Supporting Information

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Photochromic Rhodamine Amides Provide Fluorescence Nanoscopy with Optical Sectioning

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Description of the synthesis.

Methyl [N-(4-nitrophthalimid)yl]acetate (2): To a solution of 4-nitrophthalimide 1 (ALDRICH, 2.00 g, 10.4 mmol) in anhydrous DMF (22 mL), was added tBuOK (1.17 g, 10.4 mmol) with stirring at –10 °C, and the mixture was vigorously stirred at 0 °C for 30 min. Methyl bromoacetate (0.96 mL, 10.4 mmol) was added dropwise, and the reaction mixture was heated at 40 °C for 24 h. The solvent was removed in vacuo and the residue was treated with CHCl₃ (100 mL) and water (100 mL). The organic layer was separated, dried with Na₂SO₄ and concentrated to give, after recrystallization from ether, 2.14 g (78%) of the title compound with m. p. 122 – 124 °C; lit. 1 124–125 °C (MeOH).

1H NMR (CDCl₃, 200 MHz, ppm), δ = 3.75 (s, 3 H, OMe), 4.46 (s, 2 H, CH₂N), 8.07 (d, 1 H, J = 8.5 Hz, H-6), 8.62 (dd, 1 H, J = 8.5 and 1.4 Hz, H-5), 8.66 (d, 1 H, J = 1.5 Hz, H-3).

13C NMR (CDCl₃, 50.3 MHz, ppm), δ = 39.1 (CH₂), 52.8 (OMe), 119.1 (CH), 125.0 (CH), 129.6 (CH), 133.5 (C), 136.4 (C), 152.0 (C-NO₂), 165.2 (CO), 165.5 (CO), 167.3 (CO).

Methyl [N-(4-aminophthalimid)yl]acetate (3): Palladium on charcoal (MERCK, 100 mg, 10 % Pd, oxidized form) was added to the solution of the compound 2 (500 mg, 1.9 mmol) in THF (50 mL). The reaction mixture was flushed with N₂ with vigorous stirring, and then was stirred under H₂ at room temp and ambient pressure for 4 h. TLC displayed full conversion of the starting compound into a new substance with lower Rf (hexane/EtOAc, 1/1), which gave a bright blue fluorescent spot (UV-detection at 254 nm). The reaction mixture was flushed with N₂, filtered through Celite®, and the filtrate was evaporated in vacuo to give the title compound as a green powder (386 mg, 86%), which was used in the next step without further purification. Analytical sample was recrystallized from EtOAc, m. p. 208 °C. 1H NMR ([D₆]DMSO, 300 MHz, ppm), δ = 3.67 (s, 3 H, OMe), 4.31 (s, 2 H, CH₂N), 6.45 (br. s, 2 H, NH₂), 6.84 (dd, 1 H, J = 8.5 and 1.5 Hz, H-5), 6.96 (d, 1 H, J = 1.4 Hz, H-3), 7.52 (d, 1 H, J = 8.5 Hz, H-6). 13C NMR ([D₆]DMSO, 75.5 MHz, ppm), δ = 38.3 (CH₂), 52.3 (OMe), 107.2 (CH), 116.3 (C), 116.9 (CH), 125.2 (CH), 134.3 (C), 155.2 (C-NO₂), 167.0 (CO), 167.5 (CO), 168.3 (CO). ESI-MS: m/z (rel. int., %) = 257 (100) [M + Na]+. Calcd. (%) for C₁₁H₁₀N₂O₄ (234.2): C 56.41, H 4.30, N 11.96; found: C 56.22, H 4.24, N 12.10.

Rhodamine B acid chloride (4) was prepared from rhodamine B hydrochloride (FLUKA, 1.1 g, 2.3 mmol) and POCl₃ (0.6 mL, 6.3 mmol) by refluxing in 1,2-dichloroethane (16 mL) for 4 h.² Volatiles were distilled in vacuo into a cold trap (dry ice – acetone bath), and the residue was dissolved in dry MeCN (23 mL).

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To the solution of 4 in acetonitrile (prepared from 1.1 g (2.3 mmol) of rhodamine B hydrochloride), amine 3 (540 mg, 2.3 mmol) was added followed by Et$_3$N (1.0 mL, 7.1 mmol), and the mixture was refluxed for 24 h under N$_2$. Volatiles were distilled in vacuo into a trap cooled with a dry ice – acetone bath, the residue was dissolved in toluene – CHCl$_3$ mixture (1:4, 150 mL), washed with sat. aq. NaHCO$_3$ (50 mL), and dried with MgSO$_4$. The solution was concentrated to ca. 15 mL in vacuo and applied carefully on top of the silica gel layer (100 mL SiO$_2$, MERCK, 0.040 – 0.063 mm), which was placed as a suspension in hexane – EtOAc mixture (1:1) into the glass filter. The product was eluted with EtOAc – hexane mixture (1:1) into the round-bottom flask made of dark brown glass. Its colorless spots on TLC (UV-detection at 254 nm) turn red in several minutes by exposing dry plates to normal laboratory light and air. Eluent was evaporated in vacuo, and the residue was taken-up in hexane and filtered to yield 1.3 g (84%) of the title compound with m. p. 236–237 °C (yellow solid). Chromatography was accompanied by partial decomposition.

**Lithium salt 5-Li:** Methyl ester 5-Me (362 mg, 0.550 mmol) was suspended in dry EtOAc (8 mL) and anhydrous Lil (204 mg, 1.50 mmol) was added. The reaction mixture was refluxed for 24 h and cooled to room temp. The insoluble solid (70 mg) was filtered through SiO$_2$ and evaporated in vacuo at 1.5 mbar and 40 °C (bath temp.). The solid residue in the flask was taken-up in CH$_2$Cl$_2$ (10 mL) and filtered through SiO$_2$ (50 mL) with EtOAc–hexane mixture (1:1) as an eluent. Yield – 64 mg (36%) of the title compound as glass-like brownish foam. Chromatography was accompanied by partial decomposition.

**N-Hydroxysuccinimidyl ester 5-NHS:** Lithium salt 5-Li (145 mg, 0.222 mmol) was suspended in dry DMF (1 mL) under Ar, and 2-succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate (120 mg, 0.399 mmol) was added. After stirring at room temperature for 2 h, a clear solution was obtained, which slowly turned red. One drop of Pr$_2$NET was added to decolorize the solution; DMF was carefully evaporated in vacuo at 1.5 mbar and 40 °C (bath temp.). The solid residue in the flask was taken-up in CH$_2$Cl$_2$ (10 mL) and filtered through SiO$_2$ (50 mL) with EtOAc–hexane mixture (1:1) as an eluent. Yield – 64 mg (36%) of the title compound as glass-like brownish foam. Chromatography was accompanied by partial decomposition.
NMR (CDCl₃, 600 MHz, gCOSY, ppm), δ = 1.17 (t, 12 H, J = 7 Hz, Me(CH₂N)), 2.79 (s, 4 H, CH₂CH₂), 3.31 (q, 2 H, J = 7 Hz, (Me)CH₂N), 4.68 (s, 2 H, CH₂N), 6.27 (dd, 2 H, J = 2 and 8 Hz, H-2'/7'), 6.31 (d, 2 H, J = 2 Hz, H-4'/5'), 6.57 (d, 2 H, J = 8 Hz, H-1''/8''), 7.12 (br. d, 1 H, J = 6.5 Hz, H-7), 7.34 (dd, 1 H, J = 7 and 1.6 Hz, H-5'''), 7.48 (dt, 1 H, J = 6.5 and 1.5 Hz, H-5), 7.51 (dt, 1 H, J = 6.5 and 1.5 Hz, H-4), 7.62 (d, 1 H, J = 6.8 Hz, H-6''), 7.74 (d, 1 H, J = 1.8 Hz, H-3'''), 7.99 (br. d, 1 H, J = 6.3 Hz, H-4). ¹³C NMR (CDCl₃, 125.7 MHz; C–H correlation [gHSQCAD] at 600 (150.8) MHz, ppm), δ = 12.5 (Me), 25.5 (CH₂CH₂), 36.5 (CH₂CO), 44.3 (CH₂N), 95.7 (CH, C-4'/5'), 105.4 (C, C-8a'/8b''), 108.4 (CH, C-2'/7'), 120.9 (CH, C-3''), 123.6 (CH, C-4), 123.9 (CH, C-7), 124.2 (CH, C-6''), 127.3 (C), 128.2 (CH, C-1'/8''), 128.4 (CH, C-5), 129.1 (C), 130.1 (CH, C-5'''), 132.2 (C), 133.7 (CH, C-6), 143.6 (C), 148.9 (C), 152.7 (C), 153.6 (C), 163.4 (CO), 166.1 (CO), 166.3 (CO), 168.19 (CO), 168.21 (CO). ESI-MS: m/z (rel. int., %) = 1505 (17) [2M + Na]⁺, 1483 (21) [2M + H]⁺, 742 (100) [M + H]⁺. C₄₂H₇₃N₂O₁₀s HR-MS (ESI, positive mode): 742.28706 [M + H]⁺ (found), 742.28714 (calculated).

N-Hydroxysulfosuccinimidyl ester 5-NHSS: Lithium salt 5-Li (65 mg, 0.1 mmol) and 22 mg (0.1 mmol) of N-hydroxysulfosuccinimid sodium salt (FLUKA) were suspended in DMF-d₇ (1 mL) under Ar, and HATU (38 mg, 0.1 mmol) was added with stirring. A weak exothermic reaction was observed, and the mixture became clear. It gradually turned red. The reaction mixture was stirred for 2 h at room temperature [δ = 1.10 (t, 12 H, J = 7 Hz, Me(CH₂N)), 2.67 (s, 12 H, N,N,N′,N′-tetramethylurea), 3.08 (dd, 1 H, A-part of ABX-system, J₆₅ = 18 Hz, J₆₅X = 2.8 Hz), 3.24 (dd, 1 H, B-part of ABX-system, J₆₅ = 18 Hz, J₆₅X = 8.3 Hz), 3.36 (q, 8 H, J = 7 Hz, (Me)CH₂N), 4.09 (dd, 1 H, X-part of ABX-system, Jₓₓ = 2.8 Hz, JₓₓX = 8.3 Hz), 4.83 (s, 2 H, CH₂N), 6.42 (m, 4 H, H-4'/5' and 2'/7'), 6.71 (d, 2 H, J = 8.9 Hz, H-1''/8''), 7.10 (dd, 1 H, J = 1.5 and 6.7 Hz, H-7), 7.53 (dd, 1 H, 7-aza-1-hydroxy-benzotriazole), 7.6–7.8 (m, 4 H, H-5/6/5''/6''), 7.87 (d, 1 H, J = 1.5 Hz, H-3'''), 7.97 (dd, 1 H, J = 7 and 1.5 Hz, H-4'), 8.52 (dd, 1 H, 7-aza-1-hydroxy-benzotriazole), 8.78 (dd, 1 H, 7-aza-1-hydroxy-benzotriazole) and compared with the ¹³C NMR spectra of the starting lithium salt (see above), N-hydroxysulfosuccinimid sodium salt (ABX-system: δₓ = 2.85, δₓ = 2.96, 3.85; 3.52 br. s of hydrate water) and HATU [3.28 (s, 6 H), 3.65 (s, 6 H), 8.08 (dd, 1 H), 8.60 (dd, 1 H), 8.90 (dd, 1 H)]. Two singlets of HATU (at 3.28 and 3.65 ppm) and two of the (three) one-proton multiplets (at 8.08 and 8.90 ppm) transformed into one singlet of N,N,N′,N′-tetramethylurea at 2.67 ppm and two one-proton multiplets at 7.53 and 8.78 ppm, respectively. All signals of the ABX-system in N-hydroxysulfosuccinimid shifted to lower field after acylation. Diagnostically important signals of the lithium salt 5-Li shifted significantly to lower fields: 3.95 to 4.83 ppm (s, 2 H, CH₂N), 7.73 to 7.87 ppm (d, 1 H, J = 1.5 Hz, H-3''), and 7.52–7.68 to 7.6–7.8 (m, 4 H, H-5/6/5''/6''). ESI-MS (negative mode): m/z = 820 [M⁻].

Cell culture and immunocytochemistry.

The mammalian PtK2 cell line was grown as described previously.³ Cells were seeded on standard glass coverslips to a confluency of about 80% and fixed with cold methanol (−20 °C) for 4 min, followed by incubation in blocking buffer (PBS containing 1% BSA). Human glioma cell line U373MG was grown in RPMI Glutamax high glucose, supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mM sodium pyruvate (Gibco). Cells were seeded and fixed as described above. All cells were grown at 37°C in a water-saturated atmosphere of 5% CO₂. Immunostaining of microtubules of PtK2 cells was performed with anti-β-tubulin mouse IgG (Sigma) and SpArh-conjugated sheep anti-mouse IgG, as primary and secondary antibody respectively. Nuclear lamina of U373MG cells was stained using anti-lamin A mouse IgG (Abcam)

and SpaRh-conjugated sheep anti-mouse IgG. All antibodies were diluted in blocking buffer and incubated for 1 h each, followed by several washes with blocking buffer.

Localization Algorithm.

Candidates for individual fluorophores were found in each CCD image (frame), by first unsharp masking the image and then identifying all objects greater than a threshold value (typically 5 photons). For each object a surrounding region of the image was cut out and passed to a centroid localization analysis. This procedure runs in form of a fixed-point iteration with the starting point $r_0$ set at the center of mass of the object. In each iteration, the center of mass $r_n$ of the data multiplied by a Gaussian (width = 270 nm) centered at the point $r_{n-1}$ is determined. Usually, the process converges after a few iterations and the position is tabulated together with the number of detected photons. A typical histogram of the number of photons detected per emitter (recognized events) is shown in Fig. S1. If after 100 iterations the path of $r_{100} - r_{99}$ is longer than 0.01 pixel $\approx$ 1 nm this is considered as a divergence and the corresponding object is not considered as an individual emitter and discarded. We obtained the final PALMIRA image by dividing the field of view into the desired number of pixels (typical pixel size of 20 nm). Each time an activated molecule was localized within an image, the corresponding pixel value was incremented by one. Note that this image assembly differs from that used by Betzig et al. In the latter the image is formed by plotting a Gaussian around each fitted position, with the standard deviation equaling the fit uncertainty.

Figure S1. Histogram of the number of photons detected per event for Figure 3c.

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