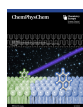


VIP Very Important Paper



A Photoswitchable Solvatochromic Dye for Probing Membrane Ordering by RESOLFT Super-resolution Microscopy**

Andrew T. Frawley,^{+, * [a]} Kathryn G. Leslie,^{+, [a]} Virginia Wycisk,^[a] Silvia Galiani,^[b] Dilip Shrestha,^[b] Christian Eggeling,^{*, [b, c, d, e]} and Harry L. Anderson^{*, [a]}

A switchable solvatochromic fluorescent dyad can be used to map ordering of lipids in vesicle membranes at a resolution better than the diffraction limit. Combining a Nile Red fluorophore with a photochromic spironaphthoxazine quencher allows the fluorescence to be controlled using visible light, via photoswitching and FRET quenching. Synthetic lipid vesicles of

varying composition were imaged with an average 2.5-fold resolution enhancement, compared to the confocal images. Ratiometric detection was used to probe the membrane polarity, and domains of different lipid ordering were distinguished within the same membrane.

Introduction

Super-resolution microscopy techniques allow fluorescence imaging of biological samples beyond the diffraction limit, revealing cellular structure with nanoscale spatial resolution.^[1–3] Recent advances such as MINFLUX and MINSTED have pushed this to the sub- or single-nanometer scale,^[4,5] however the challenge of combining super-resolution techniques with quantitative environmental sensing remains a nascent field, particularly in live-cell imaging.

An example in cellular research where quantitative environmental information as well as high resolution imaging is important is the investigation of biological membranes, such as

the plasma membrane. These membranes are made up of a lipid bilayer comprising a variety of different lipids and proteins. Consequently, the organization of these membranes varies on different spatial and temporal scales, where regions of distinct lipid compositions participate in many processes,^[6,7] such as membrane signaling^[8–11] and host-pathogen interactions.^[12–14] Different compositions of lipids, cholesterol, and proteins give more or less ordered membrane regions, which are an important factor in (local) membrane bioactivity.^[7] Consequently, it is important to have access to tools that can directly image such organization. However, such tools would require both high spatial resolution and lipid-order sensitivity. Fortunately, membrane ordering (or lipid packing) is directly correlated to the amount of water at the bilayer interface, and therefore the environment polarity.^[15–19] Polarity-sensitive dyes such as Nile Red have been used extensively to report on lipid packing through the solvatochromism of their emission.^[20–28] Usually, their emission shifts to shorter wavelengths in more ordered (liquid ordered, L_o) compared to more disordered (liquid disordered, L_d) membrane environments. Super-resolution polarity-sensitive imaging with Nile Red has been achieved using PAINT,^[29,30] STORM,^[31] and STED microscopy techniques.^[32] Stochastic techniques such as PAINT and STORM require many images to be recorded and reconstructed to produce the final super-resolution image, which limits the temporal resolution that can be achieved. However, for probing dynamic biological processes with high spatial resolution, imaging with a high temporal resolution is required. STED microscopy imaging of Nile Red can achieve higher temporal resolution, yet repetitive recordings might be limited by phototoxic effects.^[32] A remedy is RESOLFT microscopy.

'Reversible saturable optical fluorescence transitions' (RESOLFT) microscopy is a super-resolution microscopy technique in which a donut-shaped laser is overlaid with the excitation laser, switching the molecules in the donut area into a non-emissive state via a photochemical reaction (Figure 1a).^[33–36] When the excitation laser is applied, only molecules in the

[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
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, Germany
E-mail: christian.eggeling@uni-jena.de

[⁺] These authors contributed equally

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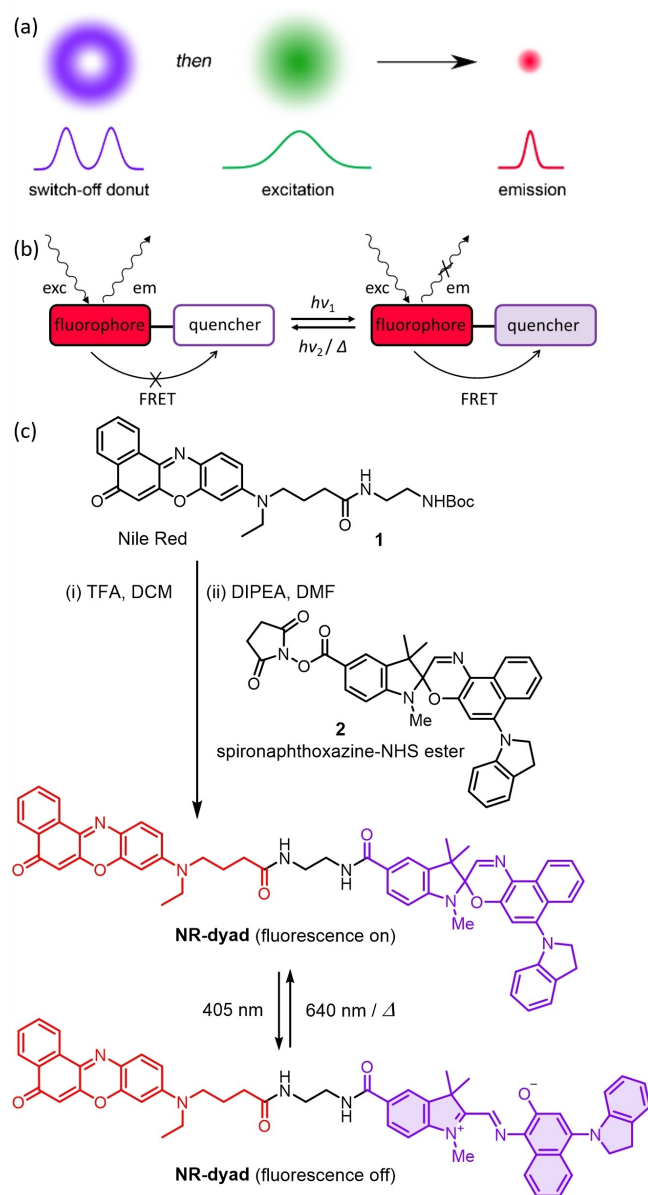


Figure 1. (a) The principle of RESOLFT microscopy: an off-switching laser (purple) with a node of zero intensity deactivates a subset of molecules such that upon excitation with a diffraction-limited fluorescence excitation laser (green), only a small diffraction-unlimited region (red) shows fluorescence emission. (b) A schematic diagram of a photoswitchable FRET dyad where a fluorophore is covalently linked to a reversibly switchable quencher and (c) structure of the Nile Red-spironaphthoxazine dyad (**NR-dyad**) and the synthetic scheme. Switching between the fluorescent and non-fluorescent forms is achieved with 405 nm and 640 nm irradiation.

diffraction-unlimited central node are emissive. RESOLFT microscopy can be used for live-cell imaging without exogenous buffers, and with lower laser powers than the analogous depletion laser used in stimulated emission techniques (STED), which is a limiting factor in their application due to photobleaching. Switchable fluorescent proteins are commonly applied as imaging agents in RESOLFT microscopy,^[37–49] however they suffer from poor photostability, low brightness, and the need to be introduced by genetic encoding. Switchable

small-molecule dyes are a promising alternative to overcome these shortcomings,^[50–55] and have the potential to incorporate environmental sensing capabilities alongside the photoswitching required for super-resolution imaging.

We have previously reported a small-molecule FRET (Förster Resonance Energy Transfer) dyad system which switches between a bright 'on' state and a dark 'off' state for use with RESOLFT donut lasers (Figure 1b).^[54] The dyad is composed of a fluorophore tethered to a spironaphthoxazine photoswitch,^[56] which can be isomerized with 405 nm light into a conjugated, broadly-absorbing colored form that turns the dyad 'off' by quenching fluorescence through FRET. In the reverse direction, the photoswitch can be closed thermally or with red light, turning the fluorescent dye back 'on'. These dyads represent an important improvement on previous small molecule RESOLFT dyes^[50–53] in that they use visible light to switch in both directions, give two-fold resolution enhancement, have high quenching efficiencies (>90%), and can be switched over >100 imaging cycles without the use of exogenous buffers.

We sought to build on this design by incorporating a polarity-sensitive Nile Red dye as the fluorescent reporter, combining RESOLFT super-resolution microscopy with environmental membrane polarity sensing (Figure 1c). We chose a short, flexible alkane linker with amide attachments, as our previous work had shown these to be effective at maximizing FRET efficiency.^[54] Previously, super-resolution polarity-sensitive imaging has not been realized with RESOLFT.

We designed **NR-dyad** (Figure 1c) as a switchable RESOLFT imaging probe, exploiting the polarity-sensing capability of the Nile Red chromophore. We predicted that the favorable spectral overlap between the Nile Red emission and open spironaphthoxazine absorption would lead to efficient FRET quenching. By recording the RESOLFT diffraction-unlimited emission in two separate spectral windows, we envisaged the dyad could act as a super-resolution ratiometric probe for membrane ordering.

Results and Discussion

The **NR-dyad** (Figure 1c) was synthesized from a Nile Red derivative bearing a carboxylic acid (Scheme S1).^[29] A Boc-protected ethylenediamine linker was added via amide coupling. Following Boc-deprotection of compound 1 with tri-fluoroacetic acid, the spironaphthoxazine switch NHS-ester^[54,56] 2 was attached with a second amide coupling giving the target **NR-dyad**. The absorption and emission spectra of the dyad are similar to those of the Nile Red dye with just the linker attached 1, demonstrating that addition of the spironaphthoxazine does not significantly affect the absorption and emission energies of Nile Red in the resting form of the dyad (Figures S1–4, Tables S1–2). In DMSO, **NR-dyad** exhibits an absorption maximum at 552 nm with a local maximum around 380 nm, corresponding to the absorption of the closed spironaphthoxazine. The emission maximum is at 635 nm. The fluorescence lifetime and quantum yield of **NR-dyad** are lower than for 1, which may be due to a small amount of photoinduced electron

transfer quenching from the closed switch to the dye (see Supporting Information Section 5).

We next sought to confirm that we could use **NR-dyad** for effective RESOLFT imaging. We predicted that the dyad would switch 'on' and 'off' through a FRET quenching mechanism (although PET is also possible; see Supporting Information Section 5). There is good spectral overlap between the merocyanine form of the spironaphthoxazine switch and the Nile Red emission, leading to a FRET distance of over 50 Å. In the fully extended conformation of the molecule, the distance of the Nile Red dye from the spironaphthoxazine switch is 21.6 Å and the calculated FRET efficiency for fluorophore quenching is >99% (see Supporting Information Section 4).

We chose to use giant unilamellar vesicles (GUVs) consisting of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) as a simple model membrane system. The membrane ordering in the vesicles can be altered by combining the DOPC lipid with varying proportions of cholesterol. Increasing the cholesterol content produces more ordered vesicles. The vesicles were prepared by electroformation^[57] and then incubated with **NR-dyad**. RESOLFT images were acquired in pixel-by-pixel mode, using a diffraction-limited Gaussian-shaped 594 nm excitation spot (providing a confocal control read-out), followed by a donut-shaped 405 nm irradiation to activate the quencher and turn fluorescence 'off'. Then, a second diffraction-limited Gaussian-shaped 594 nm irradiation spot was applied to record a RESOLFT super-resolution image. Finally, the photoswitch was

recovered to its closed form and the dye molecules switched back 'on' by irradiation at 640 nm (Figure 1a; for laser sequence see Figure S7). We define the resolution as the full-width-at-half-maximum (FWHM) of a line profile perpendicular to the membrane (see Figure S9). The confocal images are well described by Gaussian functions, and the RESOLFT line profiles were fitted by two coaxial Gaussian functions,^[54] where the width of one Gaussian matches the confocal profile, describing the background contribution of non-quenched dyad. The second component in the fit represents the RESOLFT FWHM. **NR-dyad** can be successfully applied to RESOLFT microscopy to image vesicle membranes of varying composition (DOPC, 80:20 DOPC:cholesterol, 50:50 DOPC:cholesterol; Figure 2).

In order to analyze all our images consistently, we developed a script capable of finding line profiles perpendicular to the membrane curve, and fitting them as described above (see Supporting Information Section 7 for a full description of the script and its function). Automation of the process using this script allowed us to fit 2306 line profiles across 336 images of GUVs of different lipid compositions. In all three membrane compositions, we observed resolution enhancement by RESOLFT microscopy, with a mean resolution enhancement factor of 2.5 for both DOPC (SD=0.66) and 80:20 DOPC:cholesterol (SD=0.52) membranes. Interestingly, the resolution enhancement was not as good for the 50:50 DOPC:cholesterol membranes, with a mean enhancement factor of 1.9 (SD=0.46). The average resolution enhancements achieved with **NR-**

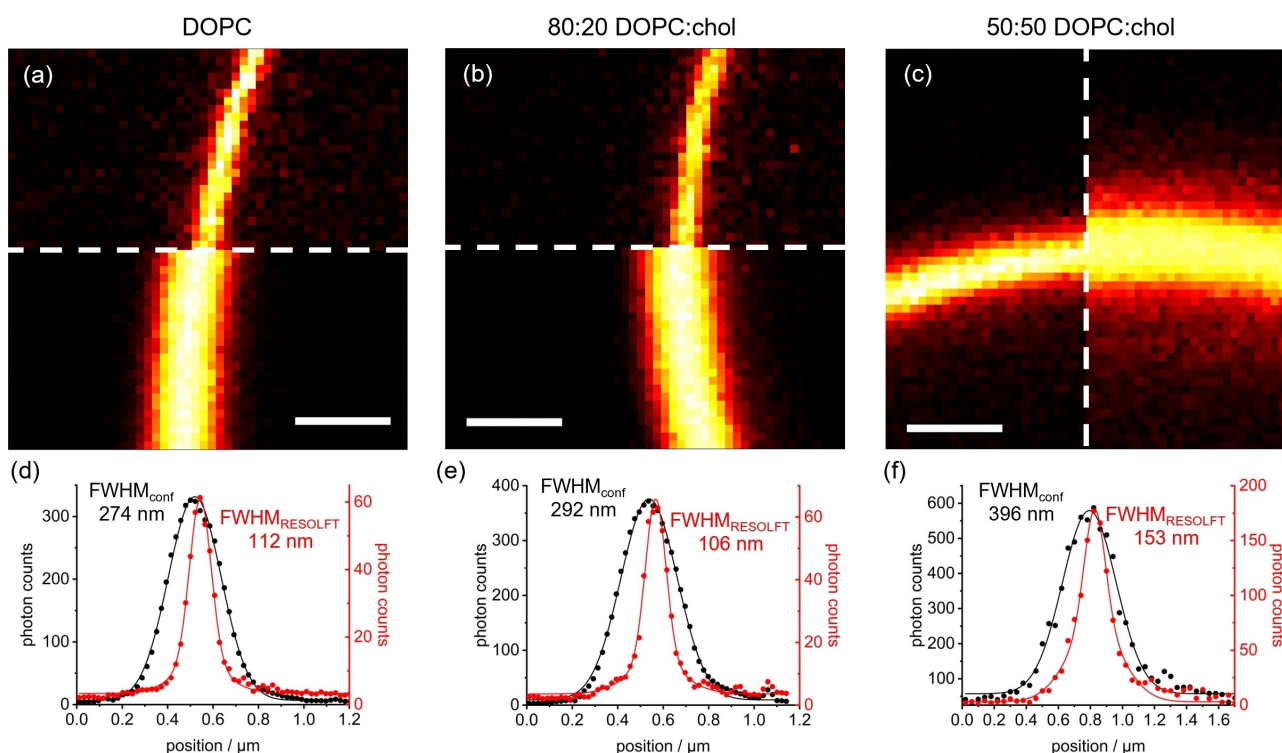


Figure 2. Demonstration of RESOLFT resolution enhancement in GUV membranes composed of (a) DOPC, (b) 80:20 DOPC:cholesterol and (c) 50:50 DOPC:cholesterol, all stained with **NR-dyad**; the fitted line profiles through the membranes used to measure FWHM are shown in (d–f). The red and black curves show the fitted line profiles from RESOLFT and confocal imaging, respectively. Excitation at 594 nm, switch-off at 405 nm, switch-on at 640 nm, scale bar 500 nm.

dyad are better than those we achieved with the previous generation of switchable spironaphthoxazine dyads bearing Atto590 or Cy3.5 fluorophores (around 2.0).^[54] We attribute this improvement to a reduction in the competing absorption by the fluorescent dye (Nile Red compared to Atto590 or Cy3.5) at the switching wavelength of 405 nm.

To further investigate why poorer resolution enhancements were observed in the 50:50 DOPC:cholesterol membranes (which are expected to be the most ordered), we considered the relative weighting of the confocal contribution to the super-resolution line profile fit. In most cases, there was a larger confocal contribution in the RESOLFT fits for the 50:50 membranes than for the DOPC or 80:20 DOPC:cholesterol membranes (Table S6). This suggests that there is poorer quenching of the dye emission in the 50:50 DOPC:cholesterol membranes. The poorer quenching cannot be attributed to the solvatochromism of the dye (see below and Figures S11–12); although the spectral overlap between the FRET donor and acceptor does change with solvent, the calculated efficiency of the FRET process is not significantly affected (SI Section 4). FRET efficiency also depends on the relative orientation of the donor and acceptor transition dipole moments. In our system, the linker is sufficiently flexible that we do not restrict the rotation of the donor and acceptor, which allows the molecule to explore a full range of transition dipole moment orientations. However, in highly ordered membrane environments, it is possible that molecular rotation is restricted, in such a way that the donor and acceptor spend time in conformations with the transition dipole moments not well-aligned for efficient FRET. Indeed, we have observed that **NR-dyad** becomes oriented in ordered membranes such as 50:50 DOPC:cholesterol. When imaging these GUVs stained with **NR-dyad** using a polarized excitation laser, the vesicles appear bright on one axis and dark on the perpendicular axis, indicating that the dyes are aligned with the excitation laser polarization on one axis but not the

other. This feature is not observed in disordered DOPC membranes (SI Section 7).

We then investigated the polarity sensitivity (i.e. solvatochromism of the emission) of **NR-dyad**. The absorption and emission spectra, as well as fluorescence quantum yields and lifetimes, of the dyad were measured in a variety of organic solvents covering a range of polarities.^[58] **NR-dyad** displays the characteristic positive solvatochromism of Nile Red in both absorption and emission, and the polarity-sensitivity of the chromophore is not significantly influenced by attachment to the photoswitching component (Figures 3a, S1–4). The emission maximum of **NR-dyad** varies between 596 nm in tetrahydrofuran and 639 nm in methanol. Note that the absorption feature centered at around 380 nm, belonging to the closed spironaphthoxazine switch, is not significantly sensitive to solvent polarity. This is a beneficial feature in our molecular design, because we can be confident that when using the 405 nm laser to switch molecules 'off', there is a similar extent of switching in membranes of different composition.

In order to demonstrate that the polarity-sensitivity of **NR-dyad** can be exploited during microscopy, we performed confocal spectral imaging experiments in GUVs of varying lipid composition. The vesicles were made by electroformation and incubated with **NR-dyad** or the Nile-Red derivative NR12A as a model solvatochromic membrane dye.^[29] We compared GUVs consisting of DOPC or the more ordered (less polar) 50:50 DOPC:cholesterol lipid composition. We also made phase-separated GUVs from a 2:2:1 DOPC:spingomyelin:cholesterol mixture, which separate into more ordered (L_d) phases, rich in sphingomyelin and cholesterol, and more disordered (L_d) phases rich in DOPC.^[59,60] Confocal spectral imaging shows that the emission from the dyad is blue-shifted in the more ordered lipid membranes, consistent with our hypothesis and with the NR12A reference (Figures S11–12).

With the knowledge that the dyad emission is sensitive to membrane composition, we next developed an imaging

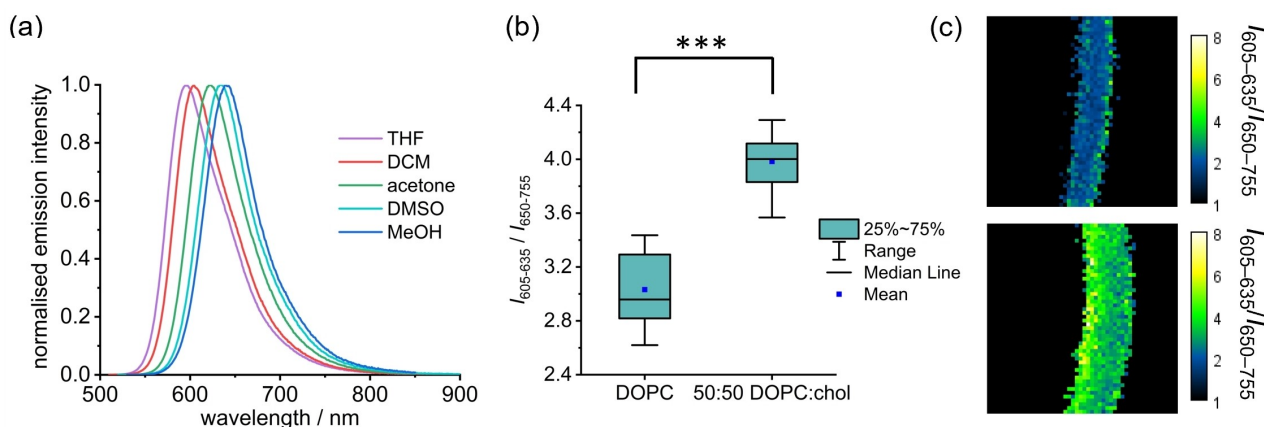


Figure 3. (a) Emission spectra of **NR-dyad** in solvents of varying polarity (Edinburgh Instruments FS5, 298 K). (b) Box-and-whisker plot of the ratio values between the pixel intensities in the 605–635 and 650–755 nm channels in DOPC and 50:50 DOPC:cholesterol GUV membranes as recorded by RESOLFT microscopy (λ_{exc} 594 nm; 50 measurements). (c) Examples of super-resolution RESOLFT microscopy images of GUV membranes with pixel-by-pixel analysis showing the ratio value between the intensities in the 605–635 and 650–755 nm channels (i.e. polarity) for DOPC (top) and 50:50 DOPC:cholesterol (bottom) membranes (higher number = more ordered; images $2 \times 2 \mu\text{m}$; excitation at 594 nm, switch-off at 405 nm, switch-on at 640 nm).

sequence capable of recording super-resolution RESOLFT microscopy images in multiple spectral regions simultaneously. Comparing the ratio of emission intensity in different spectral windows provides information about the polarity in the membranes, and hence the lipid ordering. Due to hardware restrictions, our detection windows were limited to 510–568 nm, 605–635 nm and 650–755 nm. Based on the spectral imaging data, we chose to use the 605–635 nm and 650–755 nm windows, to provide the largest discrimination between different membrane compositions. We expanded our existing RESOLFT imaging sequence to incorporate simultaneous detection in these windows (see Figure S7), and compared the output in the two channels for vesicles with different lipid compositions. Using a macro-based automated analysis process, 290 images were analyzed and the ratio of emission intensity in the two spectral windows was compared. Vesicles with different lipid compositions gave reproducible and highly statistically significant differences in the ratio of emission intensities in the two spectral windows (Figures 3b and S14). These analyses were carried out on entire images, with results that were not significantly different to analyzing just the membrane region of interest.

In order to realize our goal of being able to discriminate between regions of different membrane ordering within the same super-resolution microscopy image, an analysis of polarity in individual pixels is required. We developed a workflow for pixel-by-pixel analysis of images (Figure S17), and applied it to images recorded in multiple detection windows. The process generates polarity maps where the color scale represents the ratio of intensity in the two spectral windows (Figure 3c). Similarly to the whole-image analysis, the pixel-by-pixel analysis shows that DOPC and 50:50 DOPC:cholesterol membranes can be easily distinguished from each other.

We then sought to demonstrate the polarity sensing and RESOLFT microscopy capabilities of **NR-dyad** within a single image, using phase-separated vesicles as a model membrane. When phase-separated vesicles formed from a 2:2:1 DOPC:sphingomyelin:cholesterol lipid mixture were incubated with **NR-dyad**, we found clear preferential localization in a single phase (Figure 4a). Colocalization experiments with the

fluorescent L_d phase membrane marker FAST DiO confirmed the preference of **NR-dyad** for the disordered phase (Figures S15–16). Acquisition of RESOLFT images at the phase boundary with our standard image acquisition sequence (594 nm excitation) showed that it is difficult to measure the polarity ratio in the ordered phase due to a lack of signal from dyad accumulated there. However, excitation at 485 nm gives significantly more signal in the L_o phase (Figure 4b), probably due to more efficient excitation of the more blue-shifted dyes in this phase, indicating not just a localization preference but also selective excitation for **NR-dyad** in the disordered phase. The relative amounts of fluorescence detected in the L_o compared to the L_d phase (the % L_o partitioning coefficients^[61]) are $8 \pm 3\%$ for 594 nm excitation and $33 \pm 4\%$ for 485 nm excitation, showing that changing excitation wavelength has a large effect on the populations we are exciting in the two phases of the membranes (Figure S20).

The ratios of emission intensity collected in the 605–635 nm and 650–755 nm windows, $I_{605-635}/I_{650-755}$, are larger with excitation at 485 nm, rather than 594 nm, and this effect is preserved in phase-separated vesicles (Figure S18). Using the pixel-by-pixel analysis workflow, polarity ‘maps’ of the phase-separated vesicles show where the L_o and L_d phases of the membrane meet at resolution below the diffraction limit, thus demonstrating that the photoswitchable Nile Red dyad can be used as a super-resolution probe for membrane ordering (Figures 5 and S19).

Using our photoswitchable **NR-dyad**, we are able to acquire super-resolved RESOLFT microscopy images and probe the local membrane environment in model membranes, demonstrating that **NR-dyad** is a promising tool for biological imaging. Following our experiments in model GU V membranes, we tested the dyad both in live cells and in giant plasma membrane vesicles (GPMVs), which are cell-derived and therefore possess lipid complexity and membrane protein composition comparable to cell membranes.^[57,62] In both cells and GPMVs, **NR-dyad** was taken up efficiently into lipid membranes. In cells, it showed no discernible sub-cellular localization pattern (Figure S22); rather it was incorporated into all sub-cellular membranes. While it was taken up into GPMV membranes (Figure S21), when trying to image the vesicles, we found that the membrane movement was too fast to allow us to collect super-resolved images.

The nature of RESOLFT imaging makes it necessary to image in a pixel-by-pixel manner, applying the image acquisition sequence (Figure S7) to each pixel in succession, and this limits the speed of image acquisition. However, this technique does give super-resolved images in a single acquisition, in contrast to other recent examples of super-resolution membrane probes utilizing, for example, PAINT microscopy, which requires tens of thousands of frames to construct the final super-resolution image.^[29] As a result of the relatively slow recovery of our spironaphthoxazine switch to its closed form, the current temporal resolution of our imaging (approx. 75 s) is comparable to the timescale required by PAINT (1 min).^[29] Future development of these systems requires switches that can be transformed between their two states in a shorter time.

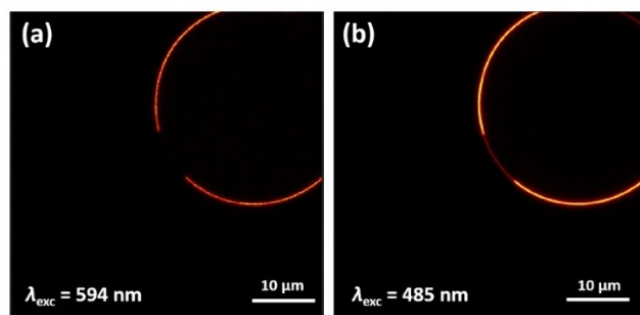


Figure 4. Confocal images of 2:2:1 DOPC:sphingomyelin:cholesterol phase-separated GUVs stained with **NR-dyad** excited with (a) 594 nm and (b) 485 nm (detection 650–755 nm, 80 nm pixels) showing selective excitation of **NR-dyad** in the larger area of disordered phase. Switch-off at 405 nm, switch-on at 640 nm.

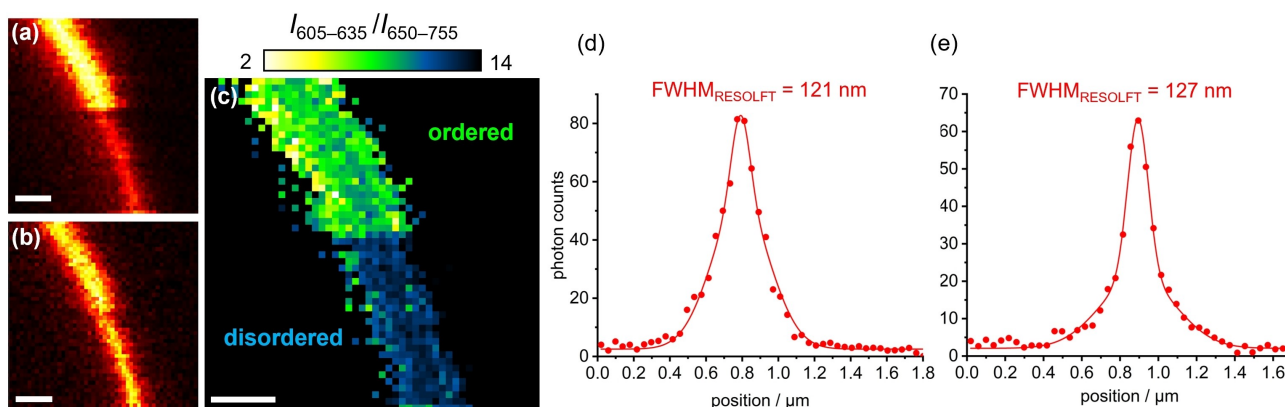


Figure 5. RESOLFT polarity sensitive imaging: RESOLFT microscopy images of phase-separated GUV membranes labelled with **NR-dyad** acquired in the detection windows (a) 605–635 nm and (b) 650–755 nm, and following excitation at 485 nm. (c) Polarity color map showing discrimination of regions of different lipid ordering. Fitted line profiles through the RESOLFT-imaged GUV membranes of the (d) ordered and (e) disordered phases with FWHMs as shown. Scale bars = 400 nm; switch-off at 405 nm, switch-on at 640 nm.

To further develop the live-cell potential of **NR-dyad**, it will be necessary to improve the design to direct localization to distinct membranes of interest within the cells. This would involve the synthesis of less-internalizing and organelle-targeted versions of **NR-dyad**. There has been much work on sub-cellular targeting of small molecule dyes,^[63–68] and we are developing strategies to incorporate targeting groups in these dyads. This will allow membrane ordering to be compared in different cellular environments below the diffraction limit. We have shown that judicious choice of excitation wavelength is required for good ratiometric imaging. Improved contrast between the L_d and L_o phases could probably be achieved with other excitation wavelengths and better tuned detection windows.

Conclusions

We have synthesized and investigated a photoswitchable dyad **NR-dyad** comprising a solvatochromic Nile Red dye covalently connected to a spironaphthoxazine quencher, which can be addressed in both directions by visible light. **NR-dyad** shows efficient quenching (94%) due to the high switching ability of the spironaphthoxazine and the excellent FRET efficiency between the Nile Red donor and switch acceptor. **NR-dyad** is incorporated efficiently into GUV and cell-derived membranes and can be used to acquire super-resolved fluorescence microscopy images using RESOLFT with resolution enhancements around 2.5-fold in GUVs. Additionally, the emission of **NR-dyad** is sensitive to polarity, and this can be used as a proxy for probing lipid ordering in membranes. Emission from the dyad can be used to distinguish regions of membranes with varying composition, both in membranes of a single composition and in phase-separated vesicles. As a result, **NR-dyad** is capable of reporting on local lipid ordering environments below the diffraction limit.

Experimental Section

Synthesis

Preparation of **NR-dyad** (Figure 1c) is described fully in the Supporting Information (Scheme S1). A Boc-protected ethylenediamine linker was added to Nile Red derivative bearing a carboxylic acid^[29] via amide coupling to give compound **1**. Following Boc-deprotection of the linker with trifluoroacetic acid, the spironaphthoxazine switch NHS-ester^[54,56] **2** was attached with a second amide coupling giving the target **NR-dyad**.

Photophysical Measurements

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. Fluorescence spectra were acquired at 298 K using an Edinburgh Instruments F55 spectrofluorometer operating Fluoracle® software, using a xenon arc lamp and a Hamamatsu R13456 PMT detector. 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 ($\lambda = 473.4$ nm). Unless otherwise stated, all spectra were recorded at 298 K.

Preparation of Giant Unilamellar Vesicles (GUVs)

GUVs were freshly prepared on the same day as imaging, according to the following electroformation procedure: 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 N_2 flow. The electrodes were placed in an electroformation chamber with sucrose (370 μL , 300 mM aqueous). A function generator (frequency 10 Hz, amplitude 5.7 $V_{\text{peak-to-peak}} = 2 V_{\text{RMS}}$) was used to apply an electrical potential 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. 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:spingomyelin:cholesterol phase separated GUVs. Room temperature GUV solution (75 μL) was transferred into an Eppendorf tube and a

solution of dyad (1 μ L, 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 μ L) in glass ibidi μ -Slide 8-well plates which had been passivated with poly-L-lysine for 1 h, and washed with PBS three times. All transfers of GUVs were carried out using pipette tips which had the end trimmed off with scissors to minimize damage to GUVs caused by shear forces.

RESOLFT Microscopy

RESOLFT microscopy was performed on a modified Abberior Instruments RESOLFT microscope, 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), 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 focal spot via a vortex phase mask (VPP-1b, RPC Photonics, Rochester, NY), placed in the path of the 405 nm laser 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 $\times$ /1.4 NA oil immersion objective lens (UPlanSApo 100 $\times$ /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 Inspector software (Abberior Instruments, Gottingen, Germany) and image analysis was carried out in Inspector and FIJI ImageJ. For solvatochromic RESOLFT images, data were acquired in all three detector ranges simultaneously, and the polarity analyzed by taking ratios of the photons detected in each range. See Figure S7 for a representative schematic of the imaging sequence.

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Conflict of Interest

The authors have no conflicts of interest to declare.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: RESOLFT • super-resolution microscopy • solvatochromism • membrane polarity • lipid ordering

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