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Supporting Information

Red-Emitting Rhodamines with Hydroxylated, Sulfonated, and Phosphorylated Dye Residues and Their Use in Fluorescence Nanoscopy

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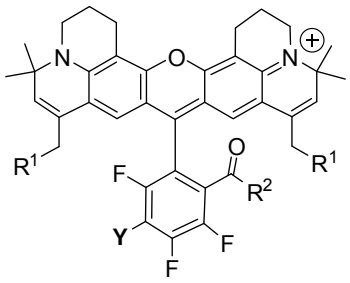
General remarks

UV-visible absorption spectra were recorded on a Varian Cary 4000 UV-Vis spectrophotometer, and fluorescence spectra on a Varian Cary Eclipse fluorescence spectrophotometer. Reactions were carried out upon magnetic stirring in Schlenk flasks equipped with septa or reflux condensers with bubble-counters under argon using a standard manifold with vacuum and argon lines. The MICROTOF spectrometer equipped with ESI ion source Apollo and direct injector with LC autosampler Agilent RR 1200 was used for obtaining high resolution mass spectra (ESI-HRMS). ESI-HRMS were obtained also on APEX IV spectrometer (Bruker). HPLC system (Knauer): Smartline pump 1000 (2×), UV detector 2500, column thermostat 4000 (25 °C), mixing chamber, injection valve with 20 and 100 µL loop for the analytical and preparative columns, respectively; 6-port-3-channel switching valve; analytical column: Eurospher-100 C18, 5 µm, 250×4 mm, 1.1 mL/min; solvent A: water + 0.1 % v/v trifluoroacetic acid (TFA); solvent B: CH_3CN + 0.1 % v/v TFA; detection at 636 or 254 nm, as specified. Analytical TLC was performed on MERCK ready-to-use plates with regular silica gel 60 (F_{254}) and UV-detector (unless specified otherwise). Preparative column chromatography was followed by stepwise filtration from SiO_2 through Rotilabo® syringe filters (45 µm and 22 µm). Capillary electrophoresis was performed on a standard capillary DNA sequencer (3130 Genetic Analyzer, Applied Biosystems) equipped with a capillary array of 50 cm length. For separation, the aqueous self-coating POP-7™ Polymer and the aqueous running buffer at pH 8.0 (ten times diluted; both from Applied Biosystems) were used. Sample injection was performed electrokinetically by applying 15 kV for 5 s to the sample. CGE separation was performed at 15 kV for 60 min. The device was equipped with a 15 mW Argon ion laser and enabled the excitation of the fluorescent dyes at 488 nm and 514.5 nm. Fluorescent emission was detected at 655 nm. Dye structures are depicted in Figures 1–4.



1 (R^1 = H, R^2 = O^-, **Y** = F)
2b (R^1 = SO_3^-, R^2 = $N(CH_3)(CH_2)_3CO_2H$, **Y** = F)
3a (R^1 = SO_3^-, R^2 = O^-, **Y** = F)
4a (R^1 = OH, R^2 = O^-, **Y** = F)
4b (R^1 = OH, R^2 = O^-, **Y** = $S(CH_2)_2SO_3^-$)
5-H (R^1 = OH, R^2 = $N(CH_3)(CH_2)_3CO_2^-$, **Y** = F)
6-H (R^1 = OH, R^2 = $N(CH_3)(CH_2)_3CO_2H$, **Y** = $S(CH_2)_2SO_3^-$)
7-H (R^1= $OP(O^-)_2$, R^2 = $N(CH_3)(CH_2)_3CO_2H$, **Y** = F)

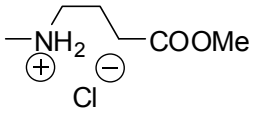
Fig 1. Structures of red-emitting rhodamine dyes.

Hydroxyl-substituted red emitting rhodamine dye (4a)

The starting rhodamine dye of structure **1** (Fig. 1) was prepared as described in our previous study [1]. The isolation of the dye was carried out in one run over regular silica gel with $CH_3CN/CH_2Cl_2/H_2O$ (10:1:1) as the mobile phase and afforded 40–55% yields. The product was then subjected to direct oxidation with SeO_2 at the allylic sites to compound **4a** (see Schemes 1 and 2 in the main text).

In a typical experiment, rhodamine **1** (0.30 g, 0.47 mmol) was refluxed overnight (12–14 h) in a solution containing 3.50 g (32 mmol) of SeO_2 in a mixture of dioxane (20 mL) and water (2 mL). The reaction was monitored by TLC on silica gel plates with $CH_3CN/CH_2Cl_2/H_2O$ (10:1:1) as a mobile phase. The solution was cooled to room temperature, stirred for 10 min with water (80 mL), CH_2Cl_2 (200 mL), silica gel (2 g), and filtered. The organic phase was separated, the aqueous phase extracted twice with CH_2Cl_2 (40 mL), the combined organic extracts washed with water (80 mL), dried over Na_2SO_4, and evaporated *in vacuo*. The residue was dissolved in anhydrous ethanol (150 mL), chilled to 0…+5 °C (ice bath), and $NaBH_4$ (0.75 g, 21 mmol) was added in few portions upon vigorous stirring, which was continued for 45 min. The solution was poured into a flask containing ice cold water (300 mL) and CH_2Cl_2 (500 mL), the mixture well stirred, aqueous phase separated and extracted with CH_2Cl_2 (3×100 mL). The combined organic solutions were evaporated, and the residue separated over 200 g of SiO_2 with $CH_3CN/CH_2Cl_2/H_2O$ (10:1:1) as the mobile phase. Pure fractions were filtered from SiO_2 through Rotilabo® syringe filters (0.22 μm) and evaporated to furnish 167 mg (53%) of rhodamine **4a** as a bronze glittering crystalline solid, slightly soluble in water, well-soluble in methanol, and soluble in most organic solvents, except alkanes. Solutions are blue and have intense red fluorescence. Analytical data on compound **4a**: t_R = 10 min (HPLC, A/B 50:50→0:100 in 25 min, HPLC area 94 %). MS (ESI): *m*/*z* (positive mode, %) = 675 (100%) $[M+H]^+$; HRMS ($C_{38}H_{34}F_4N_2O_5$): 675.2468 (found M+H), 675.2477 (calc.). ^{1}H NMR (600 MHz, CD_3OD): δ = 1.52/1.54 (s×2, 12 H), 2.06 (m, CH_2, 4 H), 3.02 (t, J = 6.5 Hz, 4 H), 3.65 (t, J = 5.5 Hz, 4 H), 4.25/4.29 (ABX-system, J_{AB} = 13 Hz, $J_{AX(BX)}$ = 1.3 Hz, 4 H), 5.84 (br. s, 2 H), 6.95 (br. s, 2 H) ppm; ^{13}C NMR (100 MHz, CD_3OD): δ = 21.5/21.9 (CH_2), 28.92/28.97 (CH_3), 44.8 (CH_2), 61.4/62.4 (CH_2), 107.5 (C), 115.2 (C), 122.0 (C), 122,2 (CH), 130.8 (C), 133.2 (CH), 147.1 (C), 152.2 (C), 155.2 (C), 167.3 (CO) ppm. ^{19}F NMR (282.4 MHz, $CDCl_3$): δ = –155.2 (dt, J = 20 and 3 Hz, 1 F), –152.3 (ddd, J = 22, 19, and 4 Hz, 1 F), –138.9 (ddd, J = 17 and 6 Hz, 1 F), –137.5 (ddd, J = 21, 12, and 4 Hz, 1 F) ppm.

Methyl *N*-methyl-4-aminobutyrate hydrochloride (the linker)



4-(Benzyloxycarbonylamino)butyric acid (VWR International) was methylated both at amino and carboxyl sites, following the general procedure described by *K. Olsen* [2] and purified by flash chromatography over regular silica gel. Purity and identity of methyl *N*-methyl-*N*-Z-4-aminobutyrate was confirmed by ^{1}H MNR and mass-spectrometry. To remove the protecting group (Z = carboxybenzyl), the methylated product (methyl *N*-methyl-*N*-Z-4-aminobutyrate) was hydrogenated as follows [3]: all the material was dissolved in a mixture of anhydrous ethanol (80 mL) and 3.6 mL of the commercial 5–6 M solution of anhydrous HCl in propanol-2 (Acros) under an argon atmosphere (the solution is hygroscopic). A 1-L Schlenk flask with a stirring bar, septum, and a bubble counter was loaded with 1 g of palladium on charcoal (10% Pd, oxidized form, VWR International) and flushed upon stirring fist with argon, then with hydrogen gas (from a special rubber balloon). The solution of the substrate was introduced via a syringe, and a vigorous stirring with a slow purge of H_2 was maintained for 6 h at r. t., until the reaction was complete (TLC-monitoring on silica gel plates with EtOAc/hexane (1:1) as a mobile phase, and 5% phosphomolybdic acid solution in ethanol (ALDRICH) as the developing reagent). The reaction solution was diluted with CH_2Cl_2 (300 mL) and filtered through Celite®, which was then thoroughly washed with CH_2Cl_2 (100 mL). The solvents were evaporated *in vacuo* at the bath temperature below 36°C, the residue dissolved in methanol (20 mL), transferred to a 50-mL flask, and evaporated *in vacuo* at r. t. till the residue started to crystallize. Dry diethyl ether (15 mL) was added, and the oil completely solidified upon trituration with a spatula at room temperature. The flask was cooled in a dry ice bath, and the liquid carefully removed with a Pasteur pipette. The bulk of the residual ether was evaporated in stream of argon, the rest – *in vacuo* to furnish 1.91 g (65%) of a colorless hygroscopic powder, (methyl *N*-methyl-4-aminobutyrate hydrochloride) which was stored at +5 °C under argon and utilized without further treatment. Purity and identity of the compound was confirmed by NMR and mass-spectrometry [3].

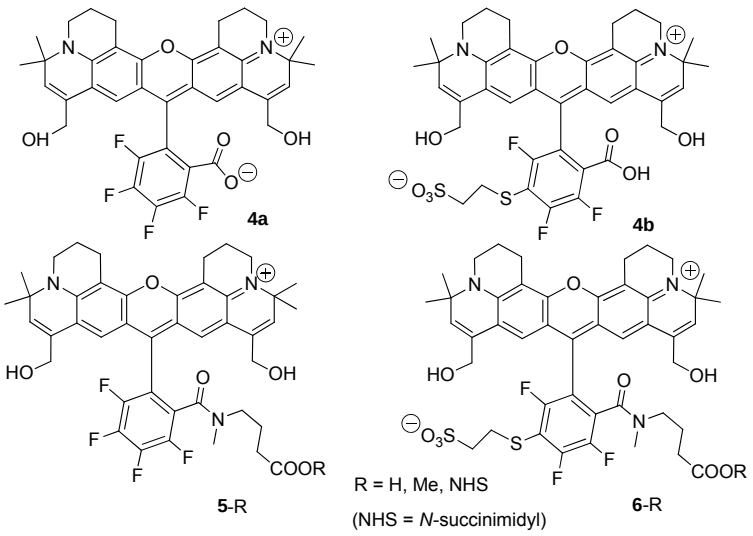


Fig. 2. Hydroxyl-substituted red emitting rhodamine dyes and intermediates.

Rhodamines 5-Me and 5-H

Amidation and saponification (for structures see Fig 2)

The amidation of the hydroxyl-substituted dye **4a** was carried out as follows: to a solution of 20 mg (0.03 mmol) of **4a** in a stirred mixture of CH_3CN (7 mL) and CH_2Cl_2 (3 mL), cooled to –7… –5 °C (bath with a mixture of ice and brine, ca. 1:1) were subsequently added: methyl *N*-methyl-4-aminobutyrate hydrochloride (65 mg, 0.39 mmol) in 3 mL CH_3CN, then HATU (91 mg, 0.24 mmol) in 2 mL CH_3CN, and, finally, Et_3N (0.2 mL, 1.3 mmol) in 2 mL CH_3CN. All reagents were introduced through a septum, and an argon atmosphere was maintained. The solution was stirred overnight in an ice-water bath, and the completion of the reaction was checked by TLC on SiO_2 plates with MeOH/CH_2Cl_2 (1:8) as the mobile phase. Then the reaction mixture was quenched with CH_2Cl_2 (30 mL) and ice cold water (50 mL). The organic phase was separated and consecutively washed with 0.5 M aq. HCl (40 mL), water (30 mL), 2% aq. $NaHCO_3$ (45 mL), again with water (3 × 50 mL), dried over Na_2SO_4, and evaporated. The crude product was chromatographed over 35 g of silica gel with MeOH/CH_2Cl_2 (1:20) as the mobile phase to furnish 63 mg of an amorphous dark blue solid, containing only rhodamine **5**-Me and *N*-methyl pyrrolidone (obtained from methyl *N*-methyl-ω-aminobutyrate in the course of spontaneous cyclization), which proved difficult to remove completely. However, this impurity caused no difficulties in the next step. Crude compound **5**-Me was saponified without further treatment. HPLC: t_R = 17 min (A/B 70:30→0:100 in 25 min; HPLC area 96%). MS (ESI): *m*/*z* (positive mode, %) = 788 (100%) $[M+H]^+$; HRMS ($C_{44}H_{46}F_4N_3O_6$): 788.3316 (found M+H), 788.3317 (calc.).

Methyl ester **5**-Me was saponified in a weak alkaline solution as follows: 12.5 mg of crude **5**-Me (1/5 of the whole amount obtained as described above) was dissolved in a mixture of 0.2% aqueous KOH solution (7.5 mL, 0.27 mmol KOH) and THF (3 mL) at room temperature upon sonication in an ultrasonic bath for few minutes, and left for 3 h at r. t. Trifluoroacetic acid (50 µL, 0.60 mmol) was added, and the solution evaporated to one-third of its initial volume (~3 mL) *in vacuo* at room temperature. The residue was diluted with equal volume of brine, extracted with CH_2Cl_2 (5×15 mL), the extract washed with sat. aqueous solution of $NaHCO_3$ (5 mL),

evaporated, and subjected to chromatography over silica gel (10 g) with CH_3CN/H_2O as the mobile phase (5:1→4:1; with 0.1 % v/v CF_3COOH added in the end of the elution to completely wash the rest of the dye from the column). The fractions were evaporated *in vacuo* to the volume of ca. 10 mL, diluted with an equal volume of brine, and extracted with CH_2Cl_2 (2×15 mL). The extract was washed with sat. aqueous solution of $NaHCO_3$ (10 mL), dried, and evaporated to furnish 4.4 mg of **5**-H (a trifluoroacetate, according to ^{19}F NMR; yield 83%, over 2 steps from 6 μmol of **4a**) as an amorphous dark blue solid. Fractions were filtered from SiO_2 through Rotilabo® syringe filters (0.22 μm). Dye **5**-H (free acid) is very slightly soluble in water, well-soluble in MeOH, CH_3CN, and chlorinated solvents. Analytical data on **5**-H: t_R = 15 min (HPLC, A/B 70:30→0:100 in 25 min, HPLC area 96%); TLC: R_f = 0.4 (regular silica gel, MeOH/ CH_2Cl_2 4:1). MS (ESI): *m*/*z* (positive mode, %) = 774(70%) $[M+H]^+$; HRMS ($C_{43}H_{44}F_4N_3O_6$): 774.3170 (found M+H) 774.3167 (calc.). ^1H NMR (300 MHz, CD_3OD, *mixture of two amide rotamers with diastereotopic methyl and CH groups; see also ref.* [1]): δ = 1.34 (m, 2 H, CH_2), 1.54/1.55/1.56 (s×3, CH_3, 12 H), 2.06 (m, CH_2, 4 H), 2.71–2.76 (s×2, NCH_3, 3 H), 2.88 (m, CH_2CO, 2 H), 3.02 (br. t, CH_2, 4 H), 3.55 (m, NCH_2, 2 H), 4.24–4.28 (m, CH_2O, 4 H), 5.85 (s, 2 H), 6.89/6.92/6.97/7.01 (s×4, 2 H) ppm; ^{19}F NMR (282.4 MHz, $CDCl_3$): δ = –151.1/–150.9 (m, 1 F), –149.3/–148.4 (m, 1 F), –137.6/–136.3 (m, 1 F), –134.6 (m, 1 F), 73,2 (s, CF_3COO^-, 3 F) ppm.

_N_-hydroxysuccinimidyl ester (5-NHS)

Rhodamine **5**-H (1.0 mg, 1.3 μmol), *N*-hydroxysucinimide (4 mg, 35 μmol), Et_3N (3 μL, 20 μmol), and HATU (4 mg, 10 μmol) were combined in dry CH_3CN (2 mL) under an argon atmosphere at 0°C. After stirring at room temperature for 15 min, the reaction was complete, as established by TLC (regular silica gel plates; CH_3CN/H_2O (5:1); R_f-values for **5**-NHS and **5**-H are 0.1 and 0.6, respectively). The reaction solution was neutralized with trifluoroacetic acid (0.15 mL of 1% (v/v) solution in CH_3CN, 20 μmol) and loaded straight onto a column with 3 g of silica gel with $CH_3CN/CH_2Cl_2/H_2O$ (10:1:1) as the mobile phase. Pure fractions with compound 5-**NHS** were filtered from SiO_2 through Rotilabo® syringe filters (0.22 μm) and evaporated *in vacuo* at room temperature to furnish 1.6 mg of an amorphous solid material containing **5**-NHS and variable amounts of *N*-hydroxysucinimide. HPLC: t_R = 18 min (A/B 70:30→0:100 in 25 min; HPLC area > 85%, detection at 636 nm). The active ester was stored under argon at –20 °C and used for immunolabeling experiments without further purification. MS (ESI): *m*/*z* (positive mode, %) = 871(100%) $[M+H]^+$; HRMS ($C_{47}H_{47}F_4N_4O_8$): 871.3326, (found M+H), 871.3325 (calc.).

Hydroxylated dye with one sulfonic acid group (4b)

The dyes with one sulfonic acid group were prepared via substitution of one fluiorine atom in **4a** with 2-mercaptoethyl sulfonic acid (Aldrich). That was followed by amidation (see Scheme 2 in the main text and Fig. 2 above). 2-Mercaptoethyl sulfonic acid (2.5 mL of 0.3 M solution in DMF

containing 10% H_2O; total amount of the thiol 0.74 mmol) was added in one portion to a solution of **4a** (100 mg, 0.148 mmol) and Et_3N (0.26 mL, 1.78 mmol) in DMF (7 mL). The solution was stirred overnight under argon at room temperature, acidified with acetic acid (1.5 mL, 26 mmol) and well-shaken with a mixture of CH_2Cl_2 (150 mL), water (40 mL), and brine (30 mL). The organic layer was separated, the aqueous layer extracted with CH_2Cl_2 (2 × 50 mL), and the combine organic solutions were washed with water (20 mL). The aqueous layer contains a colored polar compound (the di-substituted product with two sulfonic acid groups) that is not extractable. The volatile solvents were removed in a rotary evaporator at t < 36°C. The residue, containing DMF, was evaporated to dryness at room temperature *in vacuo* straight from the flask, which was connected to the distillation bridge with a receiver in a dry ice trap. Compound **4b** was purified over a column with SiO_2 (30 g) using MeOH/ CH_2Cl_2 (5:1), then MeOH/CH_2Cl_2/H_2O (10:10:1). Pure fractions were combined, 0.20 mL of Et_3N was added (to prevent esterification with MeOH, as it occurs in compound with sulfonic acid group; see ref. [1]), and the solvents evaporated. The residue was re-dissolved in 30 mL of a mixture MeOH/H_2O (1:8) and filtered from SiO_2 through Rotilabo® syringe filters (0.22 μm). Evaporation of the solvents (with 50μL of Et_3N added beforehand) afforded 103 mg (76%) of **4b** as a triethylammonium salt (as established by MNR spectroscopy) with MM = 907.

Analytical data for **4b**: t_R = 12 min (HPLC, A/B 70:30→0:100 in 25 min; HPLC area 99%). TLC: R_f = 0.3 (silica, MeOH/CH_2Cl_2 4:1). MS (ESI): *m*/*z* (positive mode, %) = 819 (100%) $[M+Na]^+$; HRMS ($C_{40}H_{39}F_3N_2O_8S_2$): 819.1987 (found M+Na), 819.1992 (calc.). ^{1}H NMR (**4b***Et_3N, 300 MHz, DMSO-d_6, *mixture of two amide rotamers with diastereotopic methyl and CH groups; the same is true for ^{13}C and ^{19}F*): δ = 0.96 (t, J = 6 Hz, 3 H, CH_3CH_2 in Et_3N), 1.49/1.51 (s×2, CH_3, 12 H), 2.06 (m, CH_2, 4 H), 2.45 (m, 2 H, CH_2), 2.67–2.75 (m, CH_2S, 2 H), 3.00 (br. t, CH_2, 4 H), 3.04–3.09 (m, CH_2SO_3, 2 H), 3.63 (m, CH_2, 4H), 4.06–4.18 (AB-system, $J_{AX(BX)}$ = 16 Hz, CH_2O, 4 H), 5.14 (br. s, OH, 1 H), 5.81 (s, 2 H), 6.83 (s, 2 H) ppm; ^{13}C NMR (75.5 MHz, CD_3OD; *due to very low intensities and splitting, the signals of C-F carbon atoms were not detected*): 11.3 (CH_2), 19.4 (CH_2), 19.9 (CH_2), 28.1/27.9 (CH_3×2), 27.0 (CH_2), 42.7 (CH_2), 45.6 (CH_2), 51.7 (CH_2), 59.3 (C), 59.7 (CH_2), 95.3 (C), 105.3 (C), 112.4 (C), 128.9 (C), 120.2 (CH), 128.9 (C), 145.1 (CH), 130.2 (C), 162.1 (C), 149.6 (C), 152.4 (C), 162.1 (CO) ppm; ^{19}F NMR (282.4 MHz, CDCl3): δ = –142.7 (m, 1 F), –125.1 (dd, J = 22 and 5 Hz, 1 F), –109.4 (dd, J = 15 and 2 Hz, 1 F) ppm.

Rhodamine dyes 6-R (for structures see Fig. 2)

Methyl ester 6-Me and acid 6-H. The amidation of the hydroxyl-substituted dye with one sulfonic acid group (**4b**) was carried out as follows: compound **4b** (90 mg, 0.11 mmol) was dissolved in dry CH_3CN (100 mL) containing Et_3N (0.5 mL, 3.5 mmol) upon sonication at r. t. in an argon-flushed flask with a magnetic bar and septum. The solution was cooled to –7…–5°C in a mixture of brine and ice (ca. 1:1). Then HATU reagent (350 mg, 0.92 mmol) in CH_3CN (5–7 mL) and *N*-methyl-4-aminobutyrate hydrochloride (180 mg, 1.08 mmol) in CH_3CN (3– 5 mL) were subsequently added through syringes upon stirring. The solution was stirred for 30 min at –7…–5°C and then left overnight at 0°C (ice bath). The solution was loaded straight onto a

column with 90 g SiO_2 and the elution was carried out with $CH_3CN/CH_2Cl_2/H_2O$ (20:1:1→10:1:1) as the mobile phase. Pure fractions with compound **6**-Me were pooled and evaporated. Analytical data on **6**-Me: t_R = 13 min (HPLC, A/B 70:30→0:100 in 25 min; HPLC area 96%). MS (ESI) *m*/*z* (positive mode, %) = 932 (100%) $[M + Na]^+$; HRMS: ($C_{46}H_{50}F_3N_3O_9S_2$) 932.2834 (found M + Na), 932.2838 (calc.). The residue in the flask (compound **6**-Me) was saponificated overnight with a solution of 4 mmol NaOH (4 mL of a 1 M aqueous solution) in a mixture of water (60 mL) and THF (30 mL) at r. t. The completion of the hydrolysis is best checked by TLC on regular silica gel plates with $CH_3CN/CH_2Cl_2/H_2O$ (10:1:1) mixture containing 0.1% Et_3N; R_f values of the starting material and the free acid are 0.1 and 0.5, respectively. The solution was neutralized with an excess of HOAc (0.6 mL, 10 mmoL) and again evaporated to the volume of 10–15 mL. Then 15 mL of CH_3CN/H_2O 1:1 mixture was added, and the flask was kept for a short time in an ultrasonic bath to completely dissolve the dark blue precipitate. The solution was loaded onto a column with reverse-phase silica gel (Polygoprep 60-50 C_{18}, 20 g; Macherey-Nagel) and water as a mobile phase. The column was eluted first with water (200 mL), then with CH_3CN/H_2O (1:1) containing 0.1% CF_3COOH. Pure fractions with acid **6**-H with were pooled, filtered from SiO_2 through Rotilabo® syringe filters (0.45 µm) and evaporated to furnish 104 mg (94%) of **6**-H as a trifluoroacetate (according to ^{19}F NMR; see below). The compound is a dark blue heavy crystalline solid, very slightly soluble in pure water, well-soluble in aq. $NaHCO_3$, alkalis, Et_3N, PBS buffer, and most organic solvents (except alkanes).

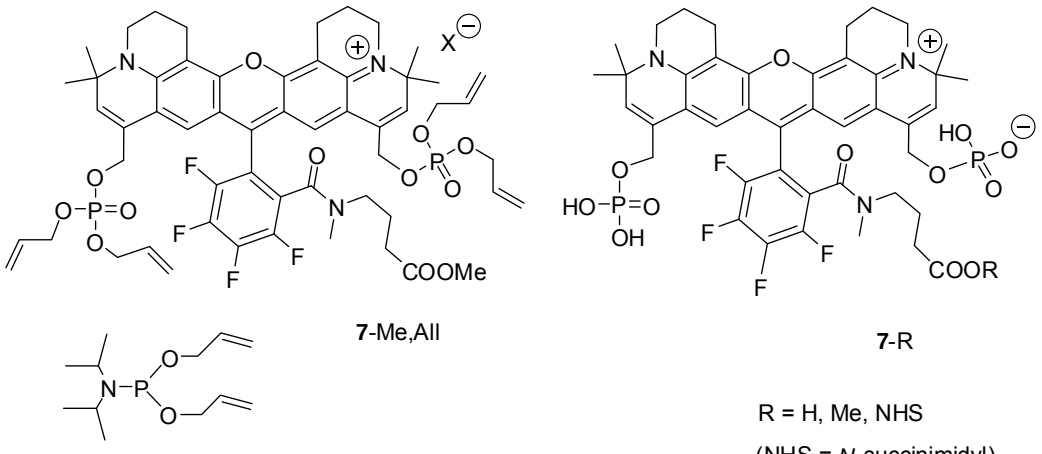
Analytical data for **6-H**: t_R = 11–12 min (HPLC, A/B 70:30→0:100 in 25 min; HPLC area 98%). TLC: R_f = 0.5 (silica gel, MeOH/CH_2Cl_2 4:1) and 0.15 (silica gel, $CH_3CN/CH_2Cl_2/H_2O$ 10:1:1); MS (ESI) *m*/*z* (positive mode, %) = 918 (100%) $[M+Na]^+$; HRMS ($C_{45}H_{48}F_3N_3O_9S_2$): 896.2864 (found M+Na), 896.2857 (calc.).

^1H NMR (300 MHz, CD_3CN, *mixture of two amide rotamers with diastereotopic methyl and CH groups; the same is true for ^{13}C and ^{19}F*): δ = 1.44/1.49/1.52/1.54 (s×4, CH_3, 12 H), 1.70 (m, CH_2, 4 H), 2.0 (m, CH_2, 4 H), 2.35 (m, CH_2, 4 H), 2.66/2.86 (s×2, NCH_3, 3 H), 2.95 (m, CH_2CO, 2 H), 3.10 (m, CH_2S, 2 H), 3.17– 3.55 (m, CH_2N + CH_2SO_3, 4 H), 3.58 (m, CH_2N, 4 H), 4.04–4.22 ($\underline{CH_2}OH$, 4 H), 5.80/5.86 (s×2, 2 H), 6.81/6.83 (s×2, 2 H) ppm; ^{13}C NMR (75.5 MHz, CD_3CN; *due to very low intensities and splitting, the signals of C-F carbon atoms were not detected*): δ = 20.5 (CH_2), 21.1 (CH_2), 21.2 (CH_2), 23.5 (CH_2), 23.7 (CH_2), 28.4/28.5/28.6/28.7 (CH_3), 28.9 (CH_2), 31.1 (CH_2), 32.5 (CH_3), 38.1 (CH_3), 40.4/40.5 (CH_3), 44.2 (CH_2), 44.2 (CH_2), 47.4 (CH_2), 50.5 (CH_2), 52 (CH_2), 53.3 (CH_2), 60.8 (C), 61.2 (CH_2), 61.5 (CH_2), 106.9 (C), 107.3 (C), 113.4 (C), 113.9 (C), 119.4 (C), 120.0 (C), 121.6 (C), 122.6 (CH), 129.0 (C), 130.3 (C), 131.3 (CH), 132.3 (CH), 142.6 (C), 151.2 (C), 151.4 (C), 154.1 (C), 162.2 (C), 164.2 (C), 173.9 (CO), 176.0 (CO) ppm; ^{19}F NMR (282.4 MHz, CD_3CN): δ = –140.8 (dd, *J* = 24.7 and 14.1 Hz, 1 F), –121.7 (dd, *J* = 24.7 and 4.7 Hz, 1 F), –108.5 (ddd, *J* = 14,1, 4.5, and 2.1 Hz, 1 F), 76,7 (s, CF_3COO^-, 3 F) ppm.

_N_-hydroxysuccinimidyl ester 6-NHS

In a typical experiment, TSTU (12 mg, 40 μmol) in dry CH_3CN (1 mL) was introduced via a syringe to a solution of rhodamine **6**-H (9 mg, 9 μmol) and Et_3N (9 μL, 62 μmol) in 3–4 mL of the same solvent at –7…–5°C upon stirring under an argon atmosphere. This temperature was maintained for additional 30–50 min by means of a mixture of brine and ice (1:1). The completion of the reaction was established by TLC (silica gel plates; CH_3CN/H_2O (7:1); R_f-values for **5**-NHS and **5**-H are 0.2 and 0.5, respectively). The solution was loaded straight onto a column with 12 g SiO_2 and $CH_3CN/CH_2Cl_2/H_2O$ (10:1:1) as the mobile phase. Pure fractions were filtered from SiO_2 through Rotilabo® syringe filters (0.45 μm) and evaporated *in vacuo* at temperatures not exceeding 20°C. The residue was re-dissolved in acetone (5 –10 mL) and filtered again through syringe filters (0.22 μm) to furnish 9 mg (94%) of **6**-NHS. The active ester was stored under argon at –20 °C and used for immunolabeling experiments without further purification. Analytical data on **6**-NHS: t_R = 14 min (HPLC, A/B 70:30→0:100 in 25 min; detection at 636 nm; HPLC area above 85%). MS (ESI) *m*/*z* (positive mode, %) = 1015 (100%) $[M+Na]^+$; HRMS ($C_{49}H_{51}F_3N_4O_{11}S_2$): 1015.2835 (found M+Na), 1015.2840 (calc.). ^{1}H NMR (300 MHz, $CDCl_3$, *mixture of two amide rotamers with diastereotopic methyl and CH groups; the same is true for ^{13}C and ^{19}F*): δ = 1.46/1.49/1.52 (s×3, CH_3, 12 H), 1.61 (m, CH_2, 4 H), 2.04 (m, CH_2, 4 H), 2.15–2.35 (m, CH_2CO, 2 H), 2.73/2.78/2.80/2.81 (s×4, NCH_3 + $COCH_2CH_2CO$, 7 H), 2.95 (m, CH_2, 4 H), 3.09 (m, CH_2S, 2 H), 3.30–3.55 (m, CH_2N + CH_2SO_3, 4 H), 3.54 (m, CH_2N, 4 H), 4.15–4.40 ($\underline{CH_2}OH$, 4 H), 5.76 (m, 2 H), 6.78/6.81/6.96/7.00 (s×4, 2 H) ppm; ^{19}F NMR (282.4 MHz, $CDCl_3$): δ = –139.6 (dd, *J* = 25.4 and 14.7 Hz), –138.8 (dd, *J* = 25.2 and 14.2 Hz), –119.1 (dd, *J* = 25.3 and 5.1 Hz), –118.9 (dd, *J* = 25.3 and 4.6 Hz), –105.2 (dd, *J* = 14,7 and 3.9 Hz), –105.1 (dd, *J* = 13,5 and 3.6 Hz) ppm.

Phosphorylated rhodamine dyes



7-Me,All

7-R

R = H, Me, NHS

(NHS = *N*-succinimidyl)

diallyl N,N-diisopropylphosphoramidite
(phosphorylating reagent)

Fig. 3. Rhodamine dyes with phosphorylated CH_2OH sites.

Compound 7-Me,All. Crude methyl ester **5**-Me (see Fig. 2 for structure), obtained as described above (40 mg, containing ca. 0.027 mmol of **5**-Me), in abs. THF (7 mL) under an argon atmosphere was treated with the following reagents: diallyl *N*,*N*-diisopropylphosphoramidite (ALDRICH; 90 mg, 0.36 mmol, for structure see Fig 3.), 0.45 M 1*H*-tetrazole solution in acetonitrile (ALDRICH, 0.80 mL, 0.36 mmol) and, after additional 2 h stirring at room temperature, 5–6 M *t*-butyl hydroperoxide solution in *n*-octane (ALDRICH, 0.15 mL, 0.75 mmol). The progress of the reactions was monitored by TLC on silica plates with MeOH/CH_2Cl_2 (1:8; R_f for **5**-Me and **7**-Me,All were 0.4 and 0.6, respectively) as the mobile phase, and HPLC (see below). The stirring was continued for 1 h, the solution was diluted with CH_2Cl_2 (70 mL), the organic phase washed consecutively with water (50 mL), 0.05 M aq. HCl (50 mL), 2% aq. $NaHCO_3$ (50 mL), dried and chromatographed over a column with 25 g of silica gel and CH_2Cl_2/MeOH (15:1→5:1) as the mobile phase. Pure fraction were combined, filtered from SiO_2 through Rotilabo® syringe filters (0.22 μm) and evaporated to furnish 26 mg (87%) of phosphorylated compound **7**-Me,All as an amorphous dark blue solid. HPLC: t_R = 22 min (A/B 70:30→0:100 in 25 min; HPLC area 94%, detection at 636 nm). MS (ESI): *m*/*z* (positive mode, %) = 1109 (100%) $[M+H]^+$; HRMS ($C_{56}H_{64}F_4N_3O_{12}P_2$): 1108.3897 (found M+H), 1108.3896 (calc.). The compound was used in the next step without further purification.

Rhodamine dyes 7-Me **and 7**-H. *Cleavege of allyl protecting groups and saponification of the Me ester*.

Crude compound **7**-Me, All (25 mg, 22 μmol) in a mixture of anhydrous THF (25 mL) and CH_3CN (10 mL) was combined with the following reagents: Et_3N*HCOOH (0.5 M in THF, 0.50 mL, 0.25 mmol), formic acid (0.5 M in THF, 0.50 mL, 0.25 mmol HCOOH), triphenylphosphane (0.1 M in THF, 1.0 mL, 100 mmol Ph_3P) and *tetrakis*(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$, 18 mg, 0.016 mmol). The solution was stirred overnight at room temperature under an argon atmosphere and at 40 °C for approx. 40 min, until the reaction was complete. The reaction course was monitored by TLC on silica plates with CH_3CN/H_2O (5:1) as the mobile phase and HPLC (see below for details). The reaction mixture was cooled to room temperature and diluted with 8 mL of water (mixed with 1.25 mL 1 M aq. HCl). A dark blue precipitate dissolved, and the solution was evaporated to the volume of 5–7 mL *in vacuo* at room temperature (to remove most of the THF). The residue was diluted with water (5 mL) and CH_3CN (5 mL), acidified with an additional amount (0.6 mL) of 1 M HCl, and loaded onto a column with reverse-phase silica gel (Macherey-Nagel, Polygoprep 60-50 C_{18}, 20 g) and water as a mobile phase. The column was eluted consecutively with water (50 mL), 0.03 M aq. HCl (100 mL), water containing 0.1% (v/v) CF_3COOH (100 mL), and CH_3CN/H_2O (1:1) containing 0.1% CF_3COOH (150 mL). Pure fractions with compound **7**-Me were combined and evaporated. The residue was further purified over a column with regular silica gel (30 g). The column was first eluted with CH_2Cl_2/MeOH (4:1) to separate the non-colored impurities, then with CH_2Cl_2/MeOH/H_2O (5:5:1) containing 0.2% (v/v) Et_3N. Pure fractions were combined, filtered from SiO_2 through Rotilabo® syringe filters (0.45 μm) and evaporated to furnish 28.5 mg of rhodamine **7**-Me as a dark-blue amorphous material (presumably, as a salt containing 4

residues of Et_3N; yield 96%, considering MW = 1351). HPLC: t_R = 11 min (A/B 70:30→0:100 in 25 min; detection at 254 nm, HPLC area 97 %). MS (ESI): *m*/*z* (negative mode, %) = 946 (100%) $[M–H]^–$. HRMS ($C_{44}H_{47}F_4N_3O_{12}P_2$): 946.2648 (found M–H), 946.2644 (calc.).

Saponification: compound **7**-Me, obtained as described above (28 mg, 0.02 mmol) was dissolved in a mixture of water (20 mL), 1 M aqueous KOH solution (0.3 mL, 0.30 mmol KOH) and THF (5 mL) at room temperature upon sonication for a few minutes in an ultrasonic bath, and left overnight. The solution was acidified with trifluoroacetic acid (0.10 mL, 1.3 mmol) and evaporated to the volume of 10 mL *in vacuo* at room temperature. The solution (with a precipitate) was diluted with CH_3CN (5 mL) and loaded onto a column containing 12 g of reverse-phase silica gel (Polygoprep 60-50 C18) with water as a mobile phase. The column was first eluted with water (100 mL) to wash out the inorganic compounds, then the dye was washed out with CH_3CN/H_2O (1:1) containing 0.1 % (v/v) of CF_3COOH. Filtration (through Rotilabo® syringe filters 0.45 and 0.22 µm) and evaporation of the pure fractions (HPLC-control, detection at 254 nm) furnished 33 mg of a solid dark blue material. That was dissolved in 10 mL of water containing $NaHCO_3$ (13 mg, 0,16 mmol) upon sonication, filtered again through a syringe filter (0.45 µm), acidified with (125 µL, 1.6 mmmol) of CF_3COOH, left overnight at +5°C and centrifuged to furnish 14 mg (68%, over 2 steps from **7**-Me, All) of compound **7**-H (free acid) as a heavy dark blue crystalline solid. The dye is well-soluble in aq. $NaHCO_3$, alkali, Et_3N, and PBS buffers (pH 7–7.4), MeOH, DMF, DMSO, insoluble in acetone, CH_3CN, chlorinated solvents.

Analytical data on compound **7**-H: t_R = 8 min (HPLC, A/B 70:30→0:100 in 25 min) and 20 min (A/B 80:20→50:50 in 25 min) with detection at 254 nm; HPLC area 98%. ^{1}H NMR of **7**-H (600 MHz, CD_3OD, *mixture of two amide rotamers with diastereotopic methyl and CH groups*): δ = 1.32–1.41 (m, 2 H, $CH_2C\underline{H}_2CH_2$), 1.55/1.56/1.57/ (s×3, 12 H, CH_3), 2.07 (m, CH_2, 4 H), 2.72/2.84 (s×2, 3 H, NCH_3), 2.82 (m, 2 H, CH_2CO), 3.03 (m, 4 H, CH_2), 3.67 (m, CH_2, 4 H), 3.75 (m, CH_2N, 2 H), 4.57/4.59 (d×2 [$^3J(^1H–\ ^{31}P)$ = 4.7/5.2 Hz], 4 H, CH_2O), 5.98 (d, [$^5J(^1H–^{31}P)$ = 4.3 Hz], 2 H), 6.96/7.01 (s×2, $2H^{ar}$); ^{31}P NMR (121.5 MHz, CD_3OD): 0.30 (s) ppm. MS (ESI): *m*/*z* (negative mode, %) = 932 (100%) $[M–H]^–$; HRMS ($C_{43}H_{45}F_4N_3O_{12}P_2$): 932.2341 (found M–H), 932.2342 (calc.).

_N_-hydroxysuccinimidyl ester 7-NHS

In a typical experiment, a 5 mg sample (5.4 µmol) of dye **7**-H was dissolved upon sonication in DMF (3 mL) containing Et_3N (50 µL, 350 µmol) and treated with TSTU (50 mg, 166 µmol) under an argon atmosphere at r. t. After the reaction was complete (30–60 min), as determined by HPLC, the solution was carefully evaporated *in vacuo* (at r. t.) and the residue dissolved in CH_3CN/H_2O (10:1; 5 mL). The solution was acidified with CF_3COOH (3 µL; 39 µmol), loaded onto a column containing 3 g of SiO_2 and eluted first with CH_3CN/H_2O mixture (10:1→3:1) as the mobile phase to remove salts, *N*-hydroxysuccinimide, and other side-products. The main fraction containing compound **7**-NHS was washed out with CH_3CN/H_2O (2/3), containing 0.1 % (v/v) of Et_3N. Pure fractions were filtered (Rotilabo® syringe filters 0.45 and 0.22 µm), concentrated in vacuo at room temperature, and freeze-dried to furnish an amorphous heavy

dark blue powder (8 – 9 mg) that contained **7**-NHS and variable amounts of silica gel. This material was stored at –20 °C and used for immunolabeling without further treatment as a stock solution in DMF (also stored at –20 °C). The NHS ester is very slightly soluble in water and most organic solvents, except DMF, DMSO and mixtures CH_3CN/H_2O; well-soluble in aqueous PBS buffer (pH 7.4) and $NaHCO_3$ solutions. Analytical data for **7**-NHS: t_R = 9–11 min (HPLC, A/B 70:30→0:100 in 25 min; a broad unresolved peak or multiple peaks, due to the equilibrium between forms with different degrees of protonation); t_R = 24 min (with A/B 80:20→50:50 in 25 min; HPLC area ca. 90 %). MS (ESI): *m*/*z* (positive mode, %) = 1031 (100%) $[M+H]^+$, 1053 (40%) $[M+Na]^+$; HRMS ($C_{47}H_{48}F_4N_4O_{14}P_2$): 1031.2645 (found M+H), 1031.2651 (calc.).

Rhodamine dye 2b with two sulfonic acid groups

Compound 3a. Compound **1** was sulfonated to **3a** (see Fig. 1 for structures) according to the method which is very similar to the procedure used for the preparation of **2a**; see ref. [1]): the substrate (600 mg, 0.93 mmol) was placed in a 250 mL round bottom flask, which was then flushed with argon and cooled to –78°C (dry ice bath). Sulfuric acid (20 mL) was added through a syringe, the flask warmed up to room temperature and gently swirled until the solid had completely dissolved. The viscous red solution was left overnight at r. t. and poured in a 1 L beaker with a mixture of Et_2O (100 mL) and 1,4-dioxane (50 mL), which was chilled to – 10…–15°C before hand (until 1,4-dioxane started to crystallize). Additional 500 mL Et_2O and hexane (200 mL) was added, the solution well stirred and kept for 30 min at 0°C. The liquid was carefully decanted from the viscous dark oil, which solidified after trituration with Et_2O (3 × 50 mL). The crude material was chromatographed over SiO_2 (300 g) with CH_3CN/H_2O (6:1→5:1) as the mobile phase. Filtration (through Rotilabo® syringe filters 0.45 and 0.22 µm) and evaporation of the pure fractions furnished 595 mg (80%) of bronze-glittering crystalline dark blue material. Compound **3a** is well-soluble in water, alcohols, DMF, very slightly soluble in CH_3CN, acetone, and insoluble in chlorinated solvents. Analytical data for **3a**: t_R = 9 min (HPLC, A/B 80:20→50:50 in 25 min; HPLC area 98%). TLC on silica with MeOH/CH_2Cl_2 (1:3): R_f = 0.3; MS (ESI): *m*/*z* (negative mode, %) = 801 (100%) $[M–H]^–$. HRMS ($C_{38}H_{34}F_4N_2O_9S_2$): 801.1639 (found M–H), 801.1642 (calc.). ^{1}H NMR (300 MHz, CD_3OD): δ = 1.52/1.54 (s, 12 H), 2.03 (m, 4 H), 3.01 (t, J = 6.6 Hz, 4 H), 3.59–3.69 (m, 4 H), 3.60/4.05 (AB-system, J_{AB} = 14 Hz, 4 H), 5.86 (s, 2 H), 7.31 (s, 2 H) ppm; ^{13}C NMR (75.5 MHz, CD_3OD; *due to very low intensities, signals of the CF carbon atoms were not detected*): δ = 21.3 (CH_2), 21.6 (CH_2), 28.4/28.7 (CH_3×2), 54.5 (CH_2), 61.1 (CH_2), 107.0 (C), 115.2 (C), 122.2 (C), 124.5 (CH), 125.0 (C), 137.8 (CH), 152.2 (C), 155.0 (C) ppm; ^{19}F NMR (282.4 MHz, CDCl3): δ = –157.8 (dt, J = 21 and 6 Hz, 1 F), –155.5 (dt, J = 21 and 6 Hz, 1 F), –143.7 (br. m, 1 F), –138.2 (dd, J = 22 and 14 Hz, 1 F) ppm.

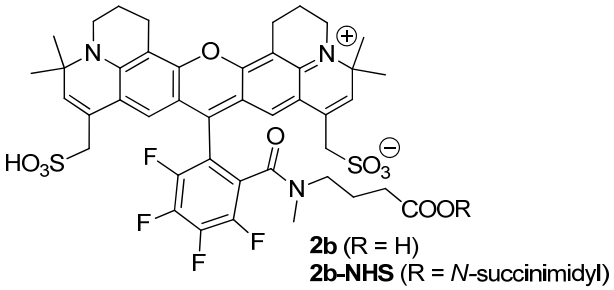


Fig. 4. Rhodamine dye with two sulfonic acid groups.

The amidation of the "sulfonated" dye **3a** was carried out as follows: compound **3a** (25 mg, 0.03 mmol) was dissolved in dry CH_3CN (30 mL) containing Et_3N (0.14 mL, 0.93 mmol) upon sonication at r. t. in an argon-flushed flask with a magnetic bar and septum. The solution was chilled to –7…–5°C by means of a mixture of brine and ice (approx.1:1). HATU reagent (98 mg, 0.26 mmol) in CH_3CN (2 mL) and methyl *N*-methyl-4-aminobutyrate hydrochloride (50 mg, 0.30 mmol) in CH_3CN (1 mL) were subsequently added through syringes upon stirring. The solution was stirred for 30 min at this temperature, and then left overnight at 0°C (ice bath). The crude methyl ester of **2b** was isolated by chromatography over a column with of SiO_2 (50 g) with CH_2Cl_2/MeOH (8:1→4:1) as a mobile phase; the reaction solution was quenched with an equal volume of the eluent and loaded straight onto a column. Pure fractions were pooled, evaporated, and the residue saponificated overnight at r. t. with a mixture of THF (10 mL) and H_2O (20 mL) containing KOH (32 mg, 0.56 mmol). The solution was acidified with CF_3COOH (0.15 mL, 2 mmol), evaporated to the volume of ca. 10 mL, and loaded onto a column with 10 g of reverse-phase silica gel (Polygoprep 60-50 C18) with water as a mobile phase. The column was first eluted with water (100 mL) to wash out all the inorganic compounds, then the elution was continued with CH_3CN/H_2O (1:4→1:1) containing 0.1 % (v/v) of CF_3COOH. Filtration through Rotilabo® syringe filters (0.45 and 0.22 μm) and freeze-drying of the pure fractions furnished 17 mg (63%) of a solid dark blue material. Compound **2b** (free acid, see Fig. 4 for structure) is well-soluble in water, alcohols, DMF, very slightly soluble in CH_3CN, acetone, and insoluble in chlorinated solvents. Analytical data for **2b**: t_R = 12 min (HPLC, A/B 80:20→50:50 in 25 min; HPLC area 98%; R_f = 0.3 (TLC on silica plates with MeOH/CH_2Cl_2 1:4). MS (ESI): *m*/*z* (positive mode, %) = 946 (100%) $[M+2Na]^+$, 924 (30%) $[M+Na]^+$; HRMS ($C_{43}H_{43}F_4N_3O_{10}S_2$): 924.2239 (found M+Na), 924.2218 (calc.).

^1H NMR (300 MHz, D_2O): δ = 1.55 (m, 2 H, CH_2), 1.70/1.74/1.78 (s×3, 12 H, CH_3), 2.12 (m, 4 H, CH_2×2), 2.72 (m, 2 H, CH_2CO), 2.91/2.99 (s×2, 3 H, NCH_3), 3.06 (m, 4 H, CH_2×2), 3.66 (m, 4 H, CH_2N×2), 3.77 (m, 2 H, CH_2N), 4.01/4.34 (AB-system, J_{AB} = 13 Hz, 4 H), 6.21/6.24 (s×2, 2 H), 7.38 (s, 2 H) ppm; ^{19}F NMR (282.4 MHz, $CDCl_3$): δ = –148.2 (dt, *J* = 21 and 6 Hz, 1 F), –146.7 (dt, *J* = 21 and 6 Hz, 1 F), –136.2 (dq, *J* = 17 and 6 Hz, 1 F), –133.5 (ddd, *J* = 22, 11, and 6 Hz, 1 F) ppm.

***N*-hydroxysuccinimidyl ester 2b-NHS**

In a typical experiment, TSTU reagent (2 mg, 6.6 μmol) was added to a solution of rhodamine **2b** (0.8 mg, 0.90 μmol) and Et_3N (50 μL of a 10% v/v solution, 35 μmol) in CH_3CN (1.5 mL) and

stirred 30 min under an argon atmosphere upon cooling to 0°C. The solution was loaded straight onto a column with 2.5 g SiO_2 and $CH_3CN/CH_2Cl_2/H_2O$ (10:1:1) as the mobile phase. The pure fractions were pooled, filtered (through Rotilabo® syringe filters 0.45 and 0.22 µm), and evaporated *in vacuo* at temperatures not exceeding 15°C. The residue was re-dissolved in water (2–3 mL) and freeze-dried. That afforded ca. 1 mg of a crystalline dark blue material (containing some SiO_2 as impurity). Compound **2b**-NHS is well-soluble in water, alcohols, DMF, very slightly soluble in CH_3CN, acetone, and insoluble in chlorinated solvents. Analytical data for **2b**-NHS: t_R = 15 min (HPLC, A/B 80:20→50:50 in 25 min; HPLC area 92%; R_f = 0.6 (TLC on regular silica with $MeOH/CH_2Cl_2$ 1:3; MS (ESI): *m*/*z* (positive mode, %) = 1043 (100%) $[M+2Na]^+$, 1021 (50%) $[M+Na]^+$; HRMS ($C_{47}H_{46}F_4N_4O_{12}S_2$): 1021.2393 (found M+Na), 1021.2382 (calc.).

Immunofluorescence labeling and mounting of the samples

For the preparation of cell samples, PtK2 cells were grown on cover slips. Cells were fixed with anhydrous methanol for 5 min at –20°C and blocked with 5% (w/v) BSA in PBS. Then the cells were incubated with a monoclonal mouse antiserum directed against the alpha-tubulin (Sigma-Aldrich, St. Louis, MO, USA). The primary antibodies were detected with secondary antibodies (sheep anti-mouse; Jackson ImmunoResearch Laboratories, West Grove; PA; USA) custom labeled with the fluorescent dyes. After several washing steps with PBS the samples were mounted in Mowiol. Staining and sample preparation were carried out according to the standard protocols, described by *C. A. Wurm et al.* [4].

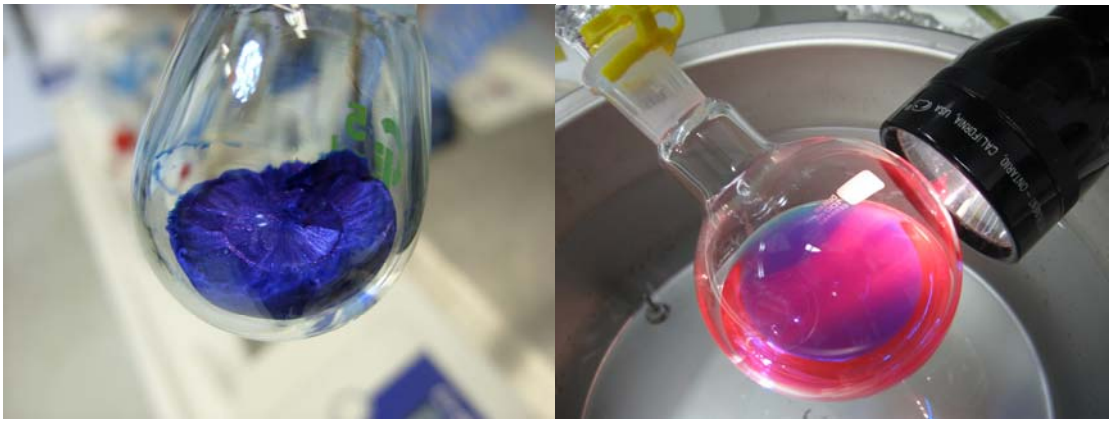


Fig. 5. A typical red-emitting dye (compound **2b**; see Fig. 4 for structure and Table 2 in the Main Text for the spectral properties). A dark blue solid (above) after freeze-drying and its blue aqueous solution with an intense red fluorescence, which is well-seen under an incandescent lamp light (below).

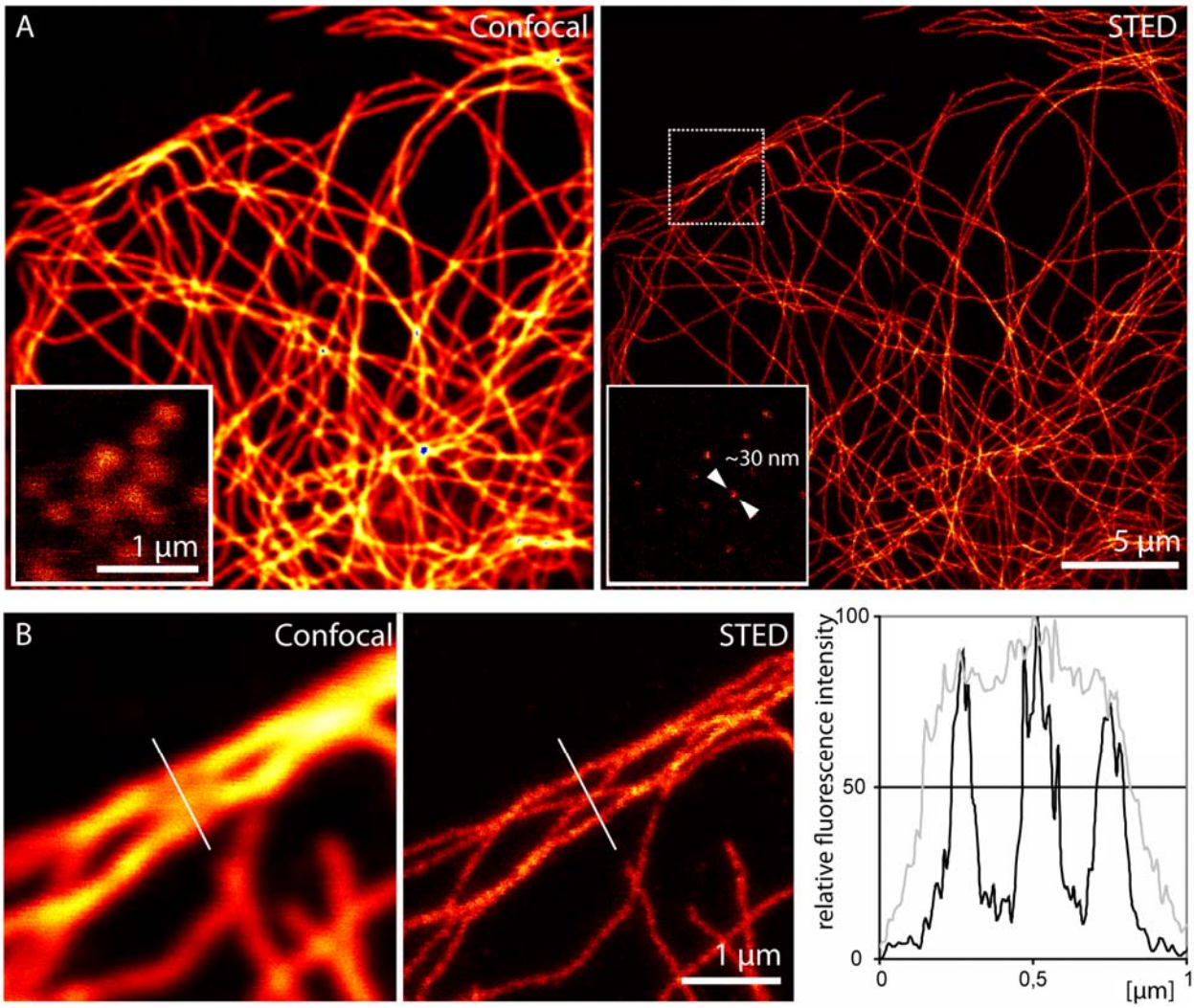


Figure 6. A. Confocal (left) and the STED (right) microscopy images of tubulin filaments in a fixed PtK2 cell. The tubulin skeleton was immunolabeled with compound **7**-NHS (Scheme 3 in this section; for spectral properties, see compound **7**-H in Table 2 of the main text); excitation with 635 nm diode laser, 17 µW. Detection at 670/40 nm; pulsed STED at 750 nm (STED power ~350 mW); optical resolution: ~300 nm (inset, left) vs. 30 nm (inset, right). **B**. Closeup of the marked region of **A**. The blurred tubulin filaments in the confocal image (left) can be distinguished by STED microscopy (right). This is further emphasized by the intensity profiles along the lines in the images.

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[1] K. Kolmakov, V. N. Belov, J. Bierwagen, C. Ringemann, V. Müller, C. Eggeling, S. W. Hell, *Chem. – Eur. J.* **2010,** *16*, 158–166 (*for details see Supporting Information therein*).

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