficiencies (using a donor-acceptor distance, $r$, of 21.6 Å) are shown in Table S3. From the spectra (Fig. S5) it can be seen that as well as Nile Red emission solvatochromism, there is positive solvatochromism for the absorption of the open form of the spironaphthoxazine, although this effect is bigger for the Nile Red emission. The data in Table S3 show that while $R_0$ is affected by the solvatochromism, there is little effect on the FRET efficiency, because the molecule is much smaller than the $R_0$ range.

Table S3. Effect of solvent on calculated FRET distances and efficiencies.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>FRET distance, $R_0$ / Å</th>
<th>FRET efficiency / %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene</td>
<td>53.1</td>
<td>99.5</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>47.2</td>
<td>99.1</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>43.4</td>
<td>98.5</td>
</tr>
</tbody>
</table>

![Figure S5. Spectra showing FRET overlap in different solvents: the absorption spectra (purple) of the open spironaphthoxazine switch and the emission spectra (red) of Nile Red 2 in 3 solvents of varying polarity (all 298 K).](image)
5. PET as an alternative quenching mechanism

Experimental redox potential measurements

Redox potentials of the Nile Red dye with linker 1 were measured using square wave voltammetry. Redox potentials for the spironaphthoxazine switch SO was previously measured in a similar analysis.[3] Electrochemical experiments were performed in acetonitrile at compound concentrations of 0.1–1.0 mM with tetrabutylammonium hexafluorophosphate as the electrolyte (0.1 M). Square wave voltammograms were collected with a modulation amplitude of 50 mV and a frequency of 2 Hz. Glassy carbon (Ø = 1 mm), platinum wire and Ag|AgNO₃ (10 mM) were used as working, counter and reference electrodes, respectively. The potentials were referenced at the end of each experiment by addition of ferrocene.

Calculated redox potentials

Density functional theory (DFT) calculations were carried out using the ORCA 4.1.1 program,[6] on simplified chemical structures, using previously-published data for the switch.[3] Tight optimization criteria were used for all geometries, and numerical frequency calculations used to confirm stationary points as minima, and to calculate thermal energy corrections. Resolution of identity was used to speed up the SCF process employing the RIJCOSX approximation. Grid6 and GridX6 were used in all calculations. Implicit solvation was introduced employing the SMD model.

![Structures of the dye derivatives and spironaphthoxazine used for calculations.](image)

Redox potentials were calculated from free energies using Equation S4, where \( z \) is the number of transferred electrons \( (z = 1) \) and \( F \) is the Faraday constant, and referenced to the SCE electrode with an absolute \( E^\circ \) of 4.429 V, corrected for the liquid junction potential in acetonitrile.[7] These values were further corrected to ferrocene (0.40 V vs. SCE)[8] for comparison with experimental values. Free energies can be calculated for redox processes as per the example of a reduction in Equation S5. Calculations were performed at the PBE0 level of theory in combination with the def2-TZVPP basis set and the SMD solvation model (acetonitrile), using the optimized PBE/def2-SVP geometries.

\[
E^\circ = -\Delta G^\circ (\text{sln, redox}) / zF \tag{Eq. S4}
\]

\[
\Delta G^\circ (\text{MeCN, redox}) = G^\circ X^- (\text{MeCN}) - G^\circ X (\text{MeCN}) \tag{Eq. S5}
\]
Results

A comparison of the calculated and experimental values for the redox potentials of each compound is shown in Table S4. The calculated values are in fairly good agreement with the obtainable experimental values. As the reduction potential of the closed switch was not seen in the observable window, and the open switch does not persist in solution long enough to be measured, we have used the calculated values in a thermodynamic analysis of PET.

Table S4. Calculated and measured oxidation and reduction potentials of the measured compounds in acetonitrile.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Process</th>
<th>Calculated $\Delta G^\circ$/ kJ mol$^{-1}$</th>
<th>Experimental $E^\circ$/ V (vs. Fc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nile Red</td>
<td>Oxidation</td>
<td>-495</td>
<td>+0.30</td>
</tr>
<tr>
<td>Nile Red</td>
<td>Reduction</td>
<td>-316</td>
<td>-1.55</td>
</tr>
<tr>
<td>SO closed</td>
<td>Oxidation</td>
<td>-476</td>
<td>+0.11</td>
</tr>
<tr>
<td>SO closed</td>
<td>Reduction</td>
<td>-231</td>
<td>-2.44</td>
</tr>
<tr>
<td>SO open</td>
<td>Oxidation</td>
<td>-472</td>
<td>+0.06</td>
</tr>
<tr>
<td>SO open</td>
<td>Reduction</td>
<td>-315</td>
<td>-1.56</td>
</tr>
</tbody>
</table>

[a] Not observable within the solvent window. [b] We cannot measure the oxidation and reduction potentials of the spironaphthoxazine in its open (merocyanine) form as it does not persist in solution.

The $\Delta G_{\text{PET}}$ of electron transfer for all possible scenarios was calculated using the Weller equation (Equation S6),\(^5\) in which $E(D/D^+)$ and $\Delta E(D)$ are the oxidation potential of the donor and singlet excitation energy of the fluorophore, respectively, and $E(A^-/A)$ is the reduction potential of the acceptor. The final term ($e^2/\varepsilon r$) accounts for the free energy gained by bringing the ions together in solution, however it is smaller than the errors in the redox potential measurements, so it can be ignored for this calculation. In this analysis, excited state energies of 2.15 eV for Nile Red was calculated as the midpoint between the absorption and emission maxima. $\Delta G_{\text{PET}}$ of the possible photoinduced electron transfer processes are shown in Table S5.

$$\Delta G_{\text{PET}} = E(D/D^+) - \Delta E(D) - E(A^-/A) - \frac{e^2}{\varepsilon r} \quad \text{(Eq. S6)}$$

Table S5. Calculated $\Delta G_{\text{PET}}$ values for different donor and acceptor pairs, compared to experimental results where possible.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>Calculated $\Delta G_{\text{PET}}$/ eV</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO closed</td>
<td>Nile Red</td>
<td>-0.49</td>
</tr>
<tr>
<td>SO open</td>
<td>Nile Red</td>
<td>-0.54</td>
</tr>
<tr>
<td>Nile Red</td>
<td>SO closed</td>
<td>+0.59</td>
</tr>
<tr>
<td>Nile Red</td>
<td>SO open</td>
<td>-0.29</td>
</tr>
</tbody>
</table>
As might be expected for the relatively electron rich photoswitch, electron transfer is more favorable when the switch acts as the donor, in both open and closed forms. The negative $\Delta G_{\text{PET}}$ values in these scenarios imply that PET quenching of fluorescence is thermodynamically favored in NR-dyad. Such a process may account for the decrease in the fluorescence quantum yield of Nile Red when it is conjugated to the switch (Tables S1 and S2).

The difference in $\Delta G_{\text{PET}}$ between open and closed switch is probably not large enough to account for the magnitude of the observed quenching when the switch is in its active form. Therefore, while PET may have a minor contribution, it is likely that FRET is the main mechanism by which the open spironaphthoxazine quenches the Nile Red.

### 6. Sample preparation

**Preparation of GUVs for imaging**

GUVs were freshly prepared on the same day as imaging, according to the following electroformation procedure:[10] The electroformation chamber was cleaned with ethanol and dried thoroughly under $\text{N}_2$ flow. A solution of lipid (5 μL of a 1 mg mL$^{-1}$ solution in CHCl$_3$) was spread evenly over two Pt electrodes and dried under $\text{N}_2$ flow. A solution of sucrose (370 μL, 300 mM aqueous) was placed in the electroformation chamber and the Pt electrodes were inserted into this solution. For single lipids, the electroformation was carried out at room temperature. For mixed DOPC/cholesterol vesicles, the chamber was heated to 45 °C and to 60 °C for 2:2:1 DOPC:sphingomyelin:cholesterol phase separated GUVs. Using a function generator (frequency 10 Hz, amplitude 5.7 V$_{\text{peak-to-peak}}$ = 2 V$_{\text{RMS}}$) an electrical potential was applied to the sample for 1 h to form the GUVs. After 1 h, the frequency was reduced to 2 Hz and left for a further 30 min to detach the GUVs from the electrodes, before being left to cool to room temperature if necessary. Meanwhile, the wells of a glass ibidi μ-Slide 8-well plates were passivated with poly-L-lysine for 1 h, before washing with PBS three times. For confocal and RESOLFT imaging, some of the GUV solution (75 μL) was transferred into an Eppendorf tube and a solution of dyad (1 μL, 1 mM in DMSO for NR-dyad, 200 nM in EtOH for NR12A) was added and left to incubate for 15 min. The vesicle solution was then placed into PBS (250 μL) in the well of the 8-well plate for imaging. All transfers of GUVs were carried out using trimmed pipette tips. Phosphate buffered saline was made by dissolution of tablets (ThermoFisher) in deionized water. The concentrations of the components are as follows: NaCl at 8.0 g L$^{-1}$, KCl at 0.2 g L$^{-1}$, Na$_2$HPO$_4$ at 1.15 g L$^{-1}$, KH$_2$PO$_4$ at 0.2 g L$^{-1}$.

**Preparation of THP-1 cells for imaging**

THP-1 (human monocytic) cells were centrifuged, and the pellet was washed with L15 three times. The cells were then re-suspended in PBS and NR-dyad was added (final concentration either 2 or 5 μM). The cells were then incubated with NR-dyad at either 37 °C or 4 °C for between 1 min and 15 min, then washed with L15 to remove the unbound dye and suspended in L15 buffer for imaging. In some examples, 0.02% sodium azide was added to the incubation step to minimize endocytosis.
Preparation of GPMVs for imaging

THP-1 cells grown in RPMI (Roswell Park Memorial Institute) medium suspended in growth medium were washed with an isotonic GPMV buffer (150 mM NaCl, 10 mM HEPES, 2 mM CaCl₂, pH 7.4), followed by incubation for 90 minutes at 37 °C with dithiothreitol (final concentration 1 mM) and paraformaldehyde (final concentration 25 mM). GPMVs were labelled in the same GPMV buffer with NR-dyad (final concentration 5 µM) and imaged directly.

7. Microscopy

RESOLFT Microscopy

RESOLFT microscopy was performed on a modified Abberior Instruments RESOLFT microscope (Abberior Instruments, Gottingen, Germany), equipped with excitation lasers at 485 nm (pulsed 100 ps at 80 MHz, LDH-D-C-485, PicoQuant), 594 nm (pulsed 80 ps at 80 MHz, LightUp594, Abberior Instruments) and 640 nm (pulsed (80 ps at 80 MHz) or CW, LDH-D-C-640P, Picoquant), and customized to provide a 405 nm doughnut shaped laser via a vortex phase mask (VPP-1b, RPC Photonics, Rochester, NY), placed in the path of the 405 nm source (Cobolt diode laser, CW). Shuttering of the lasers was achieved with acousto-optical modulators (MT110-A1.5-VIS, Photon Lines, Banbury, UK). Images were acquired with 100×/1.4 NA oil immersion objective lens (UPlanSApo 100×/1.4 oil, Olympus, Japan) and detected with photon counting avalanche photo diodes (restricted to detection ranges of 510–568 nm, 605–635 nm or 650–755 nm). The microscope was operated with Imspector software (Abberior Instruments, Gottingen, Germany) and image analysis was carried out in Imspector and FIJI ImageJ. Lookup tables for the images are based on min/max pixel intensities for each image, with no correction for background. Zero on the lookup table corresponds to a zero-photon count. Laser powers, $P$, were measured at the back focal plane of the objective. Together with the FWHM of the focal laser intensity distribution, they allow for the calculation of the time-averaged intensity $I = P / [\pi (\text{FWHM}/2)^2]$ and a pulse peak intensity $I_{\text{peak}} = I/(\tau f)$ with pulse width $\tau = 80$ ps and repetition rate $f = 80$ MHz. The FWHM of the laser spot can be calibrated from images of 100 nm TetraSpeck beads taken with each laser, and are approximately 205 nm for the 405 nm laser, 225 nm for the 594 nm laser, and 235 nm for the 640 nm laser. These measurements give intensities for each laser as follows: 405 nm ($P = 34–466 \, \mu W$ corresponds to $I = 0.10–1.41$ MW cm⁻²); 594 nm ($P = 0.46–3.96 \, \mu W$ corresponds to $I = 1.2–10.0$ kW cm⁻²); 640 nm ($P = 0.66–3.9 \, \mu W$ corresponds to $I = 1.5–8.9$ kW cm⁻²).

RESOLFT images were acquired using a previously-developed pixel-by-pixel imaging sequence. Briefly, we used sequence in which each pixel is irradiated with a number of laser beams sequentially. Each pixel was irradiated as follows: firstly, a Gaussian-shaped diffraction-limited excitation spot (either 485 or 594 nm) acquired the confocal signal; secondly, a donut-shaped 405 nm pulse converted the spironaphthoxazine to its active open form; thirdly, the Gaussian-shaped excitation spot acquired the RESOLFT signal by inducing fluorescence from the central (unquenched) region of the pixel. Finally, the spironaphthoxazine switch was
driven back to its closed form using a Gaussian-shaped 640 nm irradiation. Each laser was applied sequentially to each pixel in the image, and raster scanning was used to build up the image. Each RESOLFT image is accompanied by a confocal image acquired simultaneously which acts as an internal control. Excitation powers were set such that the detector was not saturated. For solvatochromic images, the RESOLFT image was acquired in all three detector ranges simultaneously. See Fig. S7 for a representative schematic of the imaging sequence.

**Figure S7.** Laser sequence for solvatochromic RESOLFT imaging.

Initial experiments tested how efficiently the dyad could be quenched. For this, we used the laser sequence in Fig. S7 but with the donut removed. So rather than acquiring confocal and super-resolved images, we acquire ‘bright’ and ‘dark’ images. The intensities of these images can be compared to give a quenching efficiency according to Equation S7.

\[
\text{Quenching efficiency} = \frac{(I_{\text{bright}} - I_{\text{dark}})}{I_{\text{bright}}} \quad \text{(Eq. S7)}
\]

We began with experimental parameters which were optimized for the previous generation of dyads bearing the spironaphthoxazine switch, and confirmed that they were suitable for imaging NR-dyad. Of particular importance is the optimisation of the 405 nm laser power. We tested a range of 405 nm laser powers and calculated the quenching efficiencies (Fig. S8). The quenching efficiency rises very quickly at low laser power, and then plateaus at around 92% (i.e. only 8% of the original emission remains unquenched).
Figure S8. Dependence of fluorescence quenching efficiency of NR-dyad on 405 nm laser power (pixel-by-pixel, λexc 594, 2.3 μW; λrecovery 640 nm, 3.9 μW (CW); pixel size 40 nm).

Resolution analysis

Automation

We developed a Python script to automatically analyze the full-width-at-half-maximum of the membranes in our images. Automation allowed us to explore the variation in the observed resolutions. An ImageJ macro script was used to convert images with a confocal and RESOLFT channel into .txt images (arrays of pixel intensities), and then the Python numpy module was used to handle the arrays (Figure S9). Firstly, a threshold was applied to the image arrays to discard pixels below 0.3× the maximum intensity (an empirically-determined value), and the matrix locations of the pixels above this threshold were stored as an array. Then, these locations were plotted on an x,y graph and a 2nd degree polynomial fitted to the shape of the ‘membrane’. The tangent and normal were calculated at specific points along the membrane, and the normal vectors overlaid with the original image array to sample the pixel intensities along the line. The pixel intensities were plotted against the distance along the normal and fitted with Gaussian or double Gaussian functions (see below), to extract the full-width-at-half-maximum of the line profile, which we use as a proxy for resolution.
Fitting Line Profiles

The RESOLFT images are generated simultaneously with a corresponding confocal image, which acts as the control. When fitting the line profiles (the image intensity along a line perpendicular to the membrane), both the conventional confocal image and the RESOLFT super-resolution image are used.

The confocal line profiles are well described by a simple Gaussian curve (Equation S8),

\[
I = I_0 + I_p e^{-\frac{4(ln2)(x-x_p)^2}{w_{con}^2}}
\]  

(Eq. S8)

where \( I \) is the signal intensity (photon counts), \( I_0 \) is a baseline correction, \( I_p \) is the peak intensity, \( x \) is the position along the line, \( x_p \) is the center of the peak, and \( w_{con} \) is the peak full-width-at-half-maximum (FWHM).

We have previously shown\(^3\) that RESOLFT line profiles are well described by a double Gaussian model (Equation S9), which incorporates the confocal background which remains in the RESOLFT image caused by incomplete quenching of the dyad,
\[ I = I_0 + I_{\text{con}} e^{-\frac{4(ln 2)(x - x_p)^2}{w_{\text{con}}^2}} + I_{\text{SRM}} e^{-\frac{4(ln 2)(x - x_p)^2}{w_{\text{SRM-2G}}^2}} \]  
(Eq. S9)

where \( w_{\text{con}} \) is fixed as the peak width from the corresponding confocal profile and \( w_{\text{SRM-2G}} \) is the fitted width of the RESOLFT component. We then report our observed resolution enhancement as \( \frac{w_{\text{con}}}{w_{\text{SRM-2G}}} \).

Fitting Results

We fitted the line profiles from 336 images of membranes of different polarity. In each case, line profiles were calculated that crossed the membrane at 7 locations, 3 pixels apart, distributed around the central point (for example – Figure S10, which shows the data output from a single image). Some data cleaning was performed and those with particularly bad fits, or fits where the RESOLFT Gaussian contribution was small (< 40%) were removed from the dataset. The mean resolutions for confocal and RESOLFT images, alongside mean enhancements are shown in Table S6.

<table>
<thead>
<tr>
<th>Membrane composition</th>
<th>Number of line profiles</th>
<th>Mean confocal resolution / nm</th>
<th>Mean RESOLFT resolution / nm</th>
<th>Mean RESOLFT Gaussian contribution</th>
<th>Mean enhancement</th>
<th>Enhancement standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC</td>
<td>1399</td>
<td>377</td>
<td>157</td>
<td>0.78</td>
<td>2.53</td>
<td>0.66</td>
</tr>
<tr>
<td>80:20 DOPC:cholesterol</td>
<td>315</td>
<td>322</td>
<td>146</td>
<td>0.79</td>
<td>2.52</td>
<td>0.52</td>
</tr>
<tr>
<td>50:50 DOPC:cholesterol</td>
<td>592</td>
<td>387</td>
<td>212</td>
<td>0.57</td>
<td>1.91</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Table S6. Mean confocal, RESOLFT resolutions and enhancements for the different vesicle compositions.
Figure S10. Example outputs from the automation script for multiple line profiles, showing 7 normals to the membrane and their confocal fits, and the 7 corresponding RESOLFT fits, for a single image.
Confocal microscopy (for colocalization and spectral imaging)

Confocal images were obtained using a Zeiss LSM780 inverted laser scanning confocal microscope equipped with various excitation sources: 458/488/514 nm (multiline Ar-Ion laser, CW; 458 nm: 3–7 µW at sample; 488 nm: 5–25 µW at sample) and 594 nm (HeNe laser, CW, 6.6–14 µW at sample). Detection was achieved with PMT non-descanned detectors or high sensitivity GaAsP arrays. Images were acquired with a Plan Apochromat 63×/1.40 NA DIC M27 oil immersion objective. The microscope was operated via a PC running Zen software. Image analysis was carried out in FIJI ImageJ.

For spectral imaging, images were taken with 8.9 nm windows in the range 500–696 nm using excitation at 488 nm. The intensity in each window was used to construct an emission spectrum. For each vesicle composition, multiple sets of data were recorded, and the spectra averaged. We compared the solvatochromic behavior of NR-dyad to that of NR12A (Fig. S11). In our hands NR12A performs very similarly to previously-published data.[1] The spectra acquired for NR-dyad closely match those of NR12A, confirming that NR-dyad is an effective probe for membrane ordering.

![Spectral imaging of NR12A (left) and NR-dyad (right) in GUV membranes comprising DOPC (black) or 50:50 DOPC:cholesterol (red).](image)

**Figure S11.** Spectral imaging of NR12A (left) and NR-dyad (right) in GUV membranes comprising DOPC (black) or 50:50 DOPC:cholesterol (red).

Spectral imaging of the ordered and disordered phases of phase separated vesicles containing NR-dyad showed a significant hypsochromic shift in the Nile Red emission in the ordered phase compared to the disordered phase (Fig. S12). While the NR-dyad emission maximum for the disordered phase was comparable to that observed in DOPC vesicles, the shift in emission maximum in the ordered phase was considerably...
larger than that observed for the 50:50 DOPC:cholesterol membranes, confirming that addition of sphingomyelin gives much more ordered membranes.

Figure S12. Spectral imaging of NR-dyad in the disordered (L_d, green) and ordered (L_o, orange) domains of phase separated GUV membranes comprising 2:2:1 DOPC:sphingomyelin:cholesterol.
**Molecular orientation**

In confocal images acquired with a polarized excitation laser, we observe that in 50:50 DOPC:cholesterol vesicles, and vesicles made entirely of the \( L_0 \) phase of DOPC:sphingomyelin:cholesterol, the membranes appear bright on one axis and dark on the perpendicular axis (Fig. S13), indicating that the transition dipole moments of the dyes are well-aligned with the excitation laser on one axis but not the other. This feature is not observed in disordered DOPC membranes or in vesicles made of the \( L_d \) phase of DOPC:sphingomyelin:cholesterol. This observation suggests that in the ordered membranes, the **NR-dyad** is aligned in some way with the lipid bilayer and that its rotation is not averaged over the time the molecule spends in the excited state.

![Images of various vesicle types stained with NR-dyad](Image)

**Figure S13.** Images of various vesicle types stained with **NR-dyad**; \( \lambda_{\text{exc}} \) 488 nm, \( \lambda_{\text{em}} \) 613 nm (\( L_0 \) phase), 631 nm (\( L_d \) phase), 579 nm (50:50 DOPC:Cholesterol), and 659 nm (DOPC). Emission wavelengths represent the center of an 8.9 nm spectral imaging window. Images are falsely colored to approximate their maximum emission. Scale bars represent 2 \( \mu \text{m} \) (\( L_0 \) and \( L_d \) phase) and 5 \( \mu \text{m} \) (50:50 DOPC:Cholesterol and DOPC).

**Polarity analysis**

Using the RESOLFT imaging sequence in Fig. S7, we acquired super-resolution images in multiple spectral windows. In order to analyze whether we could distinguish different membrane compositions using **NR-dyad**, we wrote a simple ImageJ macro which extracted the relevant channels from the image data and compared the mean pixel intensities, calculating the ratio between them \( (I_{605-635} / I_{650-755}) \). To check whether automated analysis of the whole image varied from analyzing just a region of interest containing the membrane, we carried out manual and automated analysis on a subset of our data \( (n = 40 \text{ images total}; 20 \text{ DOPC and } 20 \text{ 50:50 DOPC:cholesterol}) \). The intensity ratios between the two detector ranges were compared (Fig. S14). At
\( P = 0.01 \) level, the differences between the automated and manual analyses were not significant, confirming that the two methods of data analysis are comparable.

![Box-and-whisker diagram](image)

**Figure S14.** Box-and-whisker diagram showing that analyzing the intensity ratio between the two detector ranges using the automated script (pink) gives comparable results to those acquired using manual analysis (blue). The difference in ratio between DOPC and 50:50 DOPC:cholesterol GUV membranes is maintained in the automated analysis. \( \lambda_{\text{exc}} \) 594 nm, \( \lambda_{\text{em}} \) 605–635 nm and 650–755 nm.

All further polarity analysis on whole images was carried out using the automated analysis process.

**Colocalization**

Colocalization experiments were carried out to identify whether \textbf{NR-dyad} preferentially localized in the L\(_d\) or L\(_o\) domain of phase separated GUVs. The GUVs were prepared as described above, but with the addition of \textbf{FAST-DiO} as well as \textbf{NR-dyad}. \textbf{FAST-DiO} is a blue-green emitting lipophilic carbocyanine dye which is known to label the L\(_d\) phase of lipid membranes. Control experiments to set appropriate imaging parameters to avoid crosstalk were carried out (Fig. S15). The co-staining experiments (Fig. S16) showed that \textbf{NR-dyad} and \textbf{FAST-DiO} localized in the same membrane regions, confirming that \textbf{NR-dyad} preferentially localized in the L\(_d\) phase of the GUVs.
Figure S15. The structure of FAST-DIO and control images for NR-dyad/FAST-DIO colocalization experiments in 2:2:1 DOPC:sphingomyelin:cholesterol phase separated GUVs (scale bar = 10 μm). Fluorescence excitation and detected emission wavelengths as labeled.
Figure S16. Confocal images of phase separated GUVs stained with both NR-dyad and FAST-DiO showing colocalization in the Ld phase (scale bar = 10 μm). Fluorescence excitation and detected emission wavelengths as labeled.

**Pixel-by-pixel polarity analysis**

To analyze RESOLFT images for polarity at a pixel-by-pixel level we used the following method in ImageJ (Fig. S17). To calculate the intensity ratio in the region of interest without interference from the image background, it is necessary to remove the background from the analysis. Firstly, a threshold is applied to the image which converts pixels in the region of the membrane to an intensity of 1 and all others to 0. This image is then multiplied by both the original images for the two detection windows, which removes the background noise but leaves all pixels in the area of interest unchanged. Then one image is divided by the other to create a map of $I_{605-635} / I_{650-755}$ for each pixel. Finally, a LUT is applied (in this case ‘Green Fire Blue’) to show regions of different polarity in the image.
Figure S17. Example workflow for analysis of images of phase separated GUVs stained with polarity sensitive dyes. Images are 2 × 2 µm unless otherwise stated.

We found that it was difficult to measure the polarity ratio in the ordered phase of phase-separated GUV membranes using 594 nm excitation due to a lack of signal from dyad accumulated in this phase. However, excitation at 485 nm gave significantly more signal in the Lc phase. To confirm that our polarity imaging sequence is still valid with 485 nm excitation, we compared the ratios of emission intensity collected in the 605–635 nm and 650–755 nm windows, $I_{605-635}/I_{650-755}$, with excitation at 485 nm and 594 nm (Fig. S18). The effect is preserved, although the ratios are larger for excitation with 485 nm. In fact, the difference in ratio between the two phases is more pronounced with 485 nm excitation than with 594 nm.
Figure S18. Comparison of ratiometric polarity measurements in DOPC and 50:50 DOPC:cholesterol GUV membranes with 485 nm (blue) and 594 nm (orange) excitation.
Figure S19. Further examples of super-resolution polarity maps of phase separated GUV membranes stained with NR-dyad; (a) RESOLFT image in 605–635 nm detector range; (b) RESOLFT image in 650–755 nm detector range and (c) polarity color maps showing discrimination of regions of lipid ordering. All scale bars = 400 nm; $\lambda_{exc}$ 485 nm.
Determination of partitioning coefficients

The fraction of NR-dyad partitioning into the L_o phase of phase separated GUVs was measured for images obtained with both 485 nm and 594 nm excitation. Line profiles were drawn in ImageJ which pass through both lipid ordering phases on opposite sides of the membrane to eliminate any polarization effects (Fig. S20). The maximum pixel intensities along this line were compared, and the %L_o partitioning coefficient was calculated according to Equation S10.\(^{11}\) %L_o values from >10 vesicles with each excitation were measured and the mean taken.

\[
\%L_o = 100 \times \frac{F(L_o)}{F(L_o) + F(L_d)} \quad \text{(Eq. S10)}
\]

Figure S20. Representative confocal image of a phase separated GUV stained with NR-dyad, showing the line profile through both the L_d and L_o phases, and the resulting intensity profile used to calculate %L_o (\(\lambda_{\text{exc}}\) 485 nm, \(\lambda_{\text{em}}\) 605–635 nm, 80 × 80 nm pixels, scale bar 5 \(\mu\)m).

GPMV imaging

GPMVs derived from THP-1 cells were labelled with NR-dyad. The dyad was taken up by the GPMVs and labelled the membranes. However, there was also significant dye visible in the inside of the vesicles, which made fitting line profiles of membranes very challenging (Fig. S21). Additionally, because the GPMVs are not fixed to the surface of the imaging chamber (unlike GUVs), they are much more mobile. The increased mobility and membrane ‘wobbling’ meant that super-resolved images could not be acquired reliably.
Figure S21. Left: confocal image of GPMVs labelled with NR-dyad ($\lambda_{\text{exc}}$ 594 nm, $\lambda_{\text{em}}$ 650–755 nm, 80 × 80 nm pixels, scale bar = 2 µm); Right: pixel intensity along line profile shown in left panel (yellow dotted line).

Live-cell imaging

THP-1 cells were incubated with NR-dyad for a range of times, dye loadings and temperatures. In all experiments, NR-dyad was internalized quickly and showed no specific labelling of any cellular components (Fig. S22). Additional experiments were carried out with 0.02% sodium azide (a known inhibitor of endocytosis). Uptake appeared reduced but localization was not affected.

Figure S22. Representative images of THP-1 cells stained with NR-dyad ($\lambda_{\text{exc}}$ 594 nm, $\lambda_{\text{em}}$ 605–635 nm, 80 × 80 nm pixels, scale bars 5 µm). Left: 5 µM dye loading, 15 min incubation at 37 °C; right: 1 µM dye loading, 1 min incubation at 4 °C.
8. NMR Spectra and Mass Spectrometry data

Figure S23. $^1$H NMR spectrum of 1 (CDCl₃, 600 MHz, 298 K).

Figure S24. $^{13}$C NMR spectrum of 1 (CDCl₃, 151 MHz, 298 K).
Figure S25. Expanded $^{13}$C NMR spectrum of 1 (CDCl$_3$, 151 MHz, 298 K).
Figure S26. $^1$H/$^1$H COSY NMR spectrum of 1 (CDCl$_3$, 600 MHz, 298 K).

Figure S27. $^1$H/$^{13}$C HSQC NMR spectrum of 1 (CDCl$_3$, 600 MHz ($^1$H), 298 K).
Figure S28. $^1$H/$^{13}$C HMBC NMR spectrum of 1 (CDCl$_3$, 600 MHz ($^1$H), 298 K).
Figure S29. Measured (top) and theoretical (bottom) mass spectra for 1 (\([M+H]^+\) ion).
Figure S30. $^1$H NMR spectrum of NR-dyad (CDCl$_3$, 600 MHz, 298 K).
Figure S31. Expanded $^1$H NMR spectra of NR-dyad (CDCl$_3$, 600 MHz, 298 K).
Figure S32. $^{13}$C NMR spectrum of NR-dyad (CDCl$_3$, 151 MHz, 298 K).
Figure S33. Expanded $^{13}$C NMR spectra NR-dyad (CDCl$_3$, 151 MHz, 298 K).
Figure S34. $^1$H/$^1$H COSY NMR spectrum of NR-dyad (CDCl$_3$, 600 MHz, 298 K).

Figure S35. $^1$H/$^{13}$C HSQC NMR spectrum of NR-dyad (CDCl$_3$, 600 MHz $^1$H, 298 K).
Figure S36. $^1$H/$^13$C HMBC NMR spectrum of NR-dyad (CDCl$_3$, 600 MHz ($^1$H), 298 K).
Figure S37. Measured (top) and theoretical (bottom) mass spectra of NR-dyad ([M+H]^+ ion).

9. References

10. Author Contributions

V.W. synthesized the compounds and carried out mass spectrometry and NMR characterization. A.T.F. carried out photophysical characterization. K.G.L. carried out electrochemistry measurements. A.T.F. and K.G.L. prepared the vesicles and performed all microscopy experiments. A.T.F., K.G.L. and V.W. analyzed microscopy data. S.G. aligned and maintained the microscope and provided advice on microscope experimental design. K.G.L. wrote image analysis scripts. D.S. prepared cells and GPMVs for imaging. H.L.A. and C.E. acquired funding and managed the project. A.T.F. and K.G.L. wrote the manuscript. All authors discussed the experimental results and contributed to the editing of the manuscript.