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Rational design, synthesis, and characterization of highly fluorescent optical switches for high-contrast optical lock-in detection (OLID) imaging microscopy in living cells

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ABSTRACT

A major challenge in cell biology is to elucidate molecular mechanisms that underlie the spatio-temporal control of cellular processes. These studies require microscope imaging techniques and associated optical probes that provide high-contrast and high-resolution images of specific proteins and their complexes. Auto-fluorescence however, can severely compromise image contrast and represents a fundamental limitation for imaging proteins within living cells. We have previously shown that optical switch probes and optical lock-in detection (OLID) image microscopy improve image contrast in high background environments. Here, we present the design, synthesis, and characterization of amino-reactive and cell permeable optical switches that integrate the highly fluorescent fluorophore, tetramethylrhodamine (TMR) and spironaphthoxazine (NISO), a highly efficient optical switch. The NISO moiety in TMR–NISO undergoes rapid and reversible, excited-state driven transitions between a colorless spiro (SP)-state and a colored merocyanine (MC)-state in response to irradiation with 365 and >530 nm light. In the MC-state, the TMR (donor) emission is almost completely extinguished by Förster resonance energy transfer (FRET) to the MC probe (acceptor), whereas in the colorless SP-state, the quantum yield for TMR fluorescence is maximal. Irradiation of TMR–NISO with a defined sequence of 365 and 546 nm manipulates the levels of SP and MC with concomitant modulation of FRET efficiency and the TMR fluorescence signal. High fidelity optical switching of TMR fluorescence is shown for TMR–NISO probes in vitro and for membrane permeable TMR–NISO within living cells.

1. Introduction

Optical lock-in detection (OLID) microscopy allows for high-contrast imaging of a specific class of optically switchable fluorescent probe even in the presence of time-varying background signals such as cellular auto-fluorescence.[1,2] Optical switch probes undergo rapid and reversible, optically-drive transitions between two distinct states that differ in their structural, physical, and spectroscopic properties.[3,4] For example, spironaphthoxazine (NISO; Fig. 1) exists either as a colored merocyanine (MC)-state, or as the colorless and non-fluorescent spiro (SP)-state.[1,3,4] Transitions between SP- and MC-states occur via rapid (<μs), intramolecular, excited states reactions.[2] In particular, irradiation of SP with near ultraviolet (365 nm) generates the MC-state, while irradiation of MC with visible (>500 nm) light generates the SP-state. Interestingly, in the case of nitrospirobenzopyran (NitroBIPS), the excited MC-state can also decay back to the MC-ground state with the emission of red fluorescence, albeit with low quantum yield.[3,5] In general, all known synthetic optical switches including NitroBIPS, NISO and diarylethenes[6] and genetically-encoded optical switches[7] are better as optical switches than they are as fluorophores.

Here, we present herein the rational design of optical switch probes having greatly improved quantum yields for both fluorescence emission and optical switching. This property is achieved by incorporating tetramethylrhodamine (TMR), a highly fluorescent probe for fluorescent imaging, and NISO, a highly efficient optical switch,[8–10] in the same molecule.[11,12] Related probes based on a fluorescent donor such as fluorescein and GFP and a switchable acceptor based on NitroBIPS were described by our group as part of the OLID-FRET imaging technique.[1] The highly overlapping emission spectra of validated single molecule fluorophores such as TMR, Cy3, and ATTO dyes with the MC-absorption of NitroBIPS places a severe limitation on the ability to achieve orthogonal modulation of the donor probe.[1,13] In the probes detailed herein, this limitation is overcome by using a MC-state whose absorption

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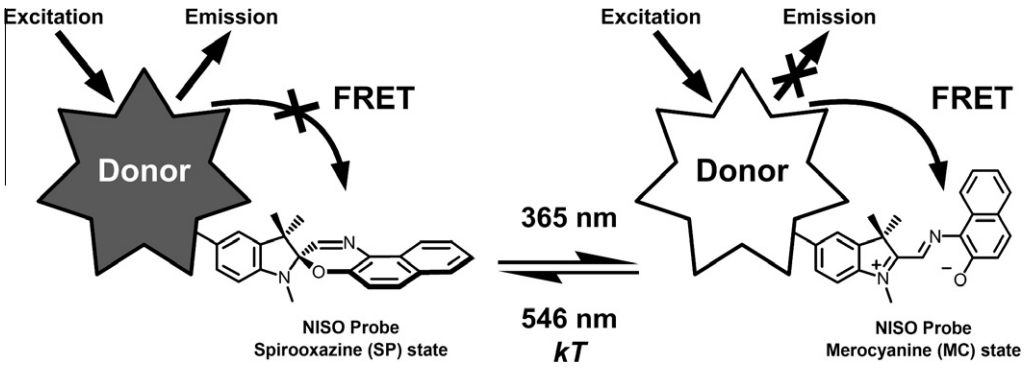


Figure 1. Optical control of TMR fluorescence intensity in TMR–NISO probes. Optical switching between SP- and MC-states of NISO generates the modulation of TMR fluorescence intensity by FRET.

spectrum is much further red shifted compared to NitroBIPS, allowing for orthogonal optical control of the TMR fluorescence.[3] Thus the absorption spectrum of MC in TMR–NISO exhibits a high spectral overlap with the emission spectrum of TMR and, given their close proximity (<1 nm), FRET efficiency is close to perfect in the MC-state of TMR–NISO and, concomitantly, TMR fluorescence is effectively extinguished. On the other hand, the absorption spectrum of SP in TMR–NISO does not overlap with TMR emission and so the FRET efficiency is zero and TMR fluorescence is maximal (Fig. 1). Thus, by irradiating a TMR–NISO probe with cycles of a defined sequence of near ultraviolet and visible light, one changes the population of the SP- and MC-states with concomitant modulation of the TMR fluorescence intensity. This property is useful in OLID imaging microscopy and is useful feature for probes employed in the stochastic optical reconstruction microscopy (STORM) technique.[1,2] We present herein the design, synthesis, spectroscopic, photochemical, and photophysical properties of various TMR–NISO probes. Most of the studies that characterize the properties of the amino-reactive and cell permeable TMR–NISO probes are performed in vitro with select examples showing they perform as high fidelity optical switches within living cells. More detailed applications of these probes within living cells will be presented elsewhere.

2. Results and discussion

2.1. Design of highly fluorescent optical switches

The low fluorescence quantum yield of the MC-state of NitroBIPS is a result of the competing loss of the MC-excited state via the MC to SP transition.[3,4,14] We therefore rationalized that the best design for highly fluorescent optical switches would involve uncoupling the fluorescence and optical switching processes. In particular, we designed a class of optical switch probes that contain TMR, a highly fluorescent and validated probe for single molecule imaging[15–17] and NISO, a highly efficient and robust optical switch.[4,8–10] The quantum yield of TMR fluorescence in TMR–NISO was reasoned to be at a maximum in the colorless SP-state while close to zero in the colored MC-state (λ_{max} ~620 nm) as a result of FRET.

2.2. Syntheses

The design features outlined above were realized through the synthesis of several TMR–NISO probes in which TMR was covalently linked to NISO through the C-4 or C-5 carbon atoms on NISO. These probes were used to investigate whether the coupling of NISO to TMR affected its optical switching properties. Both C-4 and C-5 linked TMR–NISO probes (Fig. 2; **8a–c**, **14**, and **20**) were shown to undergo successful cycles of optical switching between the SP- and MC-states with concomitant modulation of FRET efficiency and TMR fluorescence (see characterization of optical switching in the following section). The reactions employed for the preparation of the TMR–NISO probes (**8a–c**, **14**, and **20**) are shown in Schemes 1–3. The amino-substituted NISO (**7a–c** and **13**) and *N*-hydroxysuccinimide ester of NISO (**19**) were synthesized using standard methods and used for coupling to commercially available fluorophores, namely 5-carboxytetramethylrhodamine, succinimidyl ester (5-TAMRA-SE) or tetramethylrhodamine-5-carboxamide lysine (5-TAMRA-Lysine).

The reactions employed for the synthesis of several 4-amino substituted NISO derivatives are shown in Scheme 1. The condensation of indoline **3** with the corresponding 1-nitroso-2-naphthol derivatives was used to prepare several of the TMR–NISO probes (**6a–c**) with yields ranging from 20% to 40%. The amino-NISO derivatives (**7a–c**) were obtained in good yield via the reaction of the azido-NISO derivatives (**6a–c**) with triphenylphosphine (PPh_3) in a mixture of tetrahydrofuran (THF) and water. The reactions used for the synthesis of the 5-amino substituted NISO derivative (**13**) are shown in Scheme 2. A similar scheme was used to prepare the 5-amino substituted NISO. The 4- and 5-amino-NISO derivatives (**7a–c** and **13**) were allowed to react with 5-TAMRA-SE, affording the corresponding TMR–NISO probes (**8a–c** and **14**) in 20–40% yield. The 5-TMR-Lys NISO (**20**) was synthesized as shown in Scheme 3. The indoleninium salt (**17**) was prepared in three steps from commercially available 4-(4-aminophenyl)butanoic acid. Condensation of intermediate salt **17** with 1-nitroso-2-naphthol in ethanol in the presence of NEt_3 led to the NISO (**18**) with a yield of 32%. Treatment of NISO (**18**) with *N*-hydroxysuccinimide (NHS) in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) gave the *N*-hydroxysuccinimide ester derivative of NISO **19**, which was further used for coupling with 5-TAMRA-Lysine to give 5-TMR-Lys NISO (**20**) in 42% yield.

The next step in the design of fluorescent optical switches was to introduce an amino-reactive functionality into the TMR–NISO probes (Fig. 2; **28a–b**) for coupling to proteins and other biomolecules. The reactions used to synthesize the amino-reactive TMR–NISO probes are shown in Scheme 4. First, bi-functional NISO derivatives were prepared by reaction of indoleninium halide **17** and **23** with nitrosonaphthol **22** in ethanol in the present of NEt_3 to give compounds **24a** and **24b**, respectively. These NISO derivatives (**24a** and **24b**) were converted to the amino derivatives (**26a** and **26b**, respectively), in two steps. The amino-NISO derivatives **26a** and **26b** were coupled to 5-TAMRA-SE following by a reaction with NHS in the presence of DCC to generate the *N*-hydroxysuccinimide esters of the TMR–NISO probes **28a** and **28b**

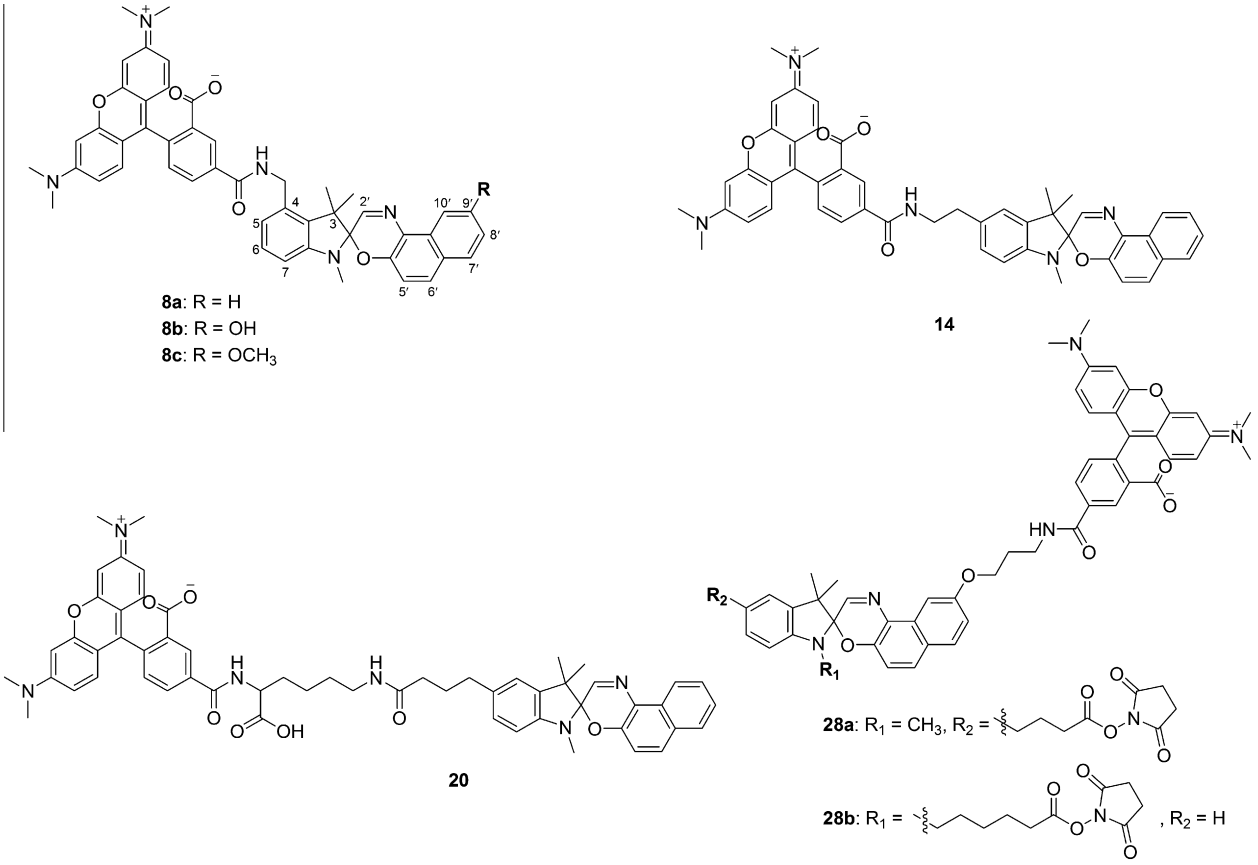
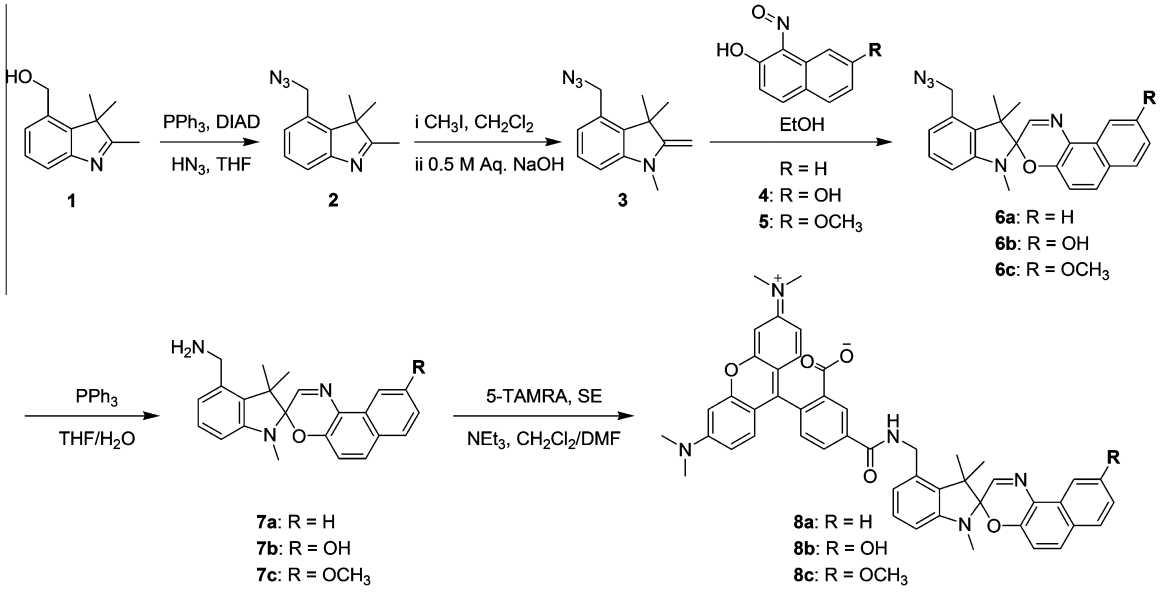


Figure 2. Structures of TMR–NISO probes.



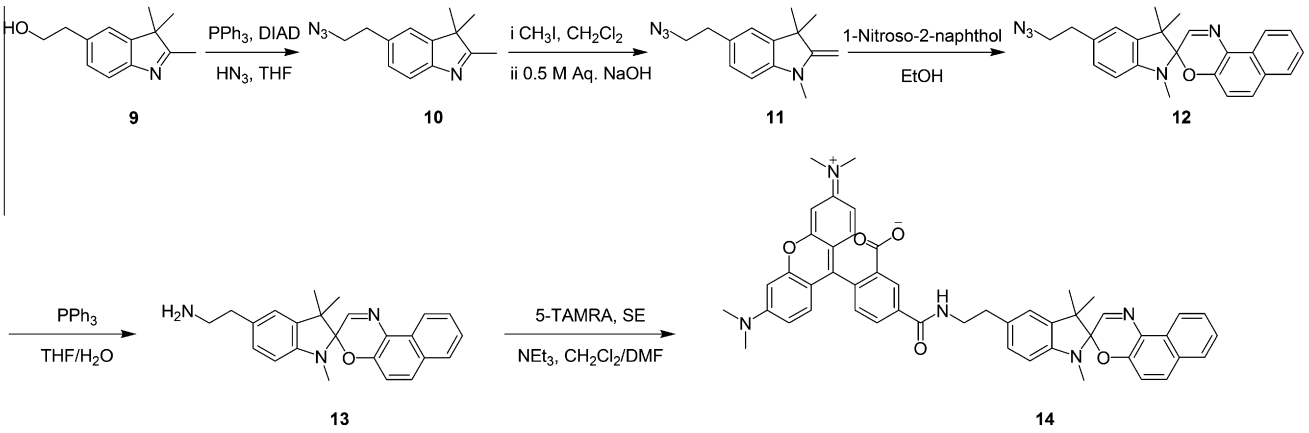
Scheme 1. Synthesis of 4-TMR NISO derivatives.

in 63 and 53% yield, respectively. These NHS ester probes are reactive with lysine residues in proteins and can be used to generate protein conjugates of TMR–NISO. The characterization and application of these conjugates will be presented elsewhere.

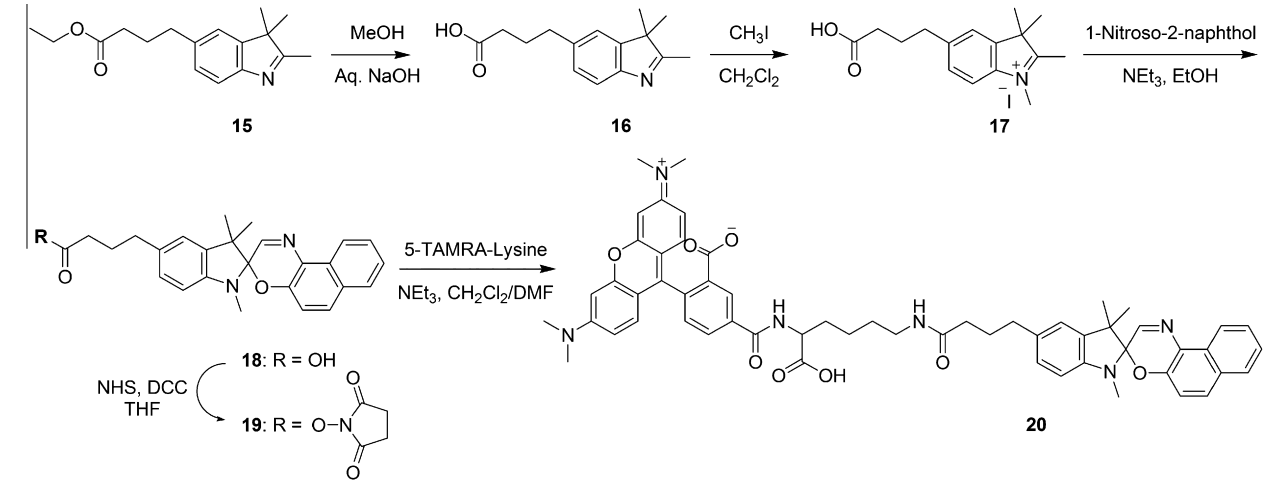
All TMR–NISO probes were confirmed from measurements of their exact molecular mass obtained from ESI mass spectra.

2.3. Spectroscopic and photophysical properties of TMR–NISO probes

Key spectroscopic and photophysical properties of TMR–NISO probes were measured in solution and compared to the same properties found for free TMR and free NISO. The SP-state of TMR–NISO



Scheme 2. Synthesis of 5-TMR NISO derivative.



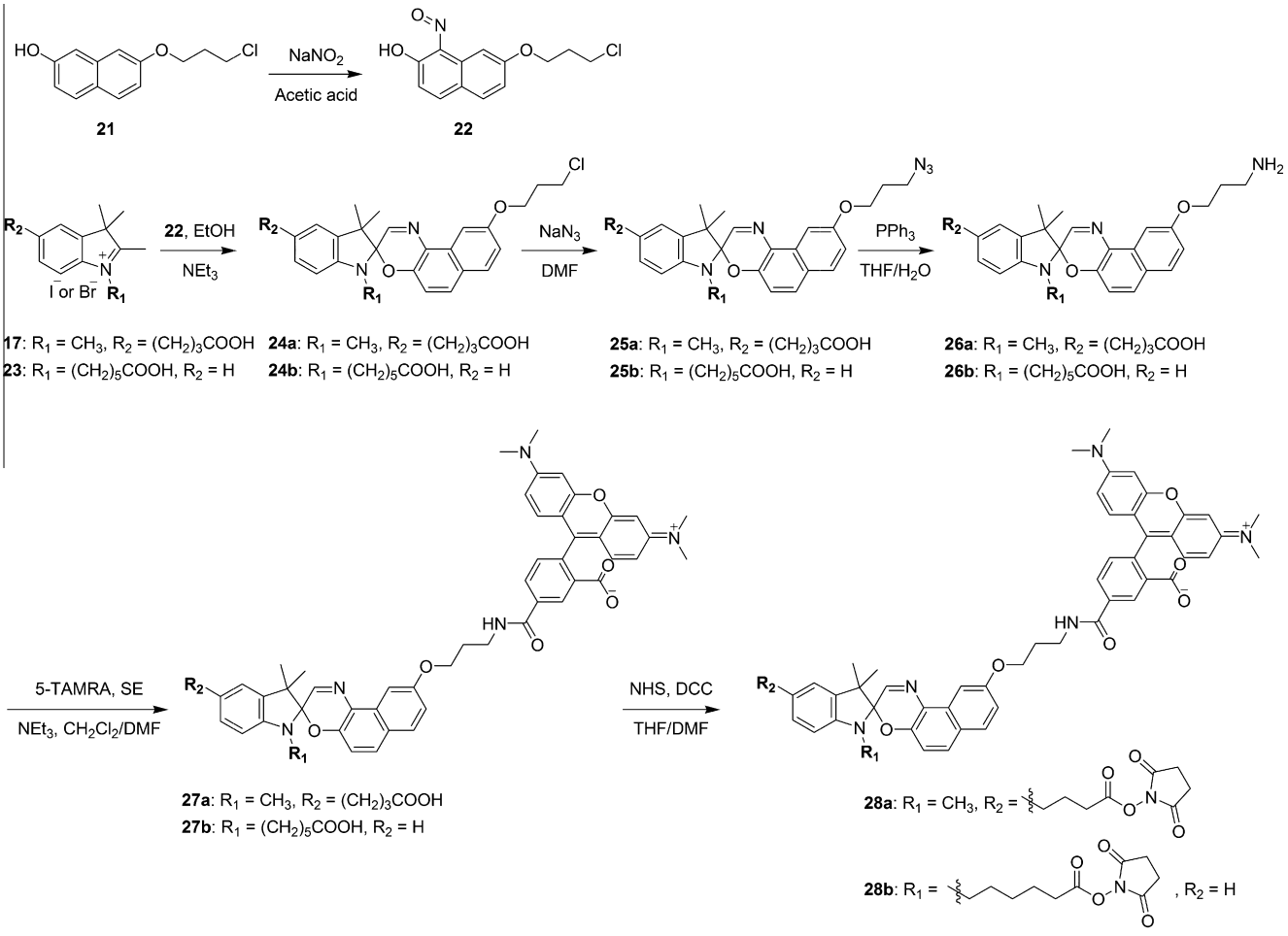
Scheme 3. Synthesis of 5-TMR-Lys NISO derivative.

proved to be the most thermodynamically-favored form at room temperature for all of the solvents used in this study (Fig. 3A). The absorption spectra of TMR–NISO probes (**8a**–**c**, **14**, **20**, and **28a**–**b**) in their SP- and MC-states in glycerol are shown in Figure 3A and B, respectively. The TMR moiety in TMR–NISO exhibits a maximum (S_0–S_1) absorption at 556–557 nm. The SP-absorption of NISO within the TMR–NISO molecule is buried within the S_0–S_2 transition of TMR making it difficult to analyze. Irradiation of TMR–NISO with 365 nm generates the corresponding MC-state having an absorption maximum of 612–625 nm (Fig. 3B). The intensity of the MC-absorption in TMR–NISO decreased with time as a result of a thermally-driven, ground state transition that formed the colorless SP-state.[4] The time constant for this thermally-driven reaction was measured for several TMR–NISO probes in glycerol at room temperature (Table 1). These results show that the time constant for the MC to SP transition is dependent on the nature of substituent groups on NISO.[18–21] The C-5 alkylated-NISO stabilizes the MC-state compared to alkylation at the 4-position (**14** and **8a**, respectively). Substituent groups at the 9′-position also stabilize the MC-state (**8a**–**c**), as does the presence of longer side chains (**8b**–**c**, **14**, and **20**).

The quantum yield for TMR fluorescence in the SP-state of TMR–NISO was measured for several TMR–NISO probes in glycerol, methanol, and water using rhodamine 101 in ethanol as a reference[22] (Table 1). The quantum yield for TMR fluorescence measured in these TMR–NISO probes is similar to that measured for free TMR. This results suggests that the high quantum efficiency of TMR in TMR–NISO is unaffected by the presence of the closely situated SP-state. On the other hand, the quantum yield for TMR fluorescence is very low in the MC-state of TMR–NISO because of the high efficiency of FRET between the TMR and MC groups. Thus, irradiation of the SP-state of TMR–NISO (**8b**) with 365 nm light in glycerol results in a significant decrease in the fluorescence intensity of TMR that is caused by formation of the MC-state and FRET (Fig. 3C). The recovery of the fluorescence signal from TMR following this UV-pulse is primarily due to the thermally-driven MC to SP transition (Fig. 3C). Optical manipulation of the SP- and MC-states of TMR–NISO over several cycles of optical switching is shown to modulate the fluorescence intensity of TMR and can be repeated over many cycles with little evidence of bleaching or secondary photochemical reactions (Fig. 3C).[3] Accurate determination of the quantum yield of TMR fluorescence in the MC-state of TMR–NISO is quite difficult using cuvette measurements such as those shown in Figure 3C owing to the large volume but it is far easier when using a fast imaging OLID microscope as is shown later.

2.4. Optical switching of TMR fluorescence in TMR–NISO in living cells

The TMR–NISO probes described in this study are readily taken up by living cells where they localize to vesicle-like structures that may include mitochondria, as shown in Figure 4A. Optical manip-



Scheme 4. Synthesis of *N*-hydroxysuccinimide ester derivatives of TMR–NISO.

ulation of TMR fluorescence in cells loaded TMR–NISO was performed using a home-built OLID microscope as detailed in the methods section. The field of view was subject to several cycles of defined excitation of the field with 546 and 365 nm light. In particular, we employed continuous irradiation of the field with 546 nm light while recording the TMR fluorescence and irradiated the same field every 2 s with a 100 ms pulse of 365 nm light. The 365 nm pulse was shown to convert almost all of the SP-state of TMR–NISO to MC in the field of view, as longer irradiation times did not appreciably change the lowest measured value of TMR fluorescence. A trace of the TMR fluorescence intensity over eight cycles of optical switching within a region of interest in a cell-loaded preparation is shown in Figure 4B. Immediately following the 365 nm pulse, the TMR intensity decreases as a result of FRET to a value that is about 65–70% below the SP level. The recovery of TMR fluorescence, seen in Figure 4B, is due to both direct excitation of the MC-state with 546 nm light (the MC has a non-zero absorption value at 546 nm) and the thermally-driven MC to SP transition. Given the presence of an auto-fluorescence background and the relatively long UV-pulse, it is difficult to arrive at the time zero value of TMR fluorescence from data shown in the trace (Fig. 4B). However, extrapolation of the intensity trace of TMR fluorescence following irradiation with 365 nm light (Fig. 4C) allows us to estimate the TMR signal is close to zero at the end of the UV-pulse. Other important properties deduced from this live cell study include; first, optical switching is robust and proceeds with high fidelity over the eight cycles; second, the intensities of TMR fluorescence are similar immediately before and immediately after the UV-pulse; third, the recovery of the TMR fluorescence intensity for each cycle of the study is best fit with a single exponential having a time constant of ~200 ms. These data strongly suggest that optical manipulation of the SP- and MC-states of TMR–NISO in living cells occurs with high fidelity and little evidence of fatigue or secondary photochemistry. The rate of recovery of TMR–NISO fluorescence during 546 nm excitation of the MC-state with 546 nm light was shown to depend on the square of the 546 nm light intensity. Thus the result shown in Figure 5A and B strongly suggests that energy used to excite the MC group in TMR–NISO results is sub-saturating. Thus the time constant measured for recovery of TMR fluorescence intensity for each cycle of optical switching shown in Figure 5B is primarily determined by the quantum yield for the MC to SP transition and the number of molecules of MC in the exited state. This time constant can be shortened by increasing the number of MC molecules in the excited state, for example, by increasing the energy of the 546 nm light, or by irradiating the MC group closer to its maximum absorption wavelength, that is, 632 nm. In the limit, that is, under a condition of saturating excitation, it should be possible to obtain a square wave modulation of TMR intensity.

The excited-state driven reactions between the MC- and SP-states are faster than 2 μs,[2] the modulation of TMR intensity is faster than any of the optical switch probes used for super-resolution imaging, including photoactivatable proteins and Cy3/Cy5 probes.[17] Perhaps more significant for studies in living cells, optical switching of TMR fluorescence in TMR–NISO occurs under normal conditions of cell culture, that is, in medium and without the need

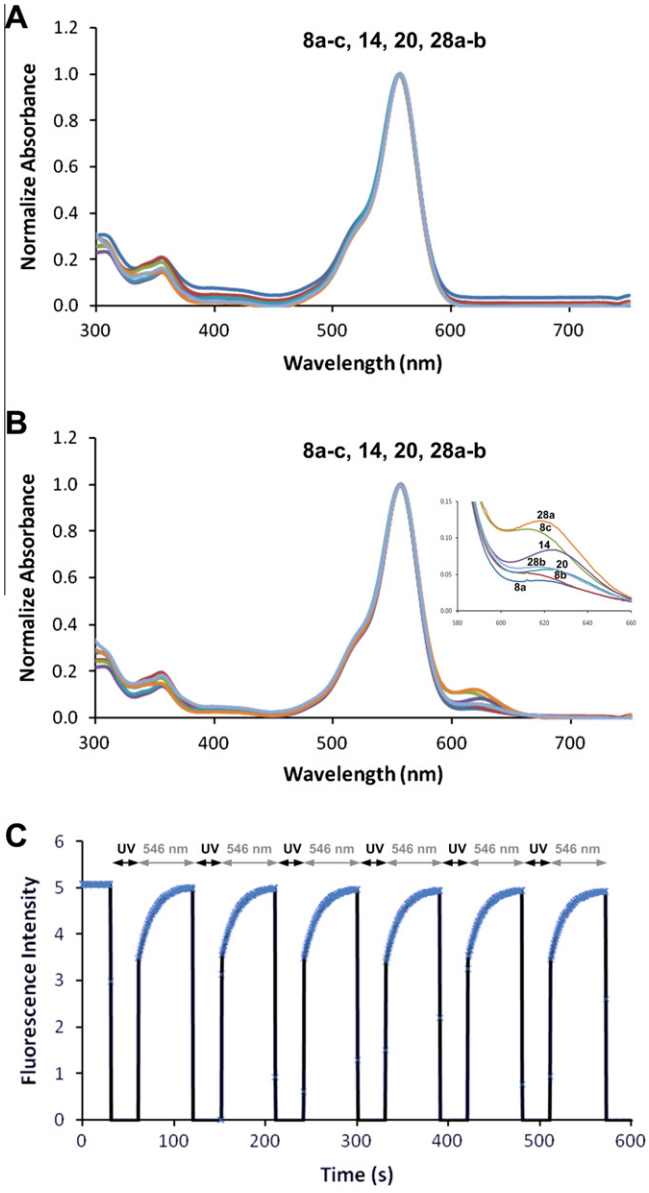


Figure 3. Absorption spectra and fluorescence intensity profile of TMR–NISO probes. Absorption spectra of TMR–NISO probes **8a–c**, **14**, **20**, and **28a–b** in SP- (A) and MC-states (B) in glycerol (inset: expand region of MC-NISO absorption between 580 and 660 nm). (C) TMR fluorescence intensity profile of **8b** over six cycles of optical switching in glycerol. A typical optical switching cycle was composed of 30 s of 365 nm light and 60 s of 546 nm light.

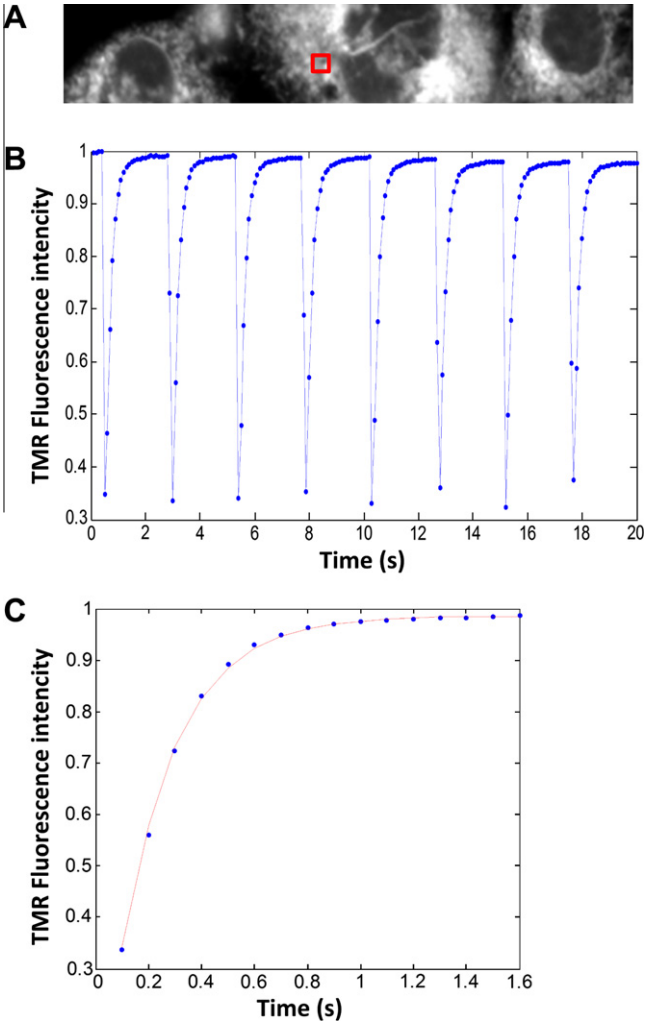


Figure 4. Optical switching of TMR–NISO (**8a**) in living cells. (A) Single image frame from a movie reflecting the distribution of **8a** within living NIH 3T3 cells. (B) A trace of TMR fluorescence intensity over eight cycles of optical switching of the **8a** within a region of interest (red square) within the image field (A). (C) The rate of return of the TMR fluorescence signal for one of the cycles shown in (B) following a 365 nm pulse was best fit by a single exponential that having a 200 ms time constant.

of high concentrations (molar) mercaptans or other reducing agents or anoxia.[17] The rapid switching of TMR fluorescence in TMR–NISO and the high quantum yield of TMR in the SP-state would be especially useful for super-resolution imaging and OLID imaging of proteins in living cells where diffusion of the probe during the acquisition period is a serious problem.

3. Conclusion

We have synthesized several members of a new class of highly fluorescent optical switch that incorporates the highly fluorescent TMR probe and NISO, a highly efficient optical switch. The NISO

Table 1
Kinetic and quantum yield of TMR–NISO derivatives in glycerol, methanol, and water at room temperature

Compound	Quantum yield			Thermal decay rate[a] (MC-NISO, $\times 10^{-2}$ s^{-1})
	Glycerol	MeOH	Water	
5-TAMRA, SE	0.76	0.65	0.43	–
8a	0.89	0.73	0.38	4.00
8b	0.91	0.71	0.29	3.14
8c	0.98	0.77	0.20	2.36
14	0.58	0.51	0.25	2.22
20	0.63	0.53	0.21	1.60

[a] In glycerol.

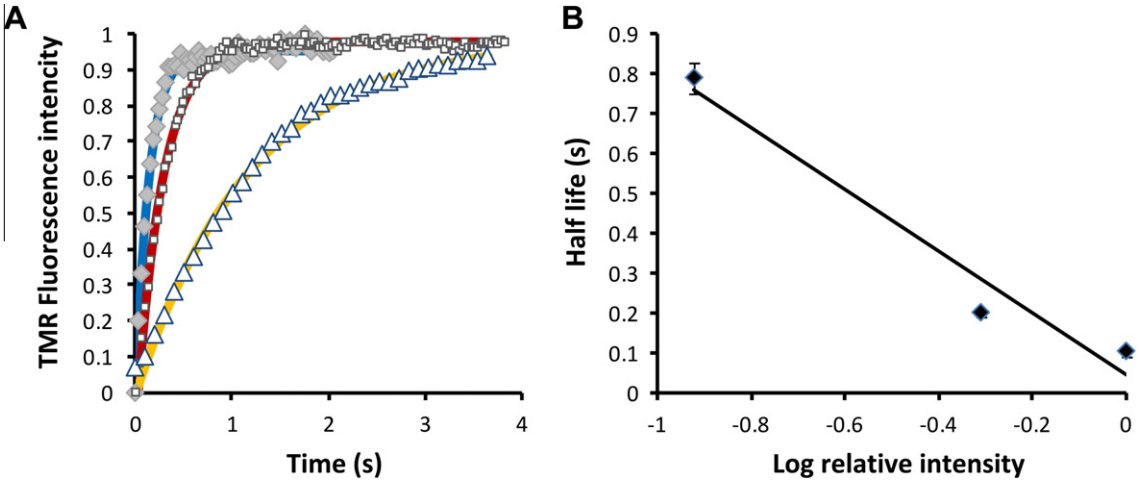


Figure 5. TMR fluorescence intensity recovery of TMR–NISO by OLID-FRET microscopy. (A) TMR fluorescence intensity recovery after 365 nm pulse using different illumination energy: 100% (◇), 50% (□), and 12.5% (△). (B) Half life of TMR fluorescence depends on illumination intensity.

group in TMR–NISO undergoes orthogonal, optically driven transitions between a colorless SP-state, and a colorful MC-state that serves as an acceptor probe in FRET with TMR. In particular, the close proximity of the TMR and MC groups in TMR–NISO results in almost perfect FRET and decreases the quantum yield of TMR fluorescence to almost zero whereas the quantum yield for TMR fluorescence in the SP-state is at a maximum. Optical switching between the SP- and MC-states was shown to result in robust modulation of the intensity of TMR fluorescence over many cycles of optical switching. The TMR–NISO probe is useful for imaging of structures in living cells and to our knowledge, optical switching of TMR fluorescence is faster than any genetically-encoded or synthetic optical switch. Moreover the TMR–NISO probes detailed herein undergo reversible optical switching without the need to exogenous reactants and do so under conditions compatible with healthy and aerobically grown cells.

4. Experimental

4.1. General procedures

Starting materials were purchased from Sigma–Aldrich, Fisher Scientific, TCI America, Invitrogen, and AnaSpec. 4-(Hydroxymethyl)-2,3,3-trimethyl-3*H*-indole (**1**) was prepared as previously reported.[3] ^{1}H NMR spectra were recorded at 300 MHz on a Bruker AC-300 spectrometer or a Varian MercuryPlus 300. Mass spectra were recorded on a Micromass LCT for ESI or a Micromass AutoSpec for EI. UV spectra were determined with a Shimadzu 1601PC instrument. Fluorescence spectroscopy was performed on an SLM-AB2 instrument (Thermoelectron, Madison, WI).

4.2. Syntheses

4.2.1. General procedure for Mitsunobu reaction

A paste of sodium azide (260 mg, 4 mmol) in water (260 μL) was stirred on an ice bath, and benzene (1.6 mL) was added. Concentrated sulfuric acid (H_2SO_4, 106 μL, 2 mmol) was carefully added dropwise to the reaction mixture. After stirred for 10 min, the organic layer containing hydrazoic acid was separated and dried over anhydrous magnesium sulfate ($MgSO_4$). To a mixture of indolenine (1 equiv) and triphenylphosphine (PPh_3, 1.5 equiv) in dry tetrahydrofuran (THF) stirred on an ice bath under a nitrogen atmosphere, the solution of hydrazoic acid in benzene (1.5 equiv) was added. After stirred for 10 min, diisopropyl azodicarboxylate (DIAD, 1.5 equiv) was added dropwise over 1 min to the reaction mixture. The reaction mixture was allowed to warm to room temperature and stirred for 4 h. The reaction mixture was concentrated and purified by a silica gel flash column using appropriate eluent to give the product.

4.2.2. General procedure for synthesis indoline or indoleninium salt

Method A: A mixture of indolenine and alkyl halide (8 equiv) in dichloromethane (CH_2Cl_2) was heated under reflux for 12 h. The reaction mixture was cooled to room temperature and then evaporated to give a residue. The residue was dissolved in an aqueous sodium hydroxide (NaOH, 0.5 M) and stirred at room temperature for 30 min and then extracted with CH_2Cl_2. The organic layer was dried over anhydrous $MgSO_4$ and evaporated to give a crude extract that was further used for next reaction without purification. *Method B:* A mixture of indolenine and alkyl halide (2–8 equiv) in CH_2Cl_2 or 1,2-dichlorobenzene was heated under reflux for 12 h. The reaction mixture was cooled to room temperature and concentrated. The precipitate was filtered and washed with hexane several times to give a residue that was used for next reaction without purification.

4.2.3. General procedure for nitrosation

To a stirred solution of 2-hydroxynaphthalene derivatives (1 equiv) in a 5:1 mixture of glacial acetic acid and water on an ice bath, an aqueous sodium nitrite ($NaNO_2$, 1 equiv) was added dropwise. After 10 min, an additional aqueous $NaNO_2$ (0.05 equiv) was added and the reaction mixture was further stirred at 0 °C for 2 h. The precipitate was collected by filtration, washed with dilute acetic acid and water, and dried in vacuo.

4.2.4. General procedure for synthesis NISO

Method A: To a solution of 1-nitroso-2-naphthol derivatives (1 equiv) in ethanol (EtOH), an equimolar solution of indoline derivatives in EtOH was added. The reaction mixture was heated under reflux for 2 h. After cooling to room temperature, the mixture was evaporated to give a residue. The residue was purified by a Sephadex LH-20 column (methanol; MeOH) and a silica gel column (hexane/ethyl acetate; EtOAc) to give a product. *Method B:* To a solution of 1-nitroso-2-naphthol derivatives (1 equiv) in EtOH, indoleninium salt derivatives (1 equiv) in EtOH and NEt_3 (1 equiv) were added. The reaction mixture was heated under reflux for 5 h. After cooling to room temperature, the mixture was

evaporated to give a residue that was further separated by a Sephadex LH-20 column (MeOH) and a silica gel column (hexane/EtOAc) to give the product.

4.2.5. General procedure for synthesis azido derivatives from chloro derivatives

To a solution of 9′-chloropropoxy derivatives of NISO in dry *N*,*N*-dimethylformamide (DMF), sodium azide (2 equiv) was added. After stirring for 24 h at 80 °C, water was added to the reaction mixture and then extracted with CH_2Cl_2. The organic layer was dried over anhydrous $MgSO_4$ and evaporated to give the product.

4.2.6. General procedure for reduction azide to amine

To a stirred solution of azido-NISO derivatives in a 10:1 mixture of THF and water, PPh_3 (2 equiv) was added. The reaction mixture was stirred at room temperature for 24 h. The solvent was removed to give a residue that was further purified by a silica gel TLC (CH_2Cl_2/MeOH) to give the product.

4.2.7. General procedure for synthesis *N*-hydroxysuccinimide ester derivatives

To a solution of *N*-hydroxysuccinimide (NHS, 1.5 equiv) and *N*,*N*′-dicyclohexylcarbodiimide (DCC, 1.5 equiv) in dry THF (1 mL), carboxyl derivative of NISO (or TMR–NISO, 1 equiv) in dry THF (or a 1:1 mixture of dry THF and dry DMF) was added. The reaction mixture was stirred at room temperature for 12 h. The precipitate was filtered out and the filtrate was evaporated and purified by silica gel TLC (CH_2Cl_2/MeOH) to give the product.

4.2.8. General procedure for synthesis TMR–NISO derivatives

A solution of 5-carboxytetramethylrhodamine, succinimidyl ester (5-TAMRA-SE) or tetramethylrhodamine-5-carboxamide lysine (5-TAMRA-Lys, 1 equiv) in dry DMF (100 μL) and triethylamine (NEt_3, 1.5 equiv) were added to a solution of amino-NISO derivatives or NHS–NISO derivative (1.5 equiv), respectively, in dry CH_2Cl_2. The reaction mixture was stirred at room temperature overnight and then evaporated to give a residue. The residue was purified by a silica gel TLC (CH_2Cl_2/MeOH; 7:3) to give product.

4.2.9. 4-(Azidomethyl)-2,3,3-trimethyl-3*H*-indole (2)

This compound was synthesized according to general procedure for Mitsunobu reaction. Yield 193 mg, 90%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.41 (s, 6H), 2.28 (s, 3H), 4.49 (s, 2H), 7.15 (dd, J = 0.9, 7.8 Hz, 1H), 7.36 (t, J = 7.8 Hz, 1H), 7.55 (dd, J = 0.9, 7.8 Hz, 1H) ppm; ESIMS: m/z 215.1285 $(M+H)^+$, calcd for $C_{12}H_{15}N_4$, 215.1291.

4.2.10. 4-(Azidomethyl)-1,3,3-trimethyl-2-methyleneindoline (3)

This compound was synthesized according to general procedure for synthesis indoline or indoleninium salt method A.

4.2.11. 1-Nitroso-2,7-dihydroxynaphthalene (4)

This compound was synthesized according to general procedure for nitrosation. Yield 116 mg, 88%. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 6.19 (br s, 1H), 6.90 (dd, J = 2.3, 7.9 Hz, 1H), 7.43 (d, J = 7.9 Hz, 1H), 7.62 (br s, 1H), 8.41 (br s, 1H) ppm; EIMS: m/z 189.0428 $(M)^+$, calcd for $C_{10}H_7NO_3$, 189.0421.

4.2.12. 7-Methoxy-1-nitroso-2-naphthol (5)

This compound was synthesized according to general procedure for nitrosation. Yield 199 mg, 98%. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 3.83 (s, 3H), 6.26 (br d, J = 9.3 Hz, 1H), 7.11 (dd, J = 2.7, 8.5 Hz, 1H), 7.55 (d, J = 8.5 Hz, 1H), 7.69 (br d, J = 9.3 Hz, 1H), 8.49 (br s, 1H) ppm; ESIMS: m/z 204.0663 $(M+H)^+$, calcd for $C_{11}H_{10}NO_3$, 204.0656.

4.2.13. 1,3,3-Trimethyl-4-(azidomethyl)spiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (6a)

This compound was synthesized according to general procedure for synthesis NISO method A. Yield 6 mg, 41%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.46 (s, 3H), 1.47 (s, 3H), 2.75 (s, 3H), 4.38 (d, J = 13.6 Hz, 1H), 4.44 (d, J = 13.6 Hz, 1H), 6.58 (dd, J = 0.8, 7.8 Hz, 1H), 6.82 (dd, J = 0.8, 7.8 Hz, 1H), 7.01 (d, J = 9.0 Hz, 1H), 7.24 (t, J = 7.8 Hz, 1H), 7.39 (ddd, J = 1.3, 8.1, 8.2 Hz, 1H), 7.58 (ddd, J = 1.4, 8.2, 8.5 Hz, 1H), 7.67 (br d, J = 9.0 Hz, 1H), 7.742 (s, 1H), 7.745 (br d, J = 8.1 Hz, 1H), 8.56 (br d, J = 8.5 Hz, 1H) ppm; EIMS: m/z 383.1741 $(M)^+$, calcd for $C_{23}H_{21}N_5O$, 383.1741.

4.2.14. 1,3,3-Trimethyl-4-(azidomethyl)-9′-hydroxyspiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (6b)

This compound was synthesized according to general procedure for synthesis NISO method A. Yield 4 mg, 20%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.455 (s, 3H), 1.46 (s, 3H), 2.75 (s, 3H), 4.38 (d, J = 13.7 Hz, 1H), 4.44 (d, J = 13.7 Hz, 1H), 6.59 (dd, J = 0.7, 7.8 Hz, 1H), 6.83 (dd, J = 0.7, 7.8 Hz, 1H), 6.84 (d, J = 8.8 Hz, 1H), 7.02 (dd, J = 2.6, 8.6 Hz, 1H), 7.25 (t, J = 7.8 Hz, 1H), 7.59 (br d, J = 8.8 Hz, 1H), 7.66 (br d, J = 8.6 Hz, 1H), 7.70 (s, 1H), 7.86 (br d, J = 2.6 Hz, 1H) ppm; ESIMS: m/z 400.1768 $(M+H)^+$, calcd for $C_{23}H_{22}N_5O_2$, 400.1769.

4.2.15. 1,3,3-Trimethyl-4-(azidomethyl)-9′-methoxyspiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (6c)

This compound was synthesized according to general procedure for synthesis NISO method A. Yield 5 mg, 22%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.46 (s, 3H), 1.47 (s, 3H), 2.76 (s, 3H), 4.01 (s, 3H), 4.38 (d, J = 13.6 Hz, 1H), 4.44 (d, J = 13.6 Hz, 1H), 6.59 (br d, J = 7.3 Hz, 1H), 6.82 (dd, J = 0.7, 7.3 Hz, 1H), 6.85 (d, J = 8.6 Hz, 1H), 7.04 (dd, J = 2.6, 9.0 Hz, 1H), 7.24 (t, J = 7.8 Hz, 1H), 7.58 (br d, J = 8.6 Hz, 1H), 7.63 (br d, J = 9.0 Hz, 1H), 7.72 (s, 1H), 7.87 (br d, J = 2.6 Hz, 1H) ppm; EIMS: m/z 413.1851 $(M)^+$, calcd for $C_{24}H_{23}N_5O_2$, 413.1847.

4.2.16. 1,3,3-Trimethyl-4-(aminomethyl)spiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (4-NH_2 NISO, 7a)

This compound was synthesized according to general procedure for reduction azide to amine. Yield 3.4 mg, 91%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.46 (s, 3H), 1.50 (s, 3H), 2.74 (s, 3H), 3.95 (m, 2H), 6.51 (br d, J = 7.9 Hz, 1H), 6.92 (br d, J = 7.9 Hz, 1H), 7.02 (d, J = 8.7 Hz, 1H), 7.25 (t, J = 7.9 Hz, 1H), 7.40 (ddd, J = 1.3, 7.7, 8.0 Hz, 1H), 7.58 (ddd, J = 1.3, 8.0, 8.5 Hz, 1H), 7.67 (br d, J = 8.7 Hz, 1H), 7.75 (s, 1H), 7.76 (br d, J = 7.7 Hz, 1H), 8.56 (br d, J = 8.5 Hz, 1H) ppm; ESIMS: m/z 358.1929 $(M+H)^+$, calcd for $C_{23}H_{24}N_3O$, 358.1914.

4.2.17. 1,3,3-Trimethyl-4-(aminomethyl)-9′-hydroxyspiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (4-NH_2, 9′-OH NISO, 7b)

This compound was synthesized according to general procedure for reduction azide to amine. Yield 2.5 mg, 89%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.44 (s, 3H), 1.46 (s, 3H), 2.74 (s, 3H), 3.91 (d, J = 14.7 Hz, 1H), 3.99 (d, J = 14.7 Hz, 1H), 6.51 (br d, J = 7.8 Hz, 1H), 6.82 (d, J = 8.9 Hz, 1H), 6.90 (br d, J = 7.8 Hz, 1H), 7.00 (dd, J = 2.7, 9.5 Hz, 1H), 7.24 (t, J = 7.8 Hz, 1H), 7.57 (br d, J = 8.9 Hz, 1H), 7.64 (br d, J = 9.5 Hz, 1H), 7.66 (s, 1H), 7.82 (br d, J = 2.7 Hz, 1H) ppm; ESIMS: m/z 374.1861 $(M+H)^+$, calcd for $C_{23}H_{24}N_3O_2$, 374.1864.

4.2.18. 1,3,3-Trimethyl-4-(aminomethyl)-9′-methoxyspiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (4-NH_2, 9′-OMe NISO, 7c)

This compound was synthesized according to general procedure for reduction azide to amine. Yield 4.6 mg, 93%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.46 (s, 3H), 1.50 (s, 3H), 2.74 (s, 3H), 3.99 (d, J = 14.7 Hz, 1H), 4.01 (s, 3H), 4.01 (d, J = 14.7 Hz, 1H), 6.50 (br d,

J = 7.4 Hz, 1H), 6.85 (d, J = 8.8 Hz, 1H), 6.90 (br d, J = 7.4 Hz, 1H), 7.03 (dd, J = 2.6, 8.8 Hz, 1H), 7.23 (t, J = 7.4 Hz, 1H), 7.57 (br d, J = 8.8 Hz, 1H), 7.64 (br d, J = 8.8 Hz, 1H), 7.66 (s, 1H), 7.87 (br d, J = 2.6 Hz, 1H) ppm; ESIMS: m/z 388.2020 $(M+H)^+$, calcd for $C_{24}H_{26}N_3O_2$, 388.2020.

4.2.19. 5-Carboxytetramethylrhodamine derivative of 1,3,3-trimethyl-4-(aminomethyl)spiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (4-TMR NISO, 8a)

This compound was synthesized according to general procedure for synthesis TMR–NISO derivatives. Yield 0.56 mg, 38%. ESIMS: m/z 770.3315 $(M+H)^+$, calcd for $C_{48}H_{44}N_5O_5$, 770.3337.

4.2.20. 5-Carboxytetramethylrhodamine derivative of 1,3,3-trimethyl-4-(aminomethyl)-9′-hydroxyspiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (4-TMR, 9′-OH NISO, 8b)

This compound was synthesized according to general procedure for synthesis TMR–NISO derivatives. Yield 0.37 mg, 25%. ESIMS: m/z 786.3281 $(M+H)^+$, calcd for $C_{48}H_{44}N_5O_6$, 786.3287.

4.2.21. 5-Carboxytetramethylrhodamine derivative of 1,3,3-trimethyl-4-(aminomethyl)-9′-methoxyspiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (4-TMR, 9′-OMe NISO, 8c)

This compound was synthesized according to general procedure for synthesis TMR–NISO derivatives. Yield 0.33 mg, 22%. ESIMS: m/z 800.3412 $(M+H)^+$, calcd for $C_{49}H_{46}N_5O_6$, 800.3443.

4.2.22. 5-(Hydroxyethyl)-2,3,3-trimethyl-3*H*-indole (9)

To a stirred solution of 4-aminophenethyl alcohol (1 g, 7.29 mmol) in a 1:1 mixture of concentrated HCl and water (4 mL) at 0 °C, an aqueous solution (1.5 mL) of $NaNO_2$ (504 mg, 7.30 mmol) was added dropwise. After 1 h, a solution of stannous chloride dihydrate ($SnCl_2{\cdot}2H_2O$, 4.93 g, 21.87 mmol) in concentrated HCl (1.5 mL) was added to a reaction mixture. The reaction mixture was stirred for additional 1 h, then neutralized with an aqueous NaOH, and filtered to give a milk white solid. The solid was dissolved in MeOH and filtered to remove an insoluble solid. The filtrate was evaporated to afford 4-hydrazinophenethyl alcohol, which was used for next reaction without purification. The obtained 4-hydrazinophenethyl alcohol was dissolved in EtOH (8 mL) and refluxed with 3-methyl-2-butanone (1.49 mL, 14.59 mmol) and concentrated H_2SO_4 (729 μL) for 12 h. After concentration, the reaction mixture was washed with diethyl ether, neutralized with a saturated aqueous sodium carbonate (Na_2CO_3), and extracted with CH_2Cl_2. The organic layer was dried over anhydrous $MgSO_4$ and evaporated to give **9** (450 mg, 30% based on 4-aminophenethyl alcohol). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.27 (s, 6H), 2.24 (s, 3H), 2.90 (t, J = 6.8 Hz, 2H), 3.86 (t, J = 6.8 Hz, 2H), 7.130 (d, J = 1.9 Hz, 1H), 7.134 (d, J = 7.9 Hz, 1H), 7.41 (br d, J = 7.9 Hz, 1H) ppm; ESIMS: m/z 204.1392 $(M+H)^+$, calcd for $C_{13}H_{18}NO$, 204.1383.

4.2.23. 5-(Azidoethyl)-2,3,3-trimethyl-3*H*-indole (10)

This compound was synthesized according to general procedure for Mitsunobu reaction. Yield 33 mg, 74%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.30 (s, 6H), 2.27 (s, 3H), 2.93 (t, J = 7.4 Hz, 2H), 3.51 (t, J = 7.4 Hz, 2H), 7.13 (s, 1H), 7.15 (d, J = 8.6 Hz, 1H), 7.47 (br d, J = 8.6 Hz, 1H) ppm; ESIMS: m/z 229.1452 $(M+H)^+$, calcd for $C_{13}H_{17}N_4$, 229.1448.

4.2.24. 5-(Azidoethyl)-1,3,3-trimethyl-2-methyleneindoline (11)

This compound was synthesized according to general procedure for synthesis indoline or indoleninium salt method A.

4.2.25. 1,3,3-Trimethyl-5-(azidoethyl)spiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (12)

This compound was synthesized according to general procedure for synthesis NISO method A. Yield 8 mg, 23%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.34 (s, 3H), 1.35 (s, 3H), 2.74 (s, 3H), 2.87 (t, J = 7.2 Hz, 2H), 3.49 (t, J = 7.2 Hz, 2H), 6.51 (d, J = 7.9 Hz, 1H), 6.93 (d, J = 1.6 Hz, 1H), 7.01 (d, J = 8.9 Hz, 1H), 7.05 (dd, J = 1.6, 7.9 Hz, 1H), 7.39 (ddd, J = 1.2, 7.8, 8.1 Hz, 1H), 7.57 (ddd, J = 1.0, 8.1, 8.5 Hz, 1H), 7.66 (br d, J = 8.9 Hz, 1H), 7.74 (dd, J = 1.0, 7.8 Hz, 1H), 7.744 (s, 1H), 8.56 (dd, J = 1.2, 8.5 Hz, 1H) ppm; EIMS: m/z 397.1910 $(M)^+$, calcd for $C_{24}H_{23}N_5O$, 397.1898.

4.2.26. 1,3,3-Trimethyl-5-(aminoethyl)spiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (5-NH_2 NISO, 13)

This compound was synthesized according to general procedure for reduction azide to amine. Yield 2.0 mg, 91%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.34 (s, 3H), 1.35 (s, 3H), 2.74 (s, 3H), 2.74 (t, J = 6.7 Hz, 2H), 2.97 (m, 2H), 6.51 (d, J = 7.8 Hz, 1H), 6.92 (d, J = 1.5 Hz, 1H), 7.02 (d, J = 8.9 Hz, 1H), 7.04 (dd, J = 1.5, 7.8 Hz, 1H), 7.39 (ddd, J = 0.8, 8.0, 8.1 Hz, 1H), 7.57 (ddd, J = 1.3, 8.0, 8.3 Hz, 1H), 7.66 (br d, J = 8.9 Hz, 1H), 7.74 (br d, J = 8.1 Hz, 1H), 7.74 (s, 1H), 8.56 (dd, J = 8.3 Hz, 1H) ppm; ESIMS: m/z 372.2067 $(M+H)^+$, calcd for $C_{24}H_{26}N_3O$, 372.2071.

4.2.27. 5-Carboxytetramethylrhodamine derivative of 1,3,3-trimethyl-5-(aminoethyl)spiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (5-TMR NISO, 14)

This compound was synthesized according to general procedure for synthesis TMR–NISO derivatives. Yield 0.31 mg, 21%. ESIMS: m/z 784.3458 $(M+H)^+$, calcd for $C_{49}H_{46}N_5O_5$, 784.3494.

4.2.28. 2,3,3-Trimethyl-5-(ethyloxycarbonylpropyl)-3*H*-indole (15)

To a stirred solution of 4-(4-aminophenyl)butanoic acid (100 mg, 0.56 mmol) in a 1:1 mixture of concentrated HCl) and water (1.4 mL) at 0 °C, an aqueous solution (500 μL) of $NaNO_2$ (42 mg, 0.61 mmol) was added dropwise. After 1 h, a solution of stannous chloride dihydrate ($SnCl_2{\cdot}2H_2O$, 377 mg, 1.67 mmol) in concentrated HCl (500 μL) was added to a reaction mixture. The reaction mixture was stirred for additional 1 h and filtered to give a milk white solid. The obtained solid was dissolved in EtOH (3 mL) and refluxed with 3-methyl-2-butanone (729 μL, 1.13 mmol) and concentrated sulfuric acid (H_2SO_4, 56 μL, 0.56 mmol) for 12 h. After concentration, the residue was neutralized with a saturated aqueous sodium carbonate (Na_2CO_3) and extracted with diethyl ether (Et_2O). The organic layer was dried over anhydrous $MgSO_4$ and then evaporated to give **15** (117 mg, 76% based on 4-(4-aminophenyl)butanoic acid). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.26 (t, J = 7.3 Hz, 3H), 1.29 (s, 6H), 1.97 (quin, J = 7.7 Hz, 2H), 2.26 (s, 3H), 2.34 (t, J = 7.7 Hz, 2H), 2.69 (t like, J = 7.7 Hz, 2H), 4.13 (q, J = 7.3 Hz, 2H), 7.09 (s, 1H), 7.10 (d, J = 7.7 Hz, 1H), 7.43 (d, J = 7.7 Hz, 1H) ppm; ESIMS: m/z 274.1815 $(M+H)^+$, calcd for $C_{17}H_{24}NO_2$, 274.1802.

4.2.29. 2,3,3-Trimethyl-5-(carboxypropyl)-3*H*-indole (16)

To a solution of **15** (68 mg, 0.25 mmol) in methanol (MeOH, 1 mL), an aqueous NaOH (1 M, 1 mL) was added and stirred at room temperature for 1 h. The reaction mixture was acidified with an aqueous HCl (1 M) and extracted with EtOAc. The organic layer was dried over anhydrous $MgSO_4$ and evaporated to give **16** (32 mg, 52%). ^{1}H NMR ($CDCl_3$,300 MHz) δ 1.32 (s, 6H), 1.99 (quin, J = 7.6 Hz, 2H), 2.36 (s, 3H), 2.39 (t, J = 7.6 Hz, 2H), 2.73 (t like, J = 7.6 Hz, 2H), 7.13 (s, 1H), 7.14 (d, J = 7.9 Hz, 1H), 7.52 (d, J = 7.9 Hz, 1H) ppm; ESIMS: m/z 246.1479 $(M+H)^+$, calcd for $C_{15}H_{20}NO_2$, 246.1489.

4.2.30. 1,2,3,3-Tetramethyl-5-(carboxypropyl)-3*H*-indoleninium iodide (17)

This compound was synthesized according to general procedure for synthesis indoline or indoleninium salt method B.

4.2.31. 1,3,3-Trimethyl-5-(carboxypropyl)spiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (5-CO NISO, 18)

This compound was synthesized according to general procedure for synthesis NISO method B. Yield 15 mg, 32%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.32 (s, 3H), 1.34 (s, 3H), 1.89 (quin, J = 7.7 Hz, 2H), 2.23 (t, J = 7.7 Hz, 2H), 2.60 (t like, J = 7.7 Hz, 2H), 2.70 (s, 3H), 6.50 (d, J = 8.0 Hz, 1H), 6.96 (d, J = 1.6 Hz, 1H), 7.01 (dd, J = 1.6, 8.0 Hz, 1H), 7.02 (d, J = 8.7 Hz, 1H), 7.38 (ddd, J = 1.1, 8.1, 8.2 Hz, 1H), 7.55 (ddd, J = 1.5, 8.1, 8.5 Hz, 1H), 7.72 (br d, J = 8.7 Hz, 1H), 7.77 (br d, J = 8.2 Hz, 1H), 7.79 (s, 1H), 8.51 (dd, J = 1.1, 8.5 Hz, 1H) ppm; ESIMS: m/z 437.1837 $(M+Na)^+$, calcd for $C_{26}H_{26}N_2O_3Na$, 437.1836.

4.2.32. 1,3,3-Trimethyl-5-(*N*-succinimidyloxycarbonylpropyl) spiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (5-NHS–NISO, 19)

This compound was synthesized according to general procedure for synthesis *N*-hydroxysuccinimide ester derivatives. Yield 15 mg, 80%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.34 (s, 3H), 1.36 (s, 3H), 2.07 (quin, J = 7.6 Hz, 2H), 2.64 (t, J = 7.6 Hz, 2H), 2.71 (t, J = 7.6 Hz, 2H), 2.74 (s, 3H), 2.85 (br s, 4H), 6.50 (d, J = 7.9 Hz, 1H), 6.92 (s, 1H), 7.034 (d, J = 8.9 Hz, 1H), 7.03 (d, J = 7.9 Hz, 1H), 7.39 (dt, J = 1.3, 7.9 Hz, 1H), 7.57 (dt, J = 1.3, 7.9 Hz, 1H), 7.66 (br d, J = 8.9 Hz, 1H), 7.741 (s, 1H), 7.745 (br d, J = 7.9 Hz, 1H), 8.56 (d, J = 7.9 Hz, 1H) ppm; ESIMS: m/z 534.2003 $(M+Na)^+$, calcd for $C_{30}H_{29}N_3O_5Na$, 534.2000.

4.2.33. Tetramethylrhodamine-5-carboxamide lysine derivative of 1,3,3-trimethyl-5-(*N*-succinimidyloxycarbonylpropyl)spiro [indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (5-TMR-Lys NISO, 20)

This compound was synthesized according to general procedure for synthesis TMR–NISO derivatives. Yield 0.73 mg, 42%. ESIMS: m/z 955.4377 $(M+H)^+$, calcd for $C_{57}H_{59}N_6O_6$, 955.4389.

4.2.34. 7-(3-Chloropropoxy)-2-naphthol (21)

To a solution of 2,7-dihydroxynaphthalene (1.6 g, 9.99 mmol) in dry acetone (50 mL), anhydrous potassium carbonate (8.3 g, 60.05 mmol) and 1-chloro-3-iodopropane (1.07 mL, 9.97 mmol) were added. The suspension was stirred at 80 °C for 20 h and then cooled down to room temperature. The solid was filtered off and the filtrate was evaporated to give a residue. The residue was further chromatographed on a silica gel flash column (hexane/EtOAc; 7:3) to give **21** (793 mg, 34%). ^{1}H NMR (CDCl3, 300 MHz) δ 2.22 (quin, J = 6.2 Hz, 2H), 3.73 (t, J = 6.2 Hz, 2H), 4.11 (t, J = 6.2 Hz, 2H), 5.80 (br s, 1H), 6.90 (d, J = 2.4 Hz, 1H), 6.93 (dd, J = 2.4, 8.9 Hz, 1H), 6.96 (dd, J = 2.4, 8.9 Hz, 1H), 7.00 (d, J = 2.4 Hz, 1H), 7.61 (d, J = 8.9 Hz, 2H) ppm; ESIMS: m/z 237.0677 $(M+H)^+$, calcd for $C_{13}H_{14}ClO_2$, 237.0677.

4.2.35. 7-(3-Chloropropoxy)-1-nitroso-2-naphthol (22)

This compound was synthesized according to general procedure for nitrosation. Yield 845 mg, 95%. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 1.75 (quin, J = 6.2 Hz, 2H), 3.36 (t, J = 6.2 Hz, 2H), 3.72 (t, J = 6.2 Hz, 2H), 5.82 (br d, J = 8.6 Hz, 1H), 6.69 (dd, J = 2.7, 8.7 Hz, 1H), 7.11 (d, J = 8.7 Hz, 1H), 7.24 (br d, J = 8.6 Hz, 1H) ppm; ESIMS: m/z 266.0578 $(M+H)^+$, calcd for $C_{13}H_{13}ClNO_3$, 266.0579.

4.2.36. *N*-Carboxypentyl-2,3,3-tetramethyl-3*H*-indoleninium bromide (23)

This compound was synthesized according to general procedure for synthesis indoline or indoleninium salt method B.

4.2.37. 1,3,3-Trimethyl-5-(carboxypropyl)-9′-chloropropoxyspiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (5-CO, 9′-Cl NISO, 24a)

This compound was synthesized according to general procedure for synthesis NISO method B. Yield 58 mg, 22%. ^{1}H NMR (CDCl3, 300 MHz) δ 1.33 (s, 3H), 1.35 (s, 3H), 1.97 (quin, J = 7.6 Hz, 2H), 2.31 (quin, J = 6.1 Hz, 2H), 2.41 (t, J = 7.6 Hz, 2H), 2.64 (t, J = 7.6 Hz, 2H), 2.73 (s, 3H), 3.78 (t, J = 6.1 Hz, 2H), 4.32 (t, J = 6.1 Hz, 2H), 6.48 (d, J = 7.7 Hz, 1H), 6.86 (d, J = 8.9 Hz, 1H), 6.90 (d, J = 1.3 Hz, 1H), 7.013 (dd, J = 1.3, 7.7 Hz, 1H), 7.014 (dd, J = 2.4, 9.2 Hz, 1H), 7.56 (d, J = 8.9 Hz, 1H), 7.62 (d, J = 9.2 Hz, 1H), 7.73 (s, 1H), 7.87 (d, J = 2.4 Hz, 1H) ppm; ESIMS: m/z 507.2043 $(M+H)^+$, calcd for $C_{29}H_{32}ClN_2O_4$, 507.2045.

4.2.38. *N*-Carboxypentyl-3,3-dimethyl-9′-chloropropoxyspiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (1-CO, 9′-Cl NISO, 24b)

This compound was synthesized according to general procedure for synthesis NISO method B. Yield 287 mg, 55%. ^{1}H NMR (CDCl3, 300 MHz) δ 1.33 (s, 3H), 1.34 (s, 3H), 1.42–1.70 (6H), 2.26–2.32 (4H), 3.19 (t, J = 7.4 Hz, 2H), 3.78 (t, J = 5.9 Hz, 2H), 4.32 (t, J = 5.9 Hz, 2H), 6.57 (d, J = 7.6 Hz, 1H), 6.82 (d, J = 9.0 Hz, 1H), 6.86 (t, J = 7.6 Hz, 1H), 7.02 (dd, J = 2.3, 9.2 Hz, 1H), 7.06 (d, J = 7.6 Hz, 1H), 7.19 (t, J = 7.6 Hz, 1H), 7.56 (d, J = 9.0 Hz, 1H), 7.61 (d, J = 9.2 Hz, 1H), 7.75 (s, 1H), 7.85 (d, J = 2.3 Hz, 1H) ppm; ESIMS: m/z 521.2195 $(M+H)^+$, calcd for $C_{30}H_{34}ClN_2O_4$, 521.2202.

4.2.39. 1,3,3-Trimethyl-5-(carboxypropyl)-9′-azidopropoxyspiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (5-CO, 9′-N_3 NISO, 25a)

This compound was synthesized according to general procedure for synthesis azido derivatives from chloro derivatives. Yield 30 mg, 77%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.30 (s, 6H), 1.94 (m, 2H), 2.12 (quin, J = 6.3 Hz, 2H), 2.38 (m, 2H), 2.60 (m, 2H), 2.69 (s, 3H), 3.55 (t, J = 6.3 Hz, 2H), 4.25 (t, J = 6.3 Hz, 2H), 6.44 (d, J = 7.6 Hz, 1H), 6.81 (d, J = 8.9 Hz, 1H), 6.88 (br s, 1H), 6.98 (br d, J = 7.6 Hz, 1H), 7.01 (dd, J = 2.2, 8.9 Hz, 1H), 7.52 (d, J = 8.9 Hz, 1H), 7.60 (d, J = 8.9 Hz, 1H), 7.69 (s, 1H), 7.85 (d, J = 2.2 Hz, 1H) ppm; ESIMS: m/z 514.2443 $(M+H)^+$, calcd for $C_{29}H_{32}N_5O_4$, 514.2449.

4.2.40. *N*-Carboxypentyl-3,3-dimethyl-9′-azidopropoxyspiro [indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (1-CO, 9′-N_3 NISO, 25b)

This compound was synthesized according to general procedure for synthesis azido derivatives from chloro derivatives. Yield 114 mg, 88%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.32 (s, 3H), 1.34 (s, 3H), 1.38–1.46 (m, 2H), 1.55–1.71 (m, 4H), 2.13 (quin, J = 6.4 Hz, 2H), 2.29 (t, J = 7.6 Hz, 2H), 3.19 (t, J = 7.2 Hz, 2H), 3.56 (t, J = 6.4 Hz, 2H), 4.27 (t, J = 6.4 Hz, 2H), 6.57 (d, J = 7.2 Hz, 1H), 6.82 (d, J = 8.6 Hz, 1H), 6.87 (t, J = 7.2 Hz, 1H), 7.02 (dd, J = 2.6, 8.8 Hz, 1H), 7.06 (dd, J = 1.8, 7.2 Hz, 1H), 7.19 (dt, J = 1.8, 7.2 Hz, 1H), 7.56 (d, J = 8.6 Hz, 1H), 7.62 (d, J = 8.8 Hz, 1H), 7.72 (s, 1H), 7.84 (d, J = 2.6 Hz, 1H) ppm; ESIMS: m/z 528.2602 $(M+H)^+$, calcd for $C_{30}H_{34}N_5O_2$, 528.2605.

4.2.41. 1,3,3-Trimethyl-5-(carboxypropyl)-9′-aminopropoxyspiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (5-CO, 9′-NH_2 NISO, 26a)

This compound was synthesized according to general procedure for reduction azide to amine. Yield 12 mg, 43%. ^{1}H NMR (MeOD-d_4, 300 MHz) δ 1.28 (s, 3H), 1.30 (s, 3H), 1.88 (quin, J = 7.7 Hz, 2H), 2.07 (quin, J = 6.3 Hz, 2H), 2.21 (t, J = 7.7 Hz, 2H), 2.59 (t, J = 7.7 Hz, 2H), 2.67 (s, 3H), 2.97 (t, J = 6.3 Hz, 2H), 4.24 (t, J = 6.3 Hz, 2H), 6.47 (d, J = 7.2 Hz, 1H), 6.81 (d, J = 8.9 Hz, 1H), 6.94 (s, 1H), 6.99 (d, J = 7.2 Hz, 1H), 7.02 (dd, J = 2.4, 9.3 Hz, 1H), 7.59 (d, J = 8.9 Hz, 1H), 7.65 (d, J = 9.3 Hz, 1H), 7.73 (s, 1H), 7.88 (d, J = 2.4 Hz, 1H) ppm; ESIMS: m/z 488.2537 $(M+H)^+$, calcd for $C_{29}H_{34}N_3O_4$, 488.2544.

4.2.42. *N*-Carboxypentyl-3,3-dimethyl-9′-aminopropoxyspiro [indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (1-CO, 9′-NH_2 NISO, 26b)

This compound was synthesized according to general procedure for reduction azide to amine. Yield 21 mg, 45%. ^{1}H NMR (MeOD-d_4,

300 MHz) δ 1.32 (s, 3H), 1.34 (s, 3H), 1.36–1.72 (m, 6H), 2.10 (m, 2H), 2.18 (t, J = 7.1 Hz, 2H), 2.86 (dd, J = 7.1, 7.4 Hz, 2H), 3.09 (t, J = 7.6 Hz, 2H), 4.29 (m, 2H), 6.57 (d, J = 7.5 Hz, 1H), 6.81 (d, J = 8.8 Hz, 1H), 6.82 (t, J = 7.5 Hz, 1H), 7.02 (dd, J = 2.6, 9.0 Hz, 1H), 7.06 (dd, J = 1.1, 7.5 Hz, 1H), 7.13 (dt, J = 1.1, 7.5 Hz, 1H), 7.61 (d, J = 8.8 Hz, 1H), 7.66 (d, J = 9.0 Hz, 1H), 7.80 (s, 1H), 7.90 (d, J = 2.6 Hz, 1H) ppm; ESIMS: m/z 502.2697 $(M+H)^+$, calcd for $C_{30}H_{36}N_3O_4$, 502.2700.

4.2.43. 5-Carboxytetramethylrhodamine derivative of 1,3,3-trimethyl-5-(carboxypropyl)-9′-aminopropoxyspiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (5-CO, 9′-TMR NISO, 27a)

This compound was synthesized according to general procedure for synthesis TMR–NISO derivatives. Yield 1.47 mg, 43%. ESIMS: m/z 900.3962 $(M+H)^+$, calcd for $C_{54}H_{54}N_5O_8$, 900.3962.

4.2.44. 5-Carboxytetramethylrhodamine derivative of *N*-carboxypentyl-3,3-dimethyl-9′-aminopropoxyspiro[indoline-2,3′-[3*H*]naphtho[2,1-*b*][1,4]oxazine] (1-CO, 9′-TMR NISO, 27b)

This compound was synthesized according to general procedure for synthesis TMR–NISO derivatives. Yield 1.55 mg, 45%. ESIMS: m/z 914.4117 $(M+H)^+$, calcd for $C_{55}H_{56}N_5O_8$, 914.4123.

4.2.45. *N*-Hydroxysuccinimide ester derivative of 5-CO, 9′-TMR NISO (5-NHS, 9′-TMR NISO, 28a)

This compound was synthesized according to general procedure for synthesis *N*-hydroxysuccinimide ester derivatives. Yield 1.03 mg, 63%. ESIMS: m/z 997.4117 $(M+H)^+$, calcd for $C_{58}H_{57}N_6O_{10}$, 997.4131.

4.2.46. *N*-Hydroxysuccinimide ester derivative of 1-CO, 9′-TMR NISO (1-NHS, 9′-TMR NISO, 28b)

This compound was synthesized according to general procedure for synthesis *N*-hydroxysuccinimide ester derivatives. Yield 0.91 mg, 53%. ESIMS: m/z 1011.4313 $(M+H)^+$, calcd for $C_{59}H_{59}N_6O_{10}$, 1011.4287.

4.3. Spectroscopic and optical switching properties of TMR–NISO derivatives in vitro

Absorption spectra of each TMR–NISO solution were recorded using a Shimadzu 1601PC spectrophotometer in solutions of glycerol, methanol, or water. The MC-state was generated from the SP-state following irradiation of the latter for 1 min with 365 nm light delivered from a hand-held lamp. The rate for the thermally-driven MC to SP transition was determined by measuring the decrease absorption intensity in glycerol at room temperature as a function of time. Fluorescence emission spectra were recorded using an SLM-AB2 instrument.[3] The SP- and MC-states of each TMR–NISO derivative were excited at 555 nm and the emission spectra recorded between 560 and 750 nm. The fluorescence quantum yield (Φ) for TMR in the SP-state of each TMR–NISO derivative was determined using rhodamine 101 in ethanol as a reference (Φ = 1 in ethanol).[22] A typical optical switching cycle was demonstrated using a 120 μL sample solution of TMR–NISO **8b** in glycerol. Each cycle of optical perturbations involved a 30 s irradiation with 365 nm light followed by excitation of the MC-solution with 550 nm laser light for 60 s. The fluorescence intensity of TMR was monitored at 580 nm. This process was repeated for six cycles. All spectra were recorded at room temperature.

4.4. Spectroscopic and optical switching properties of TMR–NISO derivatives in vivo

Two different microscope systems were used to image and manipulate TMR–NISO probes. In the first system, fluorescence images of the donor probe were collected on an Olympus FluoView500 confocal microscope using a 60×, NA = 1.45 oil immersion objective. UV-light pulses from a 100 W Hg-arc lamp filtered through a 365 ± 25 bandpass filter were controlled with a time varying shutter (Vincent Associates) to drive the SP to MC transition as described in Marriott et al.[2] In the second system, a 100 W Hg-arc lamp was used to continually excite the donor probe at 546 nm while a second 100 W Hg-arc aligned orthogonal was used to deliver 365 nm pulses whose duration was controlled by a mechanical shutter (Melles-Griot). Typically, the 100 ms pulses were used to convert SP- to MC-state. The UV-light was directed into the optical path using a dichroic mirror from a GFP filter set, which allowed for pulsed UV-irradiation of the sample while continuously illuminating the sample with 546 nm light. The donor probe images were collected using a Hamamatsu EM-CCD camera at ~30 Hz (512 × 512) or at 300 Hz using a smaller region of interest.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.07.015. These data include MOL files and InChiKeys of the most important compounds described in this article.

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