

Supplementary information

Far-red switching DNA probes for live cell nanoscopy

Jonas Bucevičius, Tanja Gilat and Gražvydas Lukinavičius*

Chromatin Labeling and Imaging group, Department of NanoBiophotonics, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Göttingen, Germany

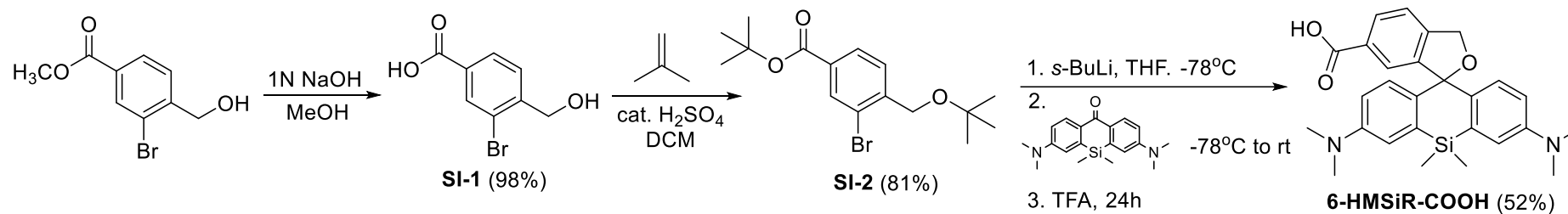
*e-mail: grazvydas.lukinavicius@mpibpc.mpg.de

Supplementary inventory

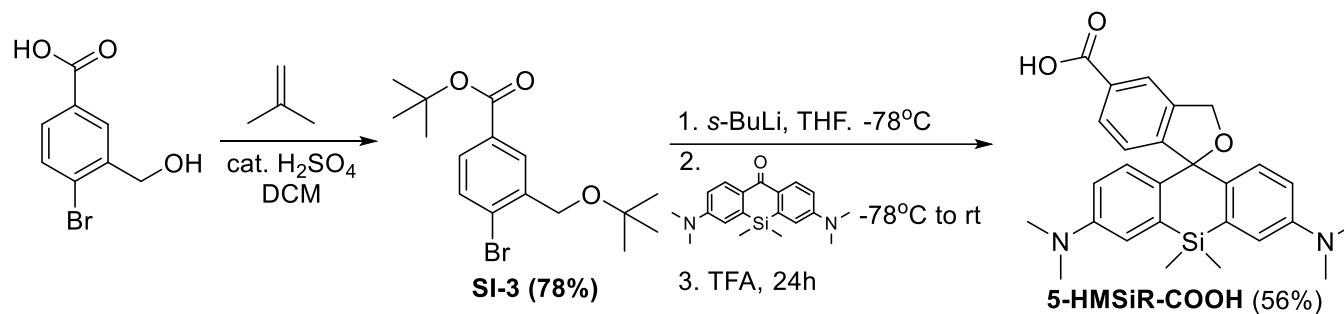
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Supplementary Schemes and figures

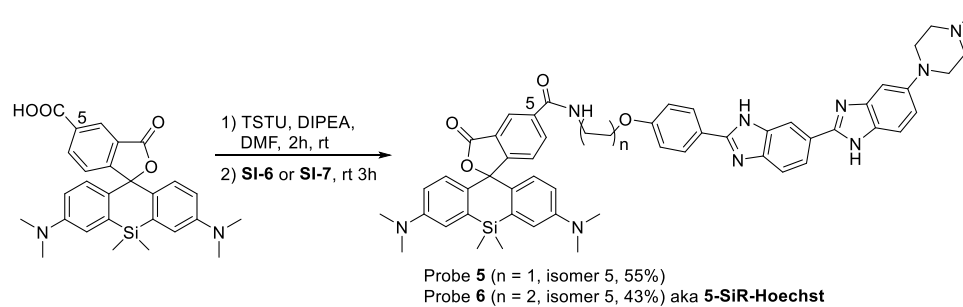
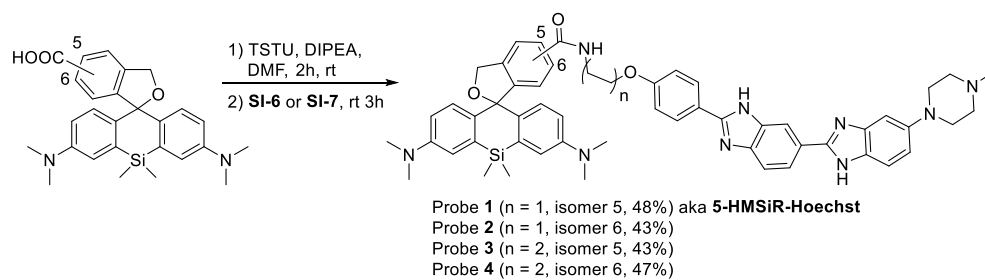
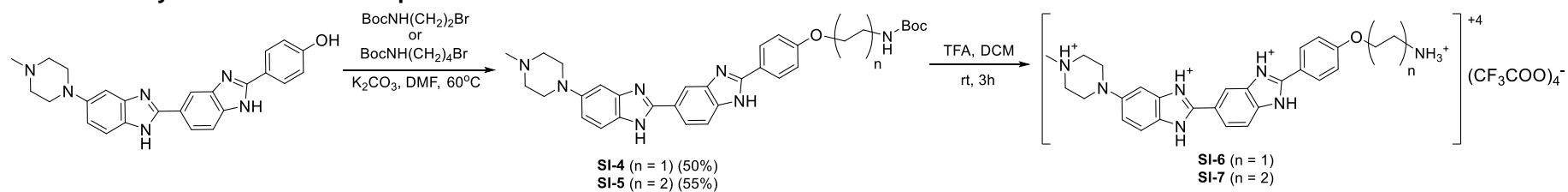
Scheme S1. Synthetic route of 6-HMSiR-COOH dye.



Scheme S2. Synthetic route of 5-HMSiR-COOH dye.



Scheme S3. Synthetic route of DNA probes.



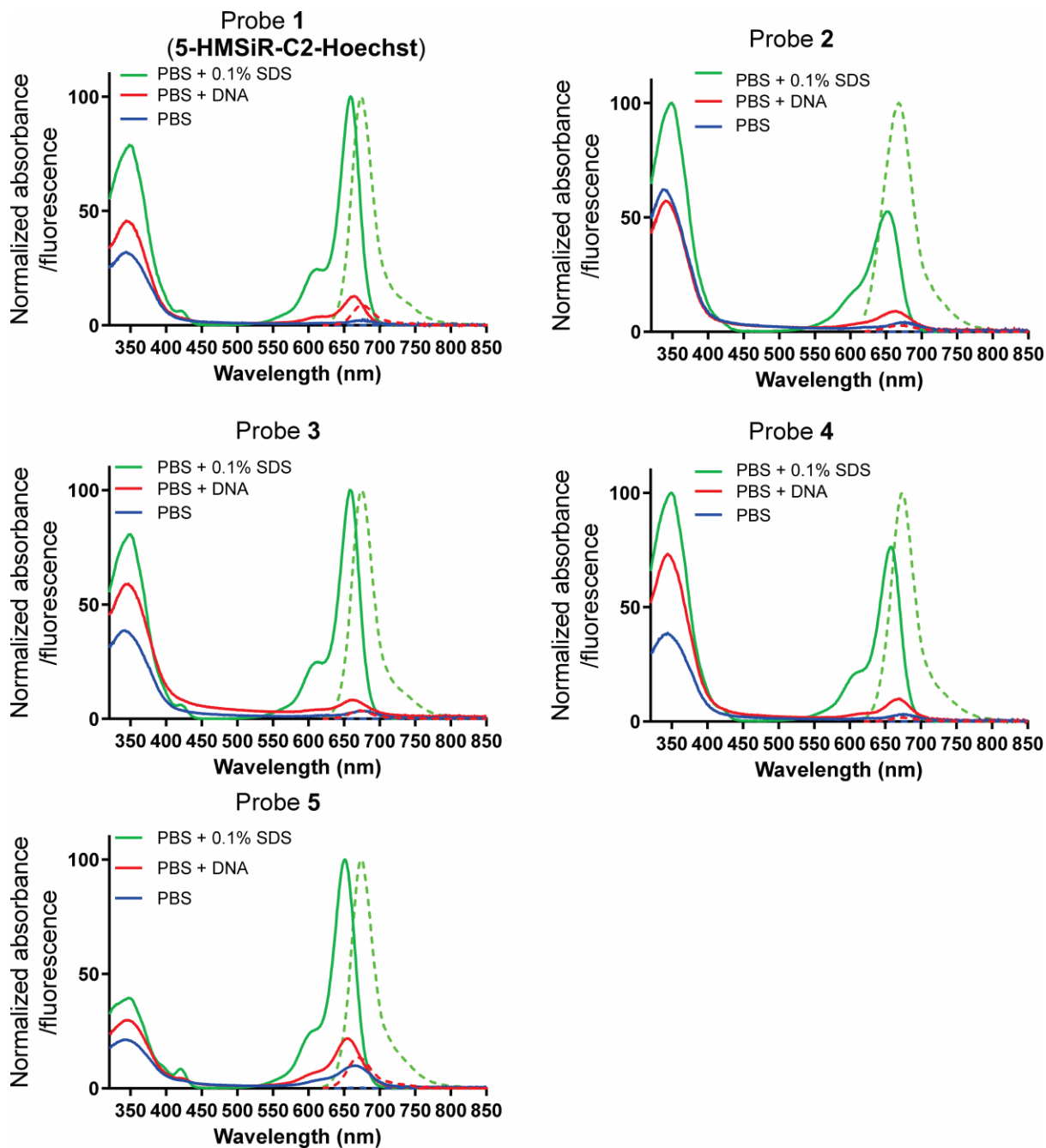


Figure S1. Spectral properties of the DNA probes 1-4. Absorbance (solid line) and fluorescence (dotted line) spectra of DNA probes 1-4 in PBS (blue), PBS containing 30 μM hpDNA oligonucleotide (red) and PBS containing 0.1% SDS (green). 10 μM probes incubated at room temperature for 3 h before measurements. Spectra normalized to the samples containing 0.1% SDS and presented as averages of three independent experiments.

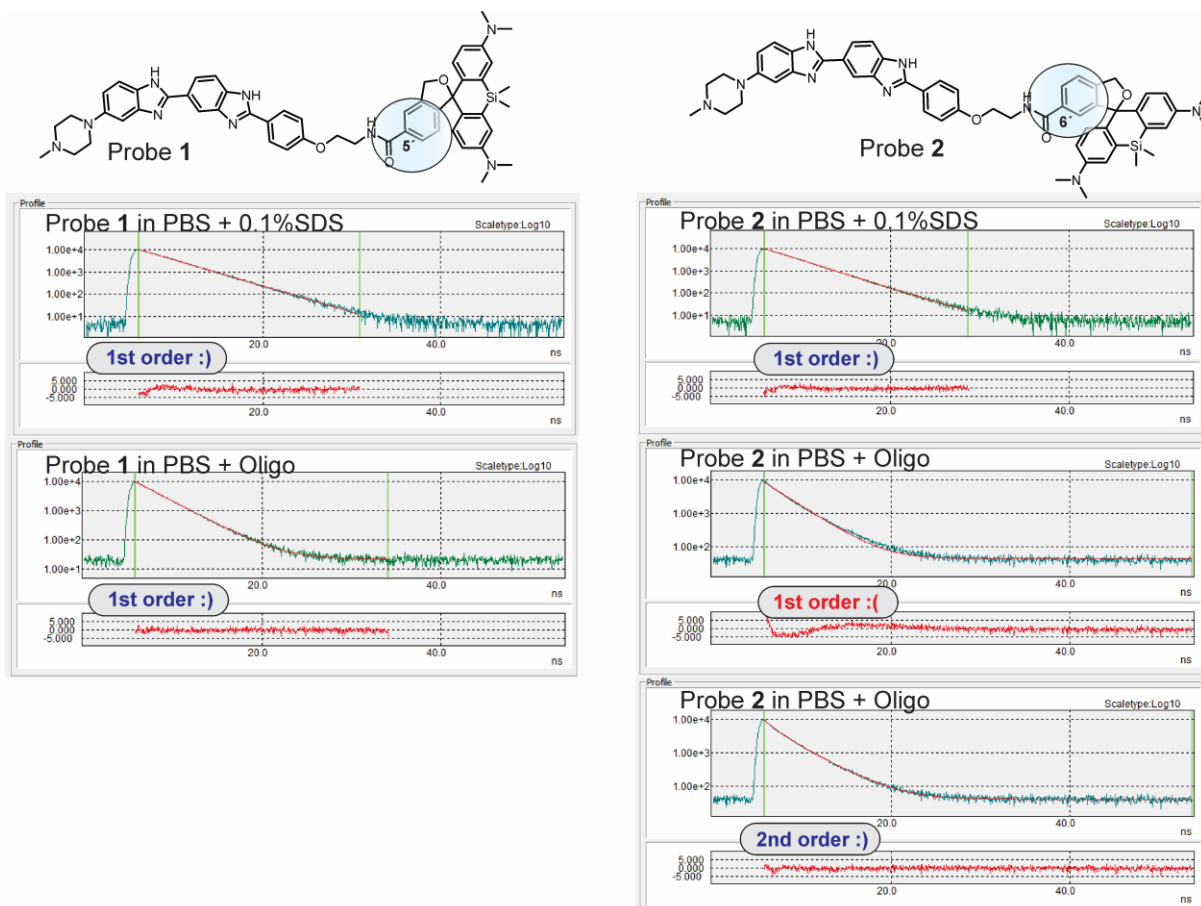


Figure S2. Fluorescence decay profiles of two HMSiR-Hoechst regioisomers. Fluorescence lifetime of probes 1 and 2 recorded in PBS + 0.1% SDS and PBS + DNA. Data points fitted to the first or second order exponential decay functions. The residuals from a fitting shown below each fitting.

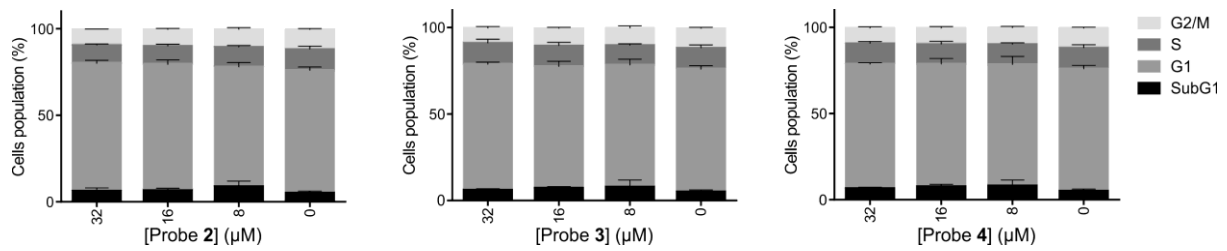


Figure S3. Cell cycle perturbation induced by HMSiR-based DNA probes. The cytotoxicity of DNA probes may result from the interference with DNA synthesis (S cell cycle phase) via topoisomerase inhibition. In addition, the probes can induce DNA damage responses because of the interference with protein-DNA interactions resulting in arrest at the G2 cell cycle phase. HeLa cells incubated with the indicated concentrations of the DNA probes at 37 °C for 24 h in a humidified 5% CO₂ incubator. Experimental data are averages of three independent experiments (N=3) and presented as means with standard deviations.

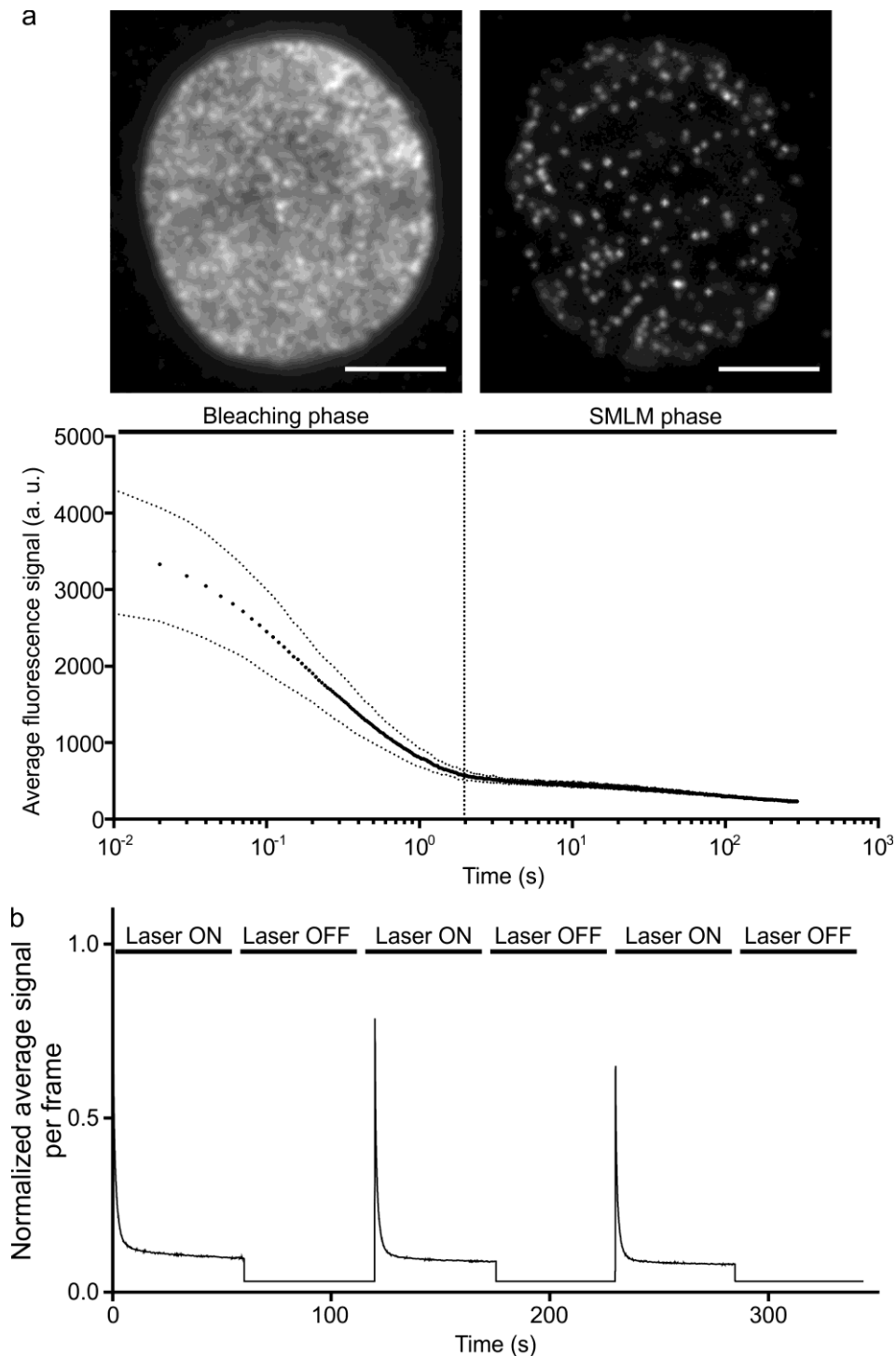


Figure S4. Two phases of TIRF SMLM data acquisition in living cells stained with HMSiR probe 1. **a.** Images show nuclear staining of living human fibroblast stained with 100 nM 5-HMSiR-Hoechst in DMEM + 10% FBS for 1h at 37°C. Initial several hundred frames can not be used for SMLM reconstruction because of the overlapping signals coming from single molecules. The exposure to excitation laser results in transition of the molecules into the dark state and allows efficient detection of single molecules. Data acquired at 100 Hz frame rate with excitation light intensity 18 kW/cm². Data presented as mean \pm s.d, N = 3 nuclei. **b.** Respective recovery of 5-HMSiR-Hoechst fluorescence in the nucleus of living human fibroblast stained with 100 nM probe in DMEM + 10% FBS for 1h at 37°C. Bleaching phase is followed by single molecule blinking phase. Fluorophores are recovering after laser is switched OFF. Data acquired at 100 Hz frame rate with excitation light intensity 18 kW/cm². Data presented as mean, N = 4 nuclei.

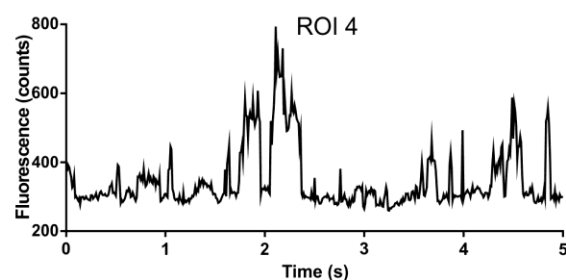
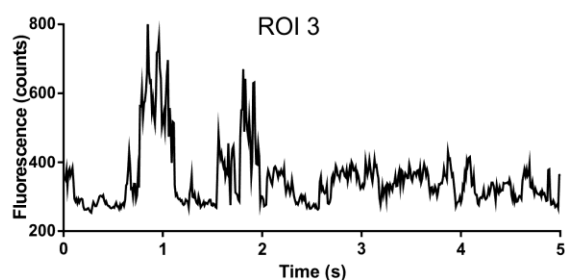
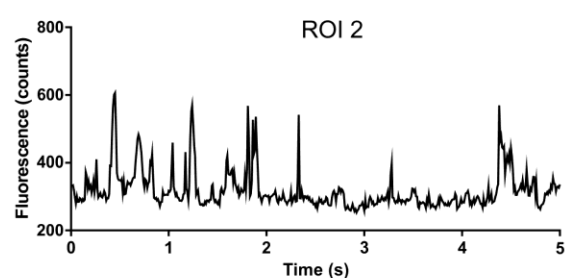
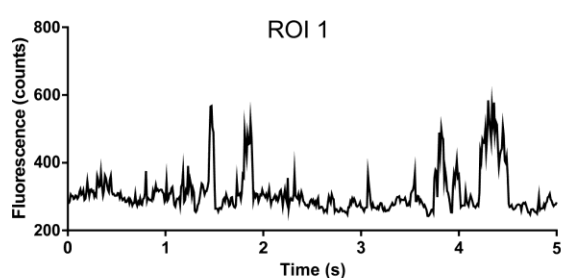
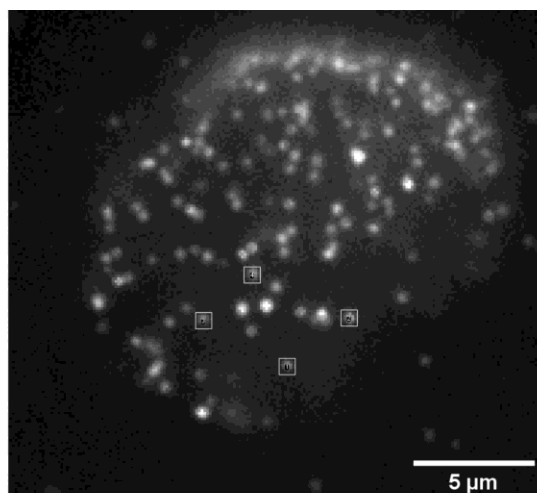


Figure S5. Single emitter intensity traces in the nucleus of the living cell stained with HMSiR probe 1. Fluorescence intensity measured in the marked regions of interest (ROI) which size is 6x6 pixels (660x660 nm). The ROIs are accommodating single emitter point spread function (PSF). Living human fibroblast stained with 100 nM 5-HMSiR-Hoechst in DMEM + 10% FBS for 1h at 37°C. Data acquired at 100 Hz frame rate with excitation light intensity 18 kW/cm².

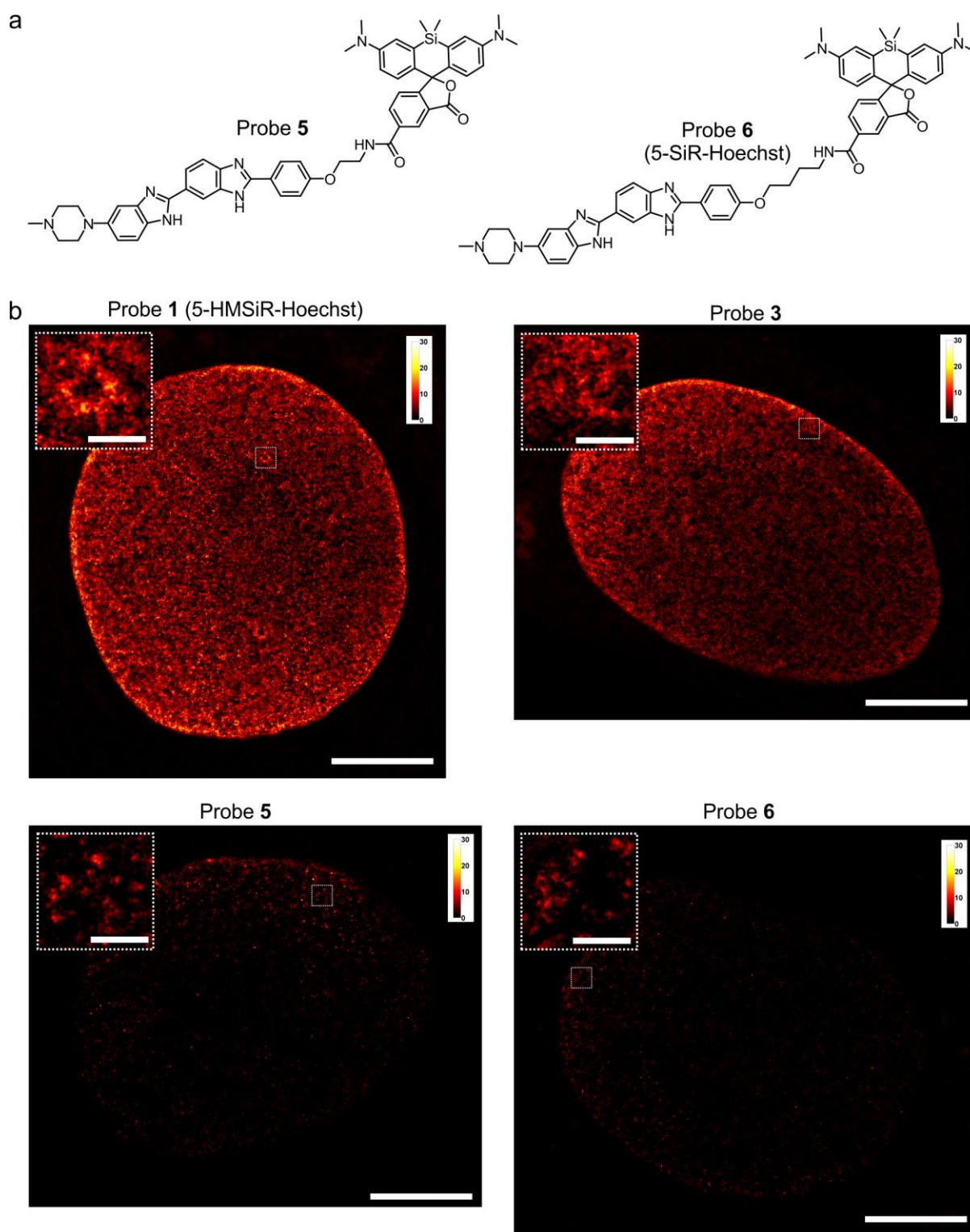


Figure S6. TIRF SMLM performance of DNA probes 1, 3, 5 and 6 in living human fibroblasts. **a.** Structures of probes 5 and 6. **b.** Reconstructions of the data acquired by imaging at 100 Hz excitation light intensity 18 kW/cm² of living human fibroblasts stained with the DNA probes at 100 nM in DMEM + 10% FBS at 37°C for 1h. No washing of the probes performed. Reconstructions performed using SVI Huygens localizer. Data set consisted of 30000 frames and initial ~300 frames removed from reconstructions because of the overlapping single emitters' signals. White dashed square marks the location of zoom-in image shown in the insert. Scale bars: large image - 5 μ m, inserts - 1 μ m.

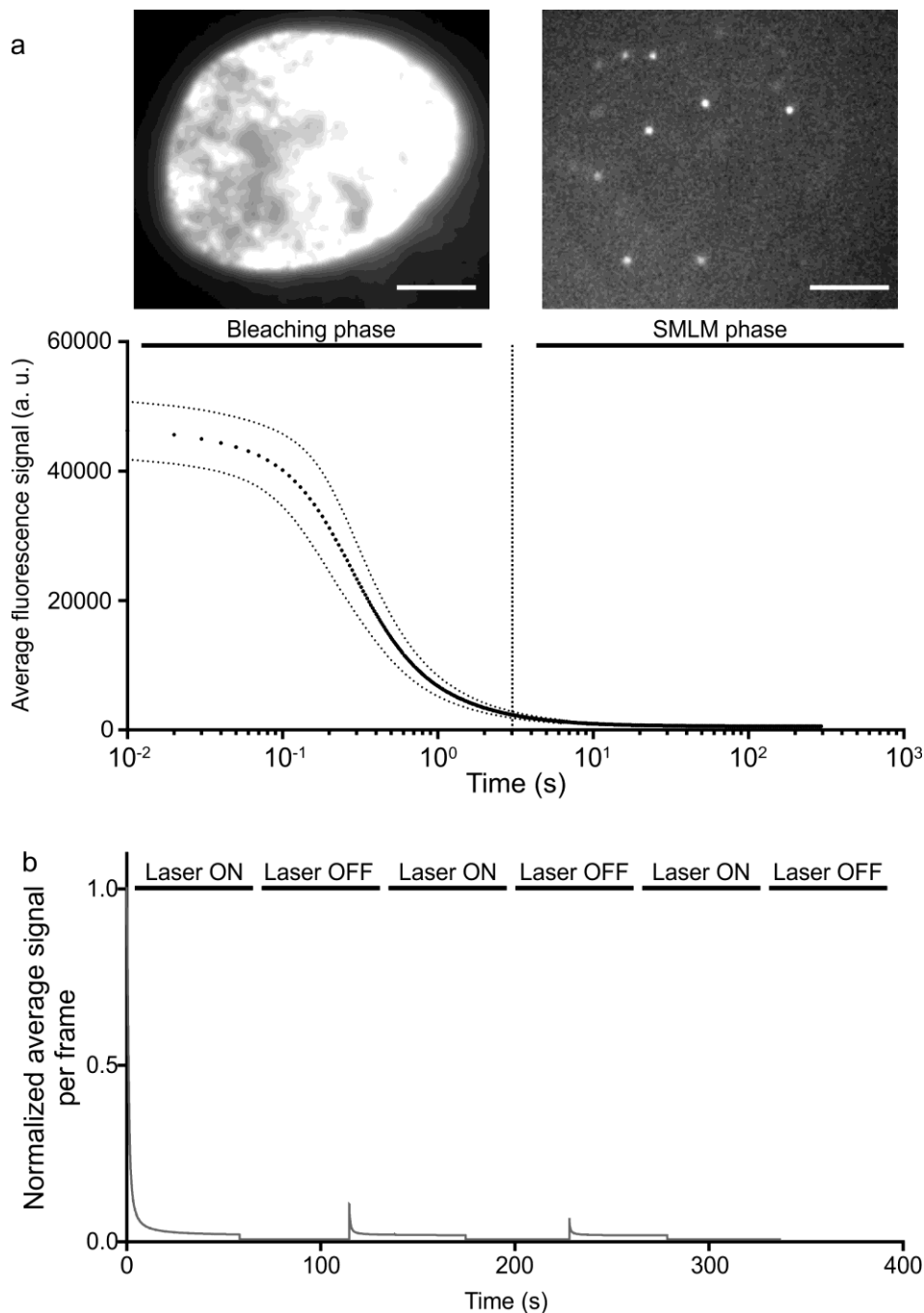


Figure S7. Two phases of TIRF SMLM data acquisition in living cells stained with SiR probe 5. **a.** Images show nuclear staining of living human fibroblast stained with 100 nM probe 5 in DMEM + 10% FBS for 1h at 37°C. Initial several hundred frames can not be used for SMLM reconstruction because of the overlapping signals coming from single molecules. The exposure to excitation laser results in transition of the molecules into the dark state and allows efficient detection of single molecules. Data acquired at 100 Hz frame rate with excitation light intensity 18 kW/cm². Data presented as mean \pm s.d, N = 3 nuclei. **b.** Respective recovery of probe 5 fluorescence in the nucleus of living human fibroblast stained with 100 nM probe in DMEM + 10% FBS for 1h at 37°C. Bleaching phase is followed by single molecule blinking phase. Fluorophores are recovering after laser is switched OFF. Data acquired at 100 Hz frame rate with excitation light intensity 18 kW/cm². Data presented as mean, N = 6 nuclei.

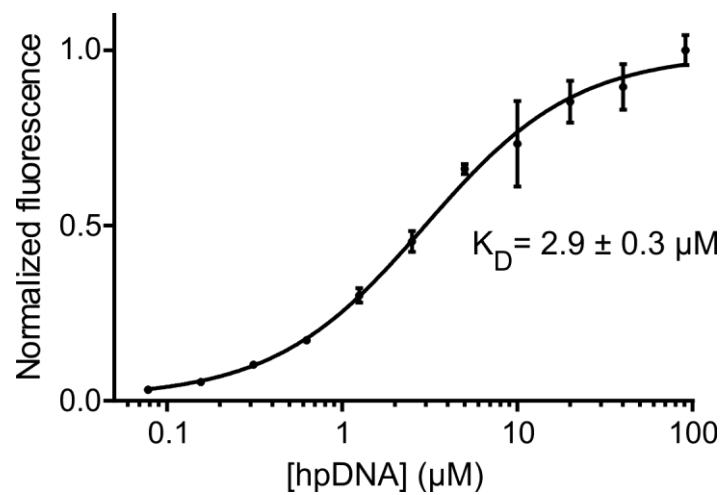


Figure S8. Determination of probe 5 and hpDNA complex K_D . Titration of 100 nM probe 5 (5-SiR-C2-Hoechst) with varying concentrations of hairpin DNA. Data points represent mean \pm s.d. measure in triplicates.

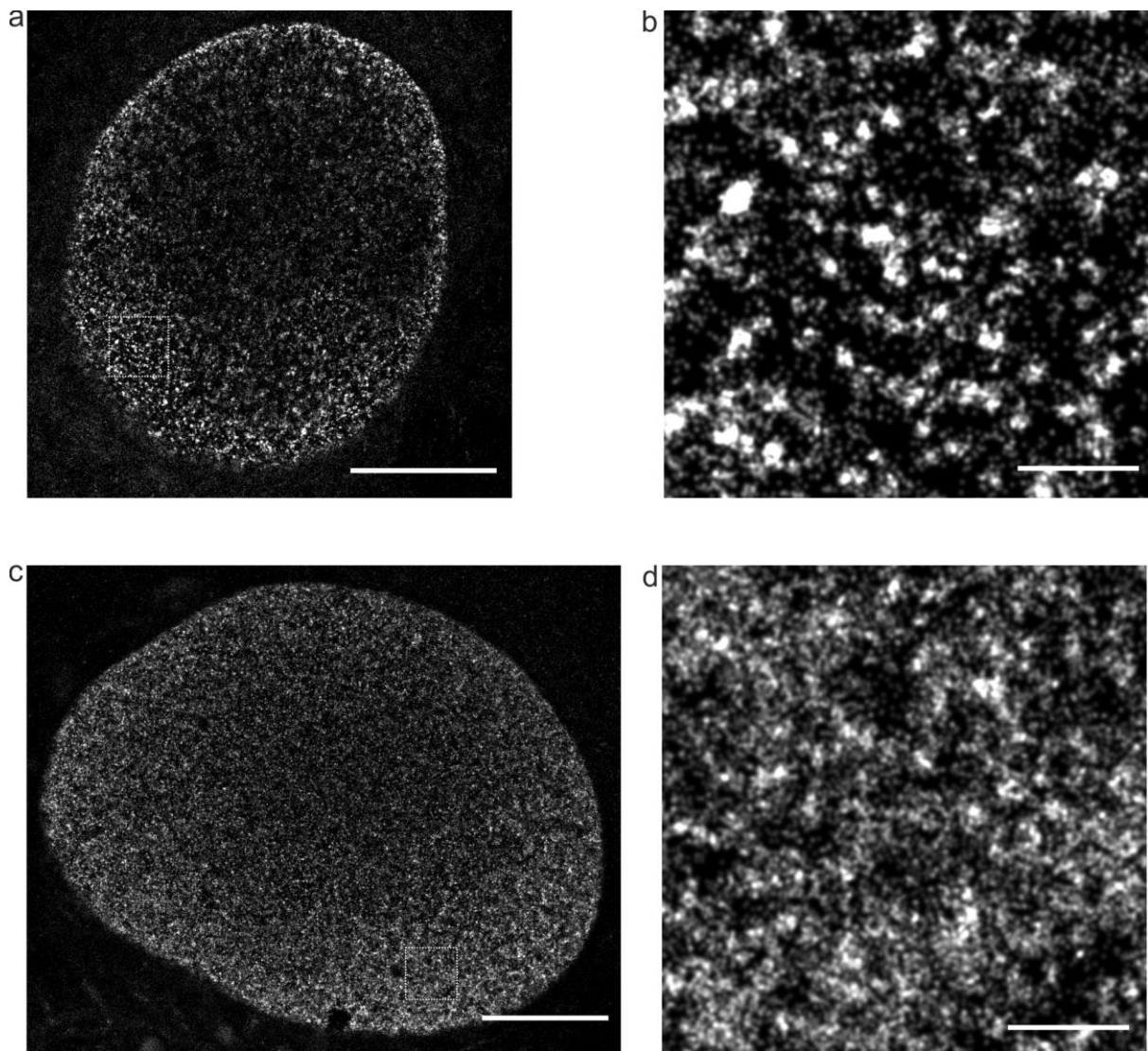


Figure S9. SMLM reconstruction images of living (a-b) HeLa or (c-d) U-2 OS cells stained with 100 nM 5-HMSiR-Hoechst. Staining performed in DMEM + 10% FBS at 37 °C for 1 h, no probe washing applied before imaging. Data acquired by imaging at 100 Hz with excitation light intensity 18 kW/cm². Reconstructions performed using SVI Huygens localizer. The data set consisted of 30000 frames and initial ~300 frames removed from reconstructions because of the overlapping single emitters' signals. Dashed line square indicates in panels a and c indicated position of zoom-in images shown in panels b and d. Scale bars: 5 μm in panels a and c, and 0.5 μm in zoom-in panels b and d.

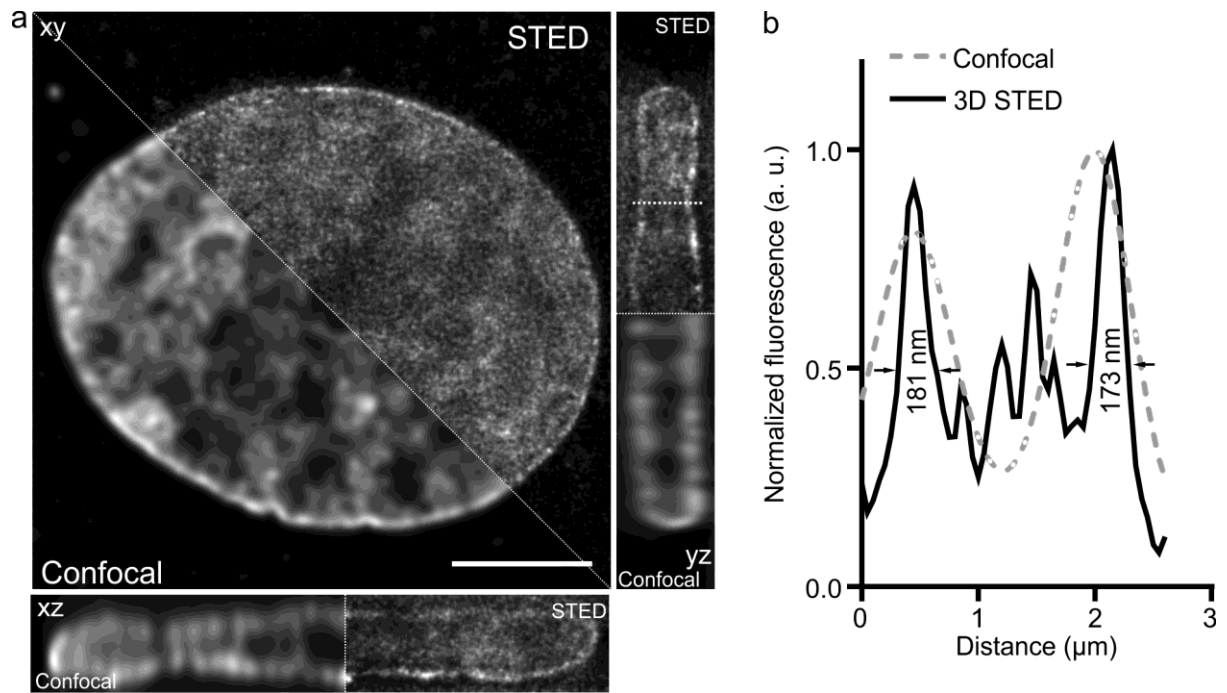


Figure S10. 3D STED nanoscopy image of living fibroblasts nucleus. **a.** Comparative confocal and 3D STED image of nucleus stained with $3 \mu\text{M}$ 5-HMSiR-Hoechst for 1 h. The image acquired without probe removal and is presented as xy, xz and yz planes. Scale bar: $5 \mu\text{m}$. **b.** Line profile of the chromatin region marked with dashed line in yz plane of panel (a). Numbers FWHM of the peripheral heterochromatin layer along z-axis.

Supplementary Movies

Video S1. Time-lapse series demonstratin switching behaviuor of 5-HMSiR-Hoechst in the of living nucleus. Living human fibroblasts stained with 100 nM probe for 1h at 37°C in DMEM + 10% FBS and no washing of the probe applied before imaging. Movie shows the first 5000 frames (50 s) acquired at 100 Hz with excitation light intensity 18 kW/cm².

Video S2. TIRF SMLM time-lapse series of the of human fibroblast nucleus stained with 100 nM 5-HMSiR-Hoechst. Living cells stained for 1h at 37°C in DMEM + 10% FBS and no washing of the probe applied before imaging. Each frame data set consisted of 10000 frames acquired at 100 Hz with excitation light intensity 18 kW/cm². Reconstruction performed with SVI Huygens Localizer.

Video S3. Rotating maximum intesity projection of human fibroblast nucleus stained with 3 µM 5-HMSiR-Hoechst. Living cells stained for 1h at 37°C in DMEM + 10% FBS and no washing of the probe applied before imaging. 3D STED image acquired using 50 x 50 x 50 nm voxel size and deconvolved with SVI Huygens Essentials package.

Supplementary Tables

Table S1. Photophysical properties of hydroxymethylsilarhodamine fluorescent dyes used in the study.

Dye	Solvent	λ_{max}^{abs} (nm)	λ_{max}^{em} (nm)	ϵ (m ⁻¹ cm ⁻¹) ^a	QY ^a	τ (ns) ^a
5-HMSiR-COOH	EtOH+0.1%TFA	655	674	119400 ± 7400	0.657 ± 0.04	3.46 ± 0.03
	PBS	651	669	3840 ± 40	0.411 ± 0.05	2.54 ± 0.01
	PBS + 0.1%SDS	657	675	63100 ± 850	0.581 ± 0.02	3.55 ± 0.03
6-HMSiR-COOH	EtOH+0.1%TFA	656	675	135900 ± 7500	0.599 ± 0.1	3.60 ± 0.02
	PBS	651	668	4850 ± 240	0.436 ± 0.02	3.59 ± 0.03
	PBS + 0.1%SDS	657	674	36500 ± 4000	0.530 ± 0.01	2.58 ± 0.01

Note: ^a Data presented as mean value with standard deviation, N = 3.

Table S2. Photophysical properties of DNA probes 1 - 6.

Probe	Solvent	λ_{max}^{abs} (nm) ^c	λ_{max}^{em} (nm)	ϵ (m ⁻¹ cm ⁻¹)	QY	τ (ns)	FL increase (fold)	Kd (μ M)	Brightness (M ⁻¹ cm ⁻¹)	Ref
1 (5-HMSiR-Hoechst)	PBS + hpDNA ^a	664	676	7750 ± 590	0.410 ± 0.01	2.71	420 ± 90	3.5 ± 0.3	3180 ± 320	This work
	PBS	672	676	1320 ± 60	<0.01	-	-	-	-	
	PBS + 0.1%SDS	659	674	61100 ± 4900	0.553 ± 0.01	3.63	-	-	-	
2	PBS + hpDNA ^a	664	670	5890 ± 120	0.189 ± 0.02	0.95 (49%) 2.93 (51%)	144 ± 39	5.9 ± 0.3	1110 ± 140	This work
	PBS	670	674	2630 ± 240	<0.01	-	-	-	-	
	PBS + 0.1%SDS	659	676	34300 ± 2250	0.497 ± 0.02	3.38	-	-	-	
3	PBS + hpDNA ^a	663	672	5440 ± 470	0.313 ± 0.01	2.53	55 ± 6	11.6 ± 0.8	1700 ± 200	This work
	PBS	670	676	2220 ± 240	<0.01	-	-	-	-	
	PBS + 0.1%SDS	659	674	66600 ± 6100	0.502 ± 0.007	3.61	-	-	-	
4	PBS + hpDNA ^a	668	674	1840 ± 110	0.139 ± 0.01	0.65 (59%) 2.55 (41%)	23 ± 3	9.5 ± 0.7	260 ± 34	This work
	PBS	670	674	5970 ± 600	<0.01	-	-	-	-	
	PBS + 0.1%SDS	659	676	46000 ± 3300	0.494 ± 0.01	3.50	-	-	-	
5	PBS + hpDNA ^a	654	670	21200 ± 360	0.349 ± 0.01	2.94	64 ± 3	2.9 ± 0.3	7400 ± 340	This work
	PBS	659	670	9400 ± 360	<0.01	-	-	-	-	
	PBS + 0.1%SDS	651	670	94000 ± 400	0.563 ± 0.01	3.98	-	-	-	
6 (5-SiR-Hoechst)	PBS + hpDNA ^b	651	672	38000 ± 3400	0.374 ± 0.006	2.99	53 ± 6	4.8 ± 0.2	14210 ± 1490	Previous work ¹
	PBS	659	671	13000 ± 300	0.007 ± 0.001	-	-	-	-	
	PBS + 0.1%SDS	651	673	101000 ± 2000	0.538 ± 0.004	3.93	-	-	-	
Hoechst 33258	PBS + hpDNA ^b	350	455	36000 ± 1600	0.823 ± 0.004	2.80	248 ± 17	2.6 *10 ⁻³ ± 4*10 ⁻⁴	32460 ± 5480	Previous work ¹
	PBS	339	508	31800 ± 1600	0.018 ± 0.001	-	-	-	-	
	PBS + 0.1%SDS	349	484	42000 ± 400	0.497 ± 0.006	2.15 (51%) 4.39 (49%)	-	-	-	

Note: ^a For the spectroscopy studies hairpin-forming oligonucleotide 5'-CGCGAATTCGCGTTTTTCGCGAATTCGCG used; probe concentration - 10 μ M in PBS buffer, oligonucleotide concentration - 30 μ M in PBS buffer; probes incubated for 2h in at room temperature before the measurements. ^b Probe concentration – 2 μ M, oligonucleotide concentration - 30 μ M; Data presented as mean value with standard deviation, N = 3. ^c All fluorophores display bathochromic shift after conjugation to the Hoechst targeting moiety which is due to the aggregation and can be reversed by addition of 0.1% SDS

Molecular biology, biochemical and microscopy methods

Preparation of hairpin DNA

For the DNA binding studies hairpin forming oligonucleotide 5'-CGCGAATTCGCGTTTTTCGCGAATTCGCG-3' (28 bp) purchased from Sigma-Aldrich. Previously, this hairpin DNA has been used for structural studies of the interaction of Hoechst 33342 with DNA². Synthetic oligonucleotides dissolved in PBS (Lonza, Cat. No. BE17-516F) at 1 mM concentration. Hairpin formed by putting the tube with hpDNA solution into boiling water bath, which slowly cooled down to room temperature.

Maintenance and preparation of cells

HeLa and U-2 OS cells purchased from American Type Culture Collection (ATCC). Adult human dermal fibroblasts purchased from Lonza. All cells cultured in high-glucose DMEM (Life Technologies, Cat. No. 31053-028) supplemented with GlutaMAX-1 (Life Technologies, Cat. No. 35050-038) and 10% foetal bovine serum (FBS, Life Technologies, Cat. No. 10270-106) in a humidified 5% CO₂ incubator at 37 °C. The cells split every 3-4 days or at confluence. Cells seeded in glass bottom 12-well plates (MatTek Corporation, Cat. No. P12G-1.0-14-F). Cells stained with the probes in DMEM (Thermo Fisher Scientific, Cat. No. 31053-028) supplemented with 10% FBS (Thermo Fisher Scientific, Cat. No. 10082139) at 37 °C and 5% CO₂. Afterwards, the cells washed 2 times with HBSS (Hanks' balanced salt solution, Lonza, Cat. No. BE10-527F). Imaging performed in DMEM with 10% FBS.

Cell cycle analysis by imaging flow cytometry

HeLa cells grown in 6-well plates (250.000 cells per well) for 24 h in the presence of the fluorescent probe in variable concentrations. The probes dissolved in DMSO to concentration corresponding to 1000 - 250x of final concentration in the growth media, the control samples prepared by accordingly adding 0.25 - 0.1% DMSO. We found that HeLa cells do not adhere strongly to the plastic bottom of the 6-well plate and thus the trypsination step could be omitted. The cells simply washed off and suspended in 1 ml of the growth medium by intensively pipetting up and down. Next, the cells processed according to the NucleoCounter® NC-3000™ two-step cell cycle analysis protocol. In particular, ~500,000 cells harvested by centrifuging at room temperature for 5 min at 400g. Afterwards, the cells resuspended in 250 µl lysis solution (Solution 10, Chemometec Cat. No. 910-3010) supplemented with 10 µg/ml DAPI (Solution 12, Chemometec Cat. No. 910-3012), incubated at 37 °C for 5 min. Then 250 µl of stabilization solution (Solution 11, Chemometec Cat. No. 910-3011) added. Cells counted on a NucleoCounter® NC-3000™ in NC-Slide A2™ slides (Chemometec, Cat. No. 942-0001) loaded with ~30 µl of each of the cell suspensions into the chambers of the slide. Each time, ~10,000 cells in total measured, and the obtained cell cycle histograms analysed with

ChemoMetec NucleoView NC-3000 software, version 2.1.25.8. All experiments repeated three times and the results presented as mean with standard deviations. The obtained mean values compared by running multiple t-tests on GraphPad Prism version 6.0 software.

TIRF SML microscope.

Wide-field and single molecule localization microscopy (SMLM) imaging was performed on an inverted microscope (Nikon Ti2) equipped with an ORBITAL-500 Series Visitron RingTIRF System (ver. 1.0). A 640 nm laser (Toptica, max. output power 200mW, measured at objective 140 mW) used for excitation of DNA probes was focused with the oil-immersion objective Apo TIRF AC 100x AC Oil NA 1.49 (MRD01996; Nikon). To increase the photon density we used a 6x beam expander in front of the laser (Visitron). The emission light detected using Prime 95B Scientific CMOS (sCMOS) camera (Photometrics) with 11 μm x 11 μm pixel area. The resulting effective pixel size is 110 x 110 nm. Typically, we acquired 30000 – 50000 images (10 ms per frame) with an excitation laser intensity of 18 kW/cm² (at maximum laser power) for a total acquisition time of 5 – 8 min for a single SMLM image. The intensity calculated for an area (~740 μm^2) estimated from a Gaussian function ($\sigma = 7.7 \mu\text{m}$) fitted to the emission profile of an evenly emitting probe that contained 95% of the illumination light. Acquisition performed using the VisiView software 4.5.0.6 (Visitron).

STED microscope with 775 nm laser

Comparative confocal and STED images acquired on an Abberior STED 775 QUAD scanning microscope (Abberior Instruments GmbH) equipped with 640 nm 40 MHz pulsed excitation lasers, a pulsed 775 nm 40 MHz STED laser, and an UPlanSApo 100x/1.40 Oil objective. The measured excitation laser intensity at focal plane was ~60kW/cm², STED laser intensity set to 4.7 MW/cm² and 90% of laser directed to the 3D donut. The 685 / 70 nm detection window used. 3D STED images acquired using pinhole set to 0.9 AU, voxel size set to 50 x 50 x 50 nm, 3D STED doughnut set to 90%, with single line accumulation and xzy scanning mode.

Processing, visualization and analysis of acquired images

All acquired or reconstructed images processed and visualized using Fiji³. Line profiles measured using the “straight line” tool with the line width set to 3 pixels.

For the signal measurements, image files converted to TIF file using Fiji and analyzed with CellProfiler 3.1.5 (ref.⁴), where the pipeline identified the nuclear region and measured the mean signal in this region. Background signal measured in the region, which is 3 pixels (450 nm) away from the nuclear border and 7 pixels (1050 nm) wide. The background subtracted signal processed with GraphPad Prism 6.

Deconvolution of 3D image z-stacks

Acquired 3D STED images processed with SVI Huygens Essential package. Drift correction applied before deconvolution. Estimated signal-to-noise ratio set to 5, deconvolution performed using GPU acceleration. Rotating projection generated using Fiji “3D project” function.

Reconstruction of SMLM images

All reconstructions performed using SVI Huygens localizer software. The peak fitting performed using maximum likelihood algorithm and GPU acceleration. Measured camera dark noise equal to 100 and detector PPU equal to 0.86. After reconstruction, objects filtered out if they are brighter than 50000 or have localization uncertainty bigger than 50 nm. Each localized emitter presented as 20 nm sized Gaussian spot with pixel size set to 5 nm. Drift correction applied at the last step before generating the final image.

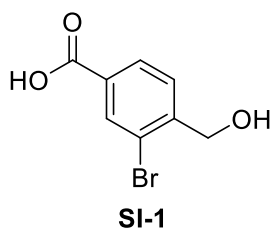
Estimation of chromatin domain FWHM

Acquired and reconstructed images were open with Fiji³ and line profiles of 3-pixel width drawn at several positions in the nuclear region. The obtained multiple peak-containing curve fitted to the corresponding number of Gaussian distributions using OriginPro 2015G 32-Bit. In order to avoid potential bias by defining chromatin domains, measurements and fitting performed on twelve different fields of view by four different persons. The statistics of fitted FWHM calculated with GraphPad Prism 6.

General experimental information and synthesis

NMR spectra recorded at 25 °C with an Agilent 400-MR spectrometer at 400.06 MHz (^1H) and 100.60 MHz (^{13}C), Bruker Avance III HD 500 spectrometer (av500) at 500.25 MHz (^1H) and 125.80 MHz (^{13}C), Varian INOVA 600 (I600) spectrometer at 599.74 MHz (^1H) and are reported in ppm. All ^1H and ^{13}C spectra are referenced to tetramethylsilane ($\delta = 0$ ppm) using the residual signals of the solvents according to the values reported in literature ⁵. Multiplicities of signals described as follows: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet or overlap of non-equivalent resonances; br = broad signal. Coupling constants (J) given in Hz. ESI-MS recorded on a Varian 500-MS spectrometer (Agilent). ESI-HRMS recorded on a MICROTOF spectrometer (Bruker) equipped with ESI ion source (Apollo) and direct injector with LC autosampler Agilent RR 1200. Liquid chromatography: Analytical HPLC performed on a Knauer Azura liquid chromatography system with a binary P 6.1L pump (Article No. EPH35, Knauer), UV diode array detector DAD 6.1L (Article No. ADC11, Knauer), an injection valve with a 20 μL loop and two electrical switching valves V 2.1S with 6-port multiposition valve head (Article No. EWA10, Knauer). Analytical columns: Knauer Eurospher II 100-5 C18, 5 μm , 150 \times 4 mm (Article No. 15DE181E2J, Knauer) or Interchim Uptisphere Strategy C18-HQ, 10 μm , 250 \times 4.6 mm (Article No. US10C18HQ-250/P46, Interchim), typical flow rate: 1.2 mL/min, unless stated otherwise. Preparative HPLC performed on an Interchim puriFlash 4250 2X preparative HPLC/Flash hybrid system (Article No. 115140, Interchim) with a 2 mL / 5 mL injection loop, a 200-600 nm UV-Vis detector and an integrated ELSD detector (Article No. 1A3640, Interchim). Preparative column: Eurospher II 100-5 C18 5 μm , 250 \times 20.0 mm (Article No.: 25PE181E2J, Knauer), typical flow rate: 25 mL/min, unless specified otherwise. Analytical TLC performed on Merck Millipore ready-to-use plates with silica gel 60 (F254) (Cat. No. 1.05554.0001). Flash chromatography performed on Biotage Isolera flash purification system using the type of cartridge and solvent gradient indicated. Methyl 3-bromo-4-(hydroxymethyl)benzoate and 4-bromo-3-hydroxymethylbenzoic acid purchased from chemPUR company.

3-Bromo-4-hydroxymethylbenzoic acid (SI-1)



Methyl 3-bromo-4-(hydroxymethyl)benzoate (600 mg, 2.45 mmol) dissolved in 15 mL of MeOH and 10 mL of 1N NaOH added. Reaction mixture stirred for 2 hours at 60 °C. The end of reaction monitored by TLC (1:1 EtOAc:Hexane). The reaction mixture cooled to room temperature, 30 mL of water added and pH adjusted to 2 by

addition of 4N HCl and the mixture extracted with EtOAc (3 \times 30 mL), The organic extracts combined and washed with water and brine, dried over Na_2SO_4 . The organic solvent

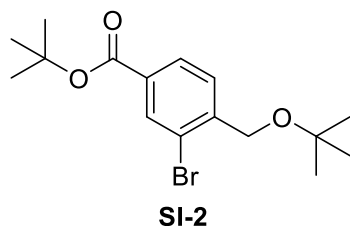
evaporated under reduced pressure and the products to furnish 555 mg (98% yield) of white solid.

^1H NMR (400 MHz, CD_3OD) δ 8.14 (d, $J = 1.5$ Hz, 1H), 8.00 (dd, $J = 8.1, 1.5$ Hz, 1H), 7.66 (d, $J = 8.0$ Hz, 1H), 4.69 (s, 2H).

^{13}C NMR (101 MHz, CD_3OD) δ 166.8, 145.6, 133.0, 131.0, 128.4, 127.4, 120.9, 63.1.

ESI-MS, negative mode: $m/z = 309.2 / 310.8$ $[\text{M}+\text{Br}]^-$.

tert-Butyl 3-bromo-4-(tert-butoxymethyl)benzoate (SI-2)

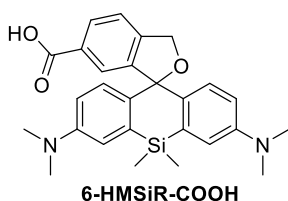


3-Bromo-4-hydroxymethylbenzoic acid (**SI-1**) (500 mg, 2.16 mmol) suspended in DCM (15 mL) in a sealable pressure glass tube. The mixture cooled in a NaCl/ice bath and ~5 mL of isobutylene gas condensed into the mixture. Catalytic amount of concentrated sulphuric acid (0.1 mL, 1.87 mmol) added to the stirred and cooled reaction mixture and the pressure tube tightly sealed. Reaction mixture stirred at room temperature for 48 h, during this time the suspension became a clear solution. Then reaction mixture cooled in ice bath and the tube carefully opened with vigorous release of pressure. The resulting solution poured to saturated NaHCO_3 solution (50 mL) and extracted with DCM (2 x 30mL). The organic extracts combined and washed with water and brine, dried over Na_2SO_4 . The product isolated by flash column chromatography (Teledyne Isco RediSep Rf 24 g, isocratic hold 10% of EtOAc in Hexane), fractions containing the product evaporated to give 600 mg (81%) of yellow oil.

^1H NMR (500 MHz, CDCl_3) δ 8.08 (d, $J = 1.7$ Hz, 1H), 7.90 (dd, $J = 8.0, 1.7$ Hz, 1H), 7.60 (d, $J = 8.1$ Hz, 1H), 4.49 (s, 2H), 1.56 (s, 9H), 1.28 (s, 9H).

^{13}C NMR (126 MHz, CDCl_3) δ 164.5, 143.9, 133.0, 132.0, 128.3, 128.1, 121.4, 81.4, 74.0, 63.4, 28.1, 27.6.

6-HMSiR-COOH:



To a solution of **SI-2** (500 mg, 1.46 mmol, 2.5 equiv) in THF (15 mL), cooled to -78°C , $s\text{-BuLi}$ (1.25 mL of 1.4 M in hexane, 1.75 mmol, 1.2 eq. in respect to bromide) added, and the mixture stirred at -78°C for 20 minutes. A solution of 3,7-bis(dimethylamino)-10,10-dimethyl-10-silaanthrone⁶ (190 mg, 0.584 mmol) in THF (20 mL) slowly added. After addition complete the mixture allowed to warm up to r.t. and stirred for 2 h. The resulting brownish solution cooled in ice-water bath, and acetic acid (1 mL) added. The dark blue reaction mixture evaporated on a rotary evaporator to a viscous residue, which

redissolved in TFA (10 mL), and the resulting dark orange solution stirred at rt for 24h. The TFA evaporated under reduced pressure and toluene-MeCN mixture added and solvents evaporated again. The cycle repeated for 5 times to get rid of all TFA. The residue suspended in DCM (with small amount of EtOAc) and deposited on celite by evaporating the solvent. Product isolated by flash column chromatography (Büchi Reveleris HP silica 40 g; gradient 20% to 100% Hexane with constant 1% v/v AcOH additive – EtOAc with constant 1% v/v AcOH additive and Büchi Reveleris HP silica 24 g; gradient 20% to 100% DCM:[DCM+10%MeOH] and lyophilised from 1,4-dioxane to furnish slightly bluish powder. Yield 140mg (52%).

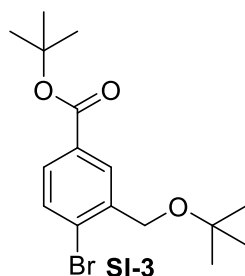
^1H NMR (600 MHz, CD_3OD) δ 8.22 (dd, $J = 8.1, 1.8$ Hz, 1H), 7.85 (d, $J = 8.1$ Hz, 1H), 7.73 (d, $J = 1.8$ Hz, 1H), 7.36 (d, $J = 2.8$ Hz, 2H), 7.02 (d, $J = 9.6$ Hz, 2H), 6.76 (dd, $J = 9.6, 2.8$ Hz, 2H), 4.36 (s, 2H), 3.34 (s, 12H), 0.62 (s, 3H), 0.60 (s, 3H).

^{13}C NMR (126 MHz, CD_3OD) δ 168.7, 167.6, 155.7, 149.3, 145.9, 142.0, 138.3, 131.2, 131.1, 130.7, 128.3, 128.2, 122.2, 115.3, 62.1, 40.9, -1.0, -1.3.

ESI-MS, positive mode: $m/z = 459.2$ $[\text{M}+\text{H}]^+$.

HRMS (ESI) calcd for $\text{C}_{27}\text{H}_{31}\text{N}_2\text{O}_3\text{Si}$ $[\text{M}+\text{H}]^+$ 459.2098, found 459.2099.

tert-Butyl 3-bromo-4-(tert-butoxymethyl)benzoate (SI-3)



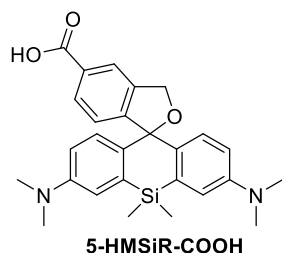
4-Bromo-3-hydroxymethylbenzoic acid (500 mg, 2.16 mmol) suspended in DCM (15 mL) in a sealable pressure glass tube. The mixture cooled in a NaCl/ice bath and ~5 mL of isobutylene gas condensed into the mixture. Catalytic amount of concentrated sulphuric acid (0.1 mL, 1.87 mmol) added to the stirred and cooled reaction mixture and the pressure tube tightly sealed. Reaction mixture stirred at room temperature for 48 h, during this time the suspension became a clear solution. Then reaction mixture cooled in ice bath and the tube carefully opened with vigorous release of pressure. The resulting solution poured to saturated NaHCO_3 solution (50 mL) and extracted with DCM (2 x 30mL). The organic extracts combined and washed with water and brine, dried over Na_2SO_4 . The product isolated by flash column chromatography (Teledyne Isco RediSep Rf 24 g, isocratic hold 10% of EtOAc in Hexane), fractions containing the product evaporated to give 575 mg (78%) of yellowish oil.

^1H NMR (400 MHz, CDCl_3) δ 8.14 (d, $J = 1.7$ Hz, 1H), 7.70 (dd, $J = 8.2, 1.7$ Hz, 1H), 7.54 (d, $J = 8.2$ Hz, 1H), 4.50 (s, 2H), 1.58 (s, 9H), 1.32 (s, 9H).

^{13}C NMR (101 MHz, CDCl_3) δ 165.2, 139.3, 132.2, 131.2, 129.9, 129.1, 127.1, 81.2, 73.9, 63.4, 28.1, 27.6.

NMR spectra corresponds to the previously reported data.⁷

5-HMSiR-COOH:



To a solution of **SI-3** (250 mg, 0.73 mmol, 2.5 equiv) in THF (10 mL), cooled to -78°C , *s*-BuLi (0.63 mL of 1.4 M in hexane, 0.88 mmol, 1.2 eq. in respect to bromide) added, and the mixture stirred at -78°C for 20 minutes. A solution of 3,7-bis(dimethylamino)-10,10-dimethyl-10-silaanthrone⁶ (95 mg, 0.29 mmol) in THF (20 mL) slowly added. After addition completed the mixture allowed to warm up to r.t. and stirred for 2 h. The resulting brownish solution cooled in ice-water bath, and acetic acid (1 mL) added. The dark blue reaction mixture evaporated on a rotary evaporator to a viscous residue, which redissolved in TFA (10 mL), and the resulting dark orange solution stirred at r.t. for 24h. The TFA evaporated under reduced pressure and toluene-MeCN mixture added and solvents evaporated again. The cycle repeated for 5 times to get rid of all TFA. The residue suspended in DCM (with small amount of EtOAc) and deposited on celite by evaporating the solvent. Product isolated by flash column chromatography (Büchi Reveleris HP silica 40 g; gradient 20% to 100% Hexane with constant 1% v/v AcOH additive – EtOAc with constant 1% v/v AcOH additive and Büchi Reveleris HP silica 24 g; gradient 20% to 100% DCM:[DCM+10%MeOH] and lyophilised from 1,4-dioxane to furnish slightly bluish powder. Yield 75 mg (56%).

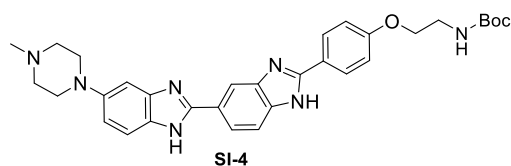
¹H NMR (400 MHz, CD₃OD) δ 8.41 (s, 1H), 8.11 (d, $J = 8.0$ Hz, 1H), 7.37 (d, $J = 2.9$ Hz, 2H), 7.28 (d, $J = 7.9$ Hz, 1H), 7.04 (d, $J = 9.6$ Hz, 2H), 6.78 (dd, $J = 9.6, 2.8$ Hz, 2H), 4.36 (s, 2H), 3.35 (s, 12H), 0.62 (s, 3H), 0.61 (s, 3H).

¹³C NMR (101 MHz, CD₃OD) δ 169.1, 167.9, 155.8, 149.4, 143.0, 142.1, 141.5, 132.8, 130.7, 129.5, 129.2, 128.1, 122.3, 115.3, 62.0, 49.0, 40.9, 40.4, 28.9, -1.1, -1.3.

ESI-MS, positive mode: $m/z = 459.2$ [M+H]⁺.

NMR spectra corresponds to the previously reported data.⁷

Tert-butyl (3-{4-[5-(4-methylpiperazin-1-yl)-1H,1'H-[2,5'-bibenzimidazol]-2'-yl]phenoxy}ethyl)carbamate (SI-4)



The free base of Hoechst 33258 prepared by dissolving commercial Hoechst 33258 trihydrochloride (100 mg, 0.187 mmol) in H₂O (10 mL) and adding a solution of K₂CO₃ (78 mg, 0.562 mmol, 3 eq.) in H₂O (10 mL). The formed precipitate isolated by filtration, washed with H₂O and lyophilised from water 1,4-dioxane mixture. The resulting Hoechst 33258 base (80 mg,

0.187 mmol, 1 eq.) suspended in dry DMF (1 mL). K_2CO_3 (78 mg, 0.562 mmol, 3 eq.) added followed by 4-(Boc-amino)ethyl bromide (63 mg, 0.28 mmol, 1.5 eq.). The reaction heated at 60 °C for 14 h. The reaction mixture cooled to room temperature and DMF evaporated under reduced pressure. The residue suspended in DCM and deposited on celite by evaporating the solvent. Product isolated by flash column chromatography (Büchi Reveleris HP silica 40 g; gradient 20% to 90% $CH_2Cl_2 - CH_2Cl_2:MeOH: NH_3(aq)$ [9:1:0.2]) as yellow solid, yield 53 mg (50%).

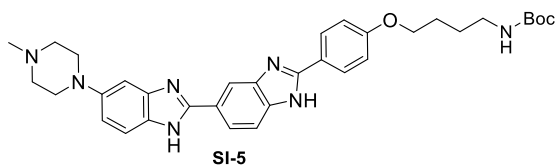
1H NMR (400 MHz, CD_3OD) δ 8.17 (s, 1H), 7.96 (d, $J = 8.8$ Hz, 2H), 7.87 (dd, $J = 8.4, 1.7$ Hz, 1H), 7.61 (d, $J = 8.4$ Hz, 1H), 7.46 (d, $J = 8.8$ Hz, 1H), 7.08 (d, $J = 2.2$ Hz, 1H), 7.05 – 6.95 (m, 3H), 4.00 (t, $J = 5.6$ Hz, 2H), 3.42 (t, $J = 5.6$ Hz, 2H), 3.21 – 3.14 (m, 4H), 2.68 – 2.60 (m, 4H), 2.35 (s, 3H), 1.45 (s, 9H).

^{13}C NMR (126 MHz, d_7 -DMF) δ 161.6, 157.3, 154.3, 149.4, 146.8, 145.9, 137.7, 137.0, 129.4, 126.3, 126.1, 124.2, 122.4, 121.6, 119.9, 117.7, 116.1, 112.5, 110.2, 79.1, 68.3, 56.6, 51.8, 46.8, 41.2, 29.2.

ESI-MS, positive mode: $m/z = 568.3$ $[M+H]^+$.

HRMS (ESI) calcd for $C_{32}H_{38}N_7O_3$ $[M+H]^+$ 568.3031, found 568.3030.

Tert-butyl (3-{4-[5-(4-methylpiperazin-1-yl)-1H,1'H-[2,5'-bibenzimidazol]-2'-yl]phenoxy}butyl)carbamate (SI-5)



The free base of Hoechst 33258 prepared by dissolving commercial Hoechst 33258 trihydrochloride (100 mg, 0.187 mmol) in H_2O (10 mL) and adding a solution of K_2CO_3 (78 mg, 0.562 mmol, 3 eq.) in H_2O (10 mL). The formed precipitate isolated by filtration, washed with H_2O and lyophilised from water 1,4-dioxane mixture. The resulting Hoechst 33258 base (80 mg, 0.187 mmol, 1 eq.) suspended in dry DMF (1 mL). K_2CO_3 (78 mg, 0.562 mmol, 3 eq.) added followed by 4-(Boc-amino)butyl bromide (57 mg, 0.24 mmol, 1.2 eq.). The reaction heated at 60 °C for 14 h. The reaction mixture cooled to room temperature and DMF evaporated under reduced pressure. The residue suspended in DCM and deposited on celite by evaporating the solvent. Product isolated by flash column chromatography (Büchi Reveleris HP silica 40 g; gradient 20% to 90% $CH_2Cl_2 - CH_2Cl_2:MeOH: NH_3(aq)$ [9:1:0.2]) as yellow solid, yield 61 mg (55%).

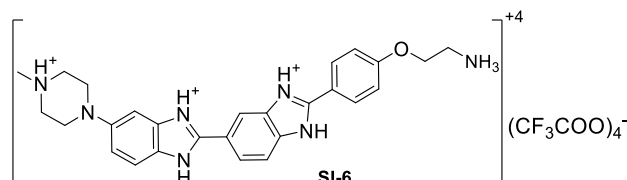
1H NMR (600 MHz, CD_3OD) δ 1.43 (s, 9H), 1.65 (p, $J = 7.2$ Hz, 2H), 1.80 (p, $J = 7.2$ Hz, 2H), 2.48 (s, 3H), 2.79 – 2.84 (m, 4H), 3.11 (t, $J = 7.0$ Hz, 2H), 3.23 – 3.33 (m, 4H), 4.03 (t, $J = 6.4$ Hz, 2H), 7.01 – 7.07 (m, 3H), 7.13 (d, $J = 2.3$ Hz, 1H), 7.50 (d, $J = 8.7$ Hz, 1H), 7.66

(d, $J = 8.4$ Hz, 1H), 7.92 (dd, $J = 8.4, 1.6$ Hz, 1H), 8.01 (d, $J = 8.8$ Hz, 2H), 8.22 (d, $J = 1.6$ Hz, 1H).

HRMS (ESI) calcd for $C_{34}H_{42}N_7O_3$ $[M+H]^+$ 596.3344, found 596.3340.

NMR and MS analytical data corresponds to previously published data.¹

4-{4-[5-(4-methylpiperazin-1-yl)-1H,1'H-[2,5'-bibenzimidazol]-2'-yl]phenoxy}butan-1-amine (SI-6)

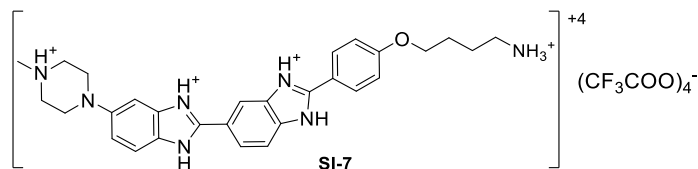


Trifluoroacetic acid (1 mL) added dropwise to a solution of **SI-4** (53 mg, 0.0935 mmol) in CH_2Cl_2 (4 mL). The resulting intense yellow solution stirred at room temperature for 3h. The reaction mixture evaporated to dryness and the residue re-evaporated three times with MeOH to remove excess trifluoroacetic acid. The residue lyophilized from aqueous dioxane. Product obtained as trifluoroacetic acid salt ($[Hoechst-(CH_2)_2NH_3]^{4+}[CF_3COO^-]_4$), with quantitative yield 86 mg (99%), yellow solid. Compound used in further step without additional purification.

ESI-MS, positive mode: $m/z = 468.3$ $[M+H]^+$.

HRMS (ESI) calcd for $C_{29}H_{30}N_7O$ $[M+H]^+$ 468.2506, found 468.2507.

4-{4-[5-(4-methylpiperazin-1-yl)-1H,1'H-[2,5'-bibenzimidazol]-2'-yl]phenoxy}butan-1-amine (SI-7)



Trifluoroacetic acid (1 mL) added dropwise to a solution of **SI-5** (61 mg, 0.103 mmol) in CH_2Cl_2 (4 mL). The resulting intense yellow solution stirred at room temperature for 3h. The reaction mixture evaporated to dryness and the residue re-evaporated three times with MeOH to remove excess trifluoroacetic acid. The residue lyophilized from aqueous dioxane. Product obtained as trifluoroacetic acid salt ($[Hoechst-(CH_2)_4NH_3]^{4+}[CF_3COO^-]_4$), with quantitative yield 97 mg (99%), yellow solid. Compound used in further step without additional purification.

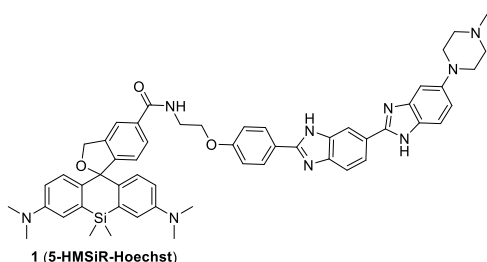
HRMS (ESI) calcd for $C_{29}H_{34}N_7O$ $[M+H]^+$ 496.2819, found 496.2820.

HRMS analytical data corresponds to previously published data.¹

General procedure for the Dye-Hoechst conjugates 1-5:

The **5-HMSiR-COOH** (for probes **1** and **3**) or **6-HMSiR-COOH** (for probes **2** or **4**) or **5-SiR-COOH**¹ (probes **5** and **6**) (0.02 mmol, 1 eq.), DIPEA (35 μ L, 0.2 mmol, 10 eq.), TSTU (9 mg, 0.031 mmol, 1.5 eq.) dissolved in 1 mL DMF and stirred at room temperature for 2 hours. A solution of **SI-6** or **SI-7** (0.03 mmol, 1.5 eq.) in 500 μ L DMF added to the reaction mixture and stirring continued for 3 h. Reaction monitored by HPLC analysis. The solvent evaporated *in vacuo* at room temperature and the products purified by preparative HPLC (preparative column: Eurospher 100 C18, 5 μ m, 250 \times 20 mm; solvent A: acetonitrile, solvent B: H₂O + 0.2% v/v HCOOH; temperature 25 $^{\circ}$ C, gradient A:B - 5 min 20:80 isocratic, 5-30 min 20:80 to 70:30 gradient) and lyophilised from 1,4-dioxane and water mixtures.

Probe 1 (5-HMSiR-Hoechst):



Obtained 8.7 mg of blue-green solid. Yield 48%.

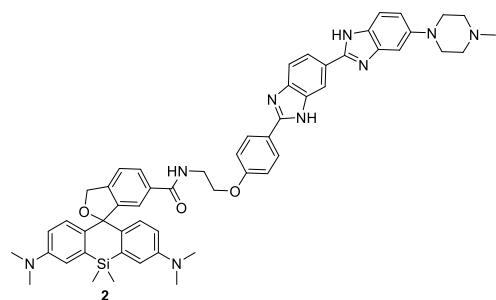
¹H NMR (400 MHz, CD₃OD + CF₃COOD) δ 10.13 (s, 1H), 9.82 – 9.74 (m, 4H), 9.63 (d, J = 8.8 Hz, 1H), 9.51 (d, J = 7.4 Hz, 1H), 9.33 (d, J = 9.1 Hz, 1H), 9.14 (d, J = 7.3 Hz, 1H), 9.01 (dd, J = 9.1, 2.2 Hz, 1H), 8.93 – 8.90 (m, 4H), 8.83 (d, J = 8.0 Hz, 1H), 8.60 (d, J = 9.6 Hz, 2H), 8.31 (dd, J = 9.7, 2.7 Hz, 2H), 5.97 (t, J = 5.4 Hz, 2H), 5.93 (s, 2H), 5.55 (d, J = 13.3 Hz, 2H), 5.48 (t, J = 5.1 Hz, 2H), 5.26 (d, J = 11.7 Hz, 2H), 4.90 (s, 12H), 4.85 – 4.80 (m, 2H), 4.80 – 4.71 (m, 2H), 4.58 (s, 3H), 2.17 (s, 3H), 2.16 (s, 3H).

¹³C NMR (101 MHz, CD₃OD + CF₃COOD) δ 169.9, 168.0, 165.1, 159.2, 155.8, 154.4, 151.0, 149.5, 149.4, 142.1, 141.8, 141.5, 136.2, 134.9, 134.5, 131.5, 130.7, 128.2, 127.9, 127.3, 126.9, 126.1, 122.3, 121.5, 119.9, 117.5, 117.2, 116.5, 115.7, 115.2, 115.0, 101.0, 62.1, 54.6, 48.4, 43.6, 40.9, 40.5, 30.9, -1.1, -1.3.

ESI-MS, positive mode: m/z = 908.5 [M+H]⁺.

HRMS (ESI) calcd for C₅₄H₅₈N₉O₃Si [M+H]⁺ 908.4426, found 908.4420.

Probe 2:



Obtained 7.8 mg of blue-green solid. Yield 43%.

¹H NMR (400 MHz, CD₃OD) δ 8.54 (s, 1H), 8.20 (dd, J = 8.6, 1.4 Hz, 1H), 8.15 (d, J = 8.9 Hz, 2H), 8.08 (d, J = 6.7 Hz, 1H), 8.03 (d, J = 8.7 Hz, 1H), 7.84 (d, J = 8.0 Hz, 1H), 7.74 (d, J = 9.1 Hz, 1H), 7.64 (s, 1H), 7.42 (dd, J = 9.1, 2.2 Hz, 1H), 7.36 – 7.32 (m, 3H), 7.27 (d, J = 8.7 Hz, 2H), 7.03 (d, J = 9.6 Hz, 2H), 6.74 (dd, J = 9.6, 2.6 Hz, 2H), 4.35 (s, 2H), 4.32 (t, J = 5.4 Hz, 2H), 3.96 (d, J =

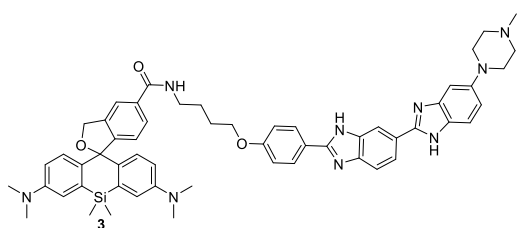
12.7 Hz, 2H), 3.83 (t, $J = 5.5$ Hz, 2H), 3.68 (d, $J = 11.7$ Hz, 2H), 3.32 (s, 12H), 3.27 – 3.18 (m, 4H), 3.00 (s, 3H), 0.60 (s, 6H).

^{13}C NMR (101 MHz, CD_3OD) δ 169.4, 167.9, 165.2, 160.9, 160.5, 160.1, 159.7, 155.8, 154.2, 152.9, 151.0, 149.5, 149.4, 142.2, 134.5, 131.5, 129.2, 128.9, 128.5, 128.3, 127.9, 126.2, 122.3, 121.7, 121.0, 120.0, 118.2, 117.2, 116.5, 115.7, 115.3, 115.3, 115.0, 114.6, 112.5, 101.0, 68.1, 68.1, 54.6, 48.4, 46.8, 43.6, 40.9, 40.4, -1.2.

ESI-MS, positive mode: $m/z = 908.4$ $[\text{M}+\text{H}]^+$.

HRMS (ESI) calcd for $\text{C}_{54}\text{H}_{58}\text{N}_9\text{O}_3\text{Si}$ $[\text{M}+\text{H}]^+$ 908.4426, found 908.4419.

Probe 3:



Obtained 8.0 mg of blue-green solid. Yield

43%.

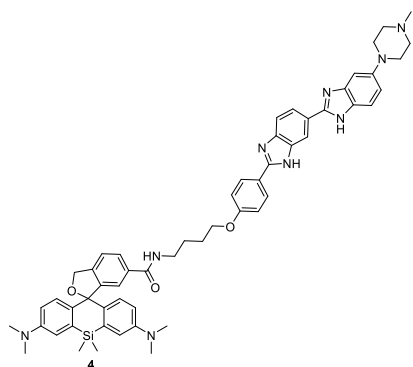
^1H NMR (400 MHz, CD_3OD) δ 8.44 (d, $J = 1.7$ Hz, 1H), 8.23 (s, 1H), 8.16 – 8.09 (m, 3H), 7.94 (d, $J = 8.6$ Hz, 1H), 7.91 (d, $J = 7.7$ Hz, 1H), 7.73 (d, $J = 9.0$ Hz, 1H), 7.41 (dd, $J = 9.1, 2.2$ Hz, 1H), 7.34 (dd, $J = 8.1, 2.5$ Hz, 3H), 7.26 (d, $J = 7.9$ Hz, 1H), 7.22 (d, $J = 8.8$ Hz, 2H), 7.04 (d, $J = 9.6$ Hz, 2H), 6.75 (dd, $J = 9.6, 2.8$ Hz, 2H), 4.37 (s, 2H), 4.21 (t, $J = 5.9$ Hz, 2H), 4.03 – 3.88 (m, 2H), 3.76 – 3.67 (m, 2H), 3.57 (t, $J = 6.6$ Hz, 2H), 3.34 (s, 12H), 3.30 – 3.10 (m, 4H), 3.02 (s, 3H), 1.99-1.90 (m, 4H), 0.61 (s, 3H), 0.60 (s, 3H).

^{13}C NMR (101 MHz, CD_3OD) δ 169.5, 168.1, 164.4, 155.8, 155.5, 150.7, 150.4, 149.4, 142.1, 141.6, 141.5, 136.5, 134.8, 130.8, 130.6, 128.4, 128.3, 127.2, 126.8, 124.7, 122.3, 120.3, 119.5, 118.7, 116.7, 116.5, 115.6, 115.3, 115.2, 111.4, 101.1, 69.2, 68.1, 62.1, 54.6, 48.5, 43.6, 40.9, 40.7, 27.7, 27.2, -1.1, -1.3.

ESI-MS, positive mode: $m/z = 936.5$ $[\text{M}+\text{H}]^+$.

HRMS (ESI) calcd for $\text{C}_{56}\text{H}_{62}\text{N}_9\text{O}_3\text{Si}$ $[\text{M}+\text{H}]^+$ 936.4739, found 936.4731.

Probe 4:



Obtained 8.8 mg of blue-green solid. Yield 47%.

^1H NMR (400 MHz, CD_3OD) δ 8.37 (d, $J = 1.7$ Hz, 1H), 8.16 – 7.96 (m, 4H), 7.88 (d, $J = 8.6$ Hz, 1H), 7.83 (d, $J = 8.2$ Hz, 1H), 7.69 (d, $J = 9.0$ Hz, 1H), 7.62 (s, 1H), 7.36 (dd, $J = 9.2, 2.4$ Hz, 3H), 7.29 (d, $J = 2.2$ Hz, 1H), 7.13 (d, $J = 8.6$ Hz, 2H), 7.04 (d, $J = 9.6$ Hz, 2H), 6.74 (dd, $J = 9.7, 2.8$ Hz, 2H), 4.35 (s, 2H), 4.12 (t, $J = 5.9$ Hz, 2H), 4.04 – 3.64 (m, 4H), 3.47

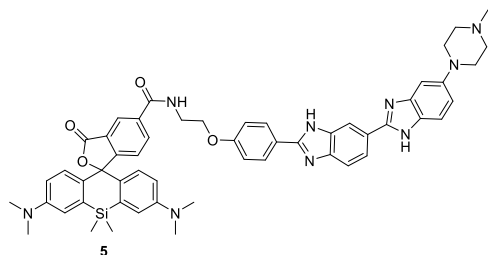
(t, $J = 6.6$ Hz, 2H), 3.32 (s, 12H), 3.28 – 3.05 (m, 4H), 3.00 (s, 3H), 1.96 – 1.73 (m, 4H), 0.60 (s, 6H).

^{13}C NMR (101 MHz, CD_3OD) δ 169.0, 168.0, 163.9, 158.3, 155.9, 155.8, 150.8, 150.6, 149.4, 144.5, 142.2, 138.5, 134.9, 134.3, 130.4, 129.1, 128.8, 128.6, 128.5, 128.3, 124.1, 122.3, 119.9, 119.8, 119.3, 116.5, 115.6, 115.5, 115.3, 111.4, 101.2, 69.1, 68.1, 62.1, 54.6, 43.6, 41.0, 40.9, 40.7, 30.9, 27.7, 27.1, -1.2, -1.2.

ESI-MS, positive mode: $m/z = 936.5$ $[\text{M}+\text{H}]^+$.

HRMS (ESI) calcd for $\text{C}_{56}\text{H}_{62}\text{N}_9\text{O}_3\text{Si}$ $[\text{M}+\text{H}]^+$ 936.4739, found 936.4726.

Probe 5:



Obtained 10.1 mg of blue-green solid. Yield

55%

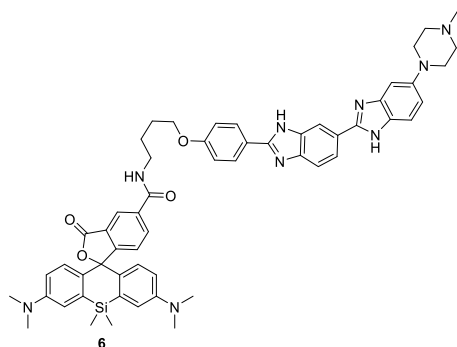
^1H NMR (400 MHz, CD_3OD) δ 8.39 (d, $J = 1.6$ Hz, 1H), 8.26 – 8.16 (m, 2H), 8.01 (d, $J = 8.8$ Hz, 2H), 7.91 (dd, $J = 8.5, 1.6$ Hz, 1H), 7.65 (d, $J = 8.5$ Hz, 1H), 7.50 (d, $J = 8.8$ Hz, 1H), 7.33 (d, $J = 7.8$ Hz, 1H), 7.15 (d, $J = 2.2$ Hz, 1H), 7.10 (d, $J = 8.9$ Hz, 2H), 7.03 (dd, $J = 8.8, 2.2$ Hz, 1H), 7.00 (d, $J = 2.8$ Hz, 2H), 6.66 (d, $J = 8.9$ Hz, 2H), 6.56 (dd, $J = 9.0, 2.9$ Hz, 2H), 4.26 (t, $J = 5.5$ Hz, 2H), 3.84 (t, $J = 5.4$ Hz, 2H), 3.40 – 3.31 (m, 4H), 3.22 – 3.13 (m, 4H), 2.91 (s, 12H), 2.75 (s, 3H), 0.61 (s, 3H), 0.53 (s, 3H).

^{13}C NMR (101 MHz, CD_3OD) δ 172.0, 168.8, 162.3, 158.8, 155.3, 154.0, 151.2, 148.6, 140.5, 138.1, 136.9, 134.5, 132.0, 129.6, 129.3, 129.3, 128.3, 128.0, 126.1, 125.5, 125.3, 123.2, 122.5, 117.8, 116.6, 116.4, 116.2, 114.7, 111.4, 103.1, 95.3, 68.1, 67.5, 55.4, 50.5, 44.5, 40.4, 0.3, -1.4.

ESI-MS, positive mode: $m/z = 922.4$ $[\text{M}+\text{H}]^+$.

HRMS (ESI) calcd for $\text{C}_{54}\text{H}_{56}\text{N}_9\text{O}_4\text{Si}$ $[\text{M}+\text{H}]^+$ 922.4219, found 922.4221.

Probe 6:



Was synthesized according to previously published procedure ¹.

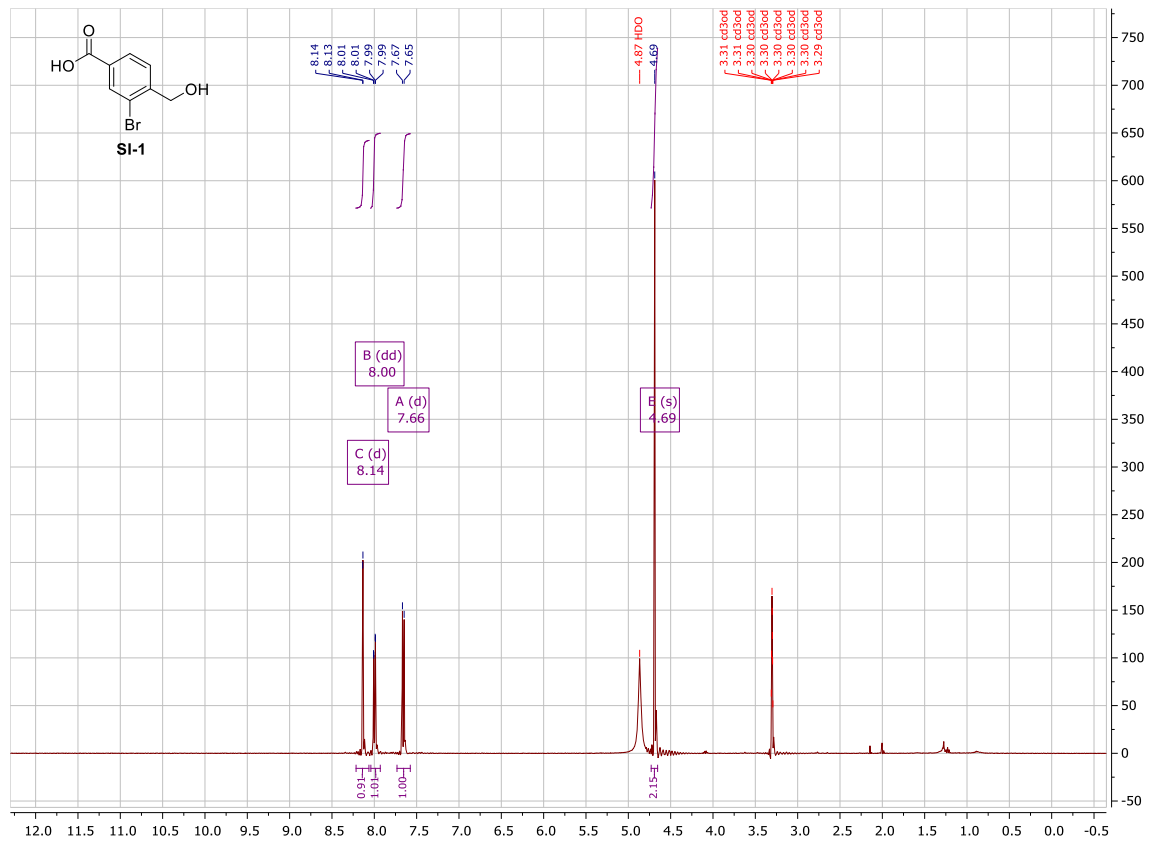
^1H NMR (400 MHz, $\text{CD}_3\text{OD}+\text{CF}_3\text{COOD}$) δ 8.64 (d, $J = 1.8$ Hz, 1H), 8.25 (d, $J = 1.6$ Hz, 1H), 8.17 (dd, $J = 8.0, 1.8$ Hz, 1H), 8.00 (d, $J = 8.8$ Hz, 2H), 7.96 (dd, $J = 8.6, 1.6$ Hz, 1H), 7.80 (d, $J = 8.5$ Hz, 1H), 7.64 (d, $J = 9.0$ Hz, 1H), 7.37 (d, $J = 8.0$ Hz, 1H), 7.31 (dd, $J = 9.1, 2.2$ Hz, 1H), 7.20 – 7.28 (m, 3H), 7.08 (d, $J = 8.9$ Hz, 2H), 6.90 (d, $J = 9.4$ Hz, 2H), 6.69 (dd, $J = 9.5, 2.8$ Hz, 2H), 4.09 (t, $J = 5.8$ Hz, 2H), 3.85 – 3.98 (m, 2H), 3.64 – 3.78 (m, 2H), 3.54 (t, $J = 6.4$ Hz, 2H), 3.30 – 3.41 (m, 2H), 3.21 (s, 12H), 3.09 – 3.20 (m, 2H), 3.00 (s, 3H), 1.83 – 1.94 (m, 4H), 0.61 (s, 3H), 0.54 (s, 3H).

Supplementary references

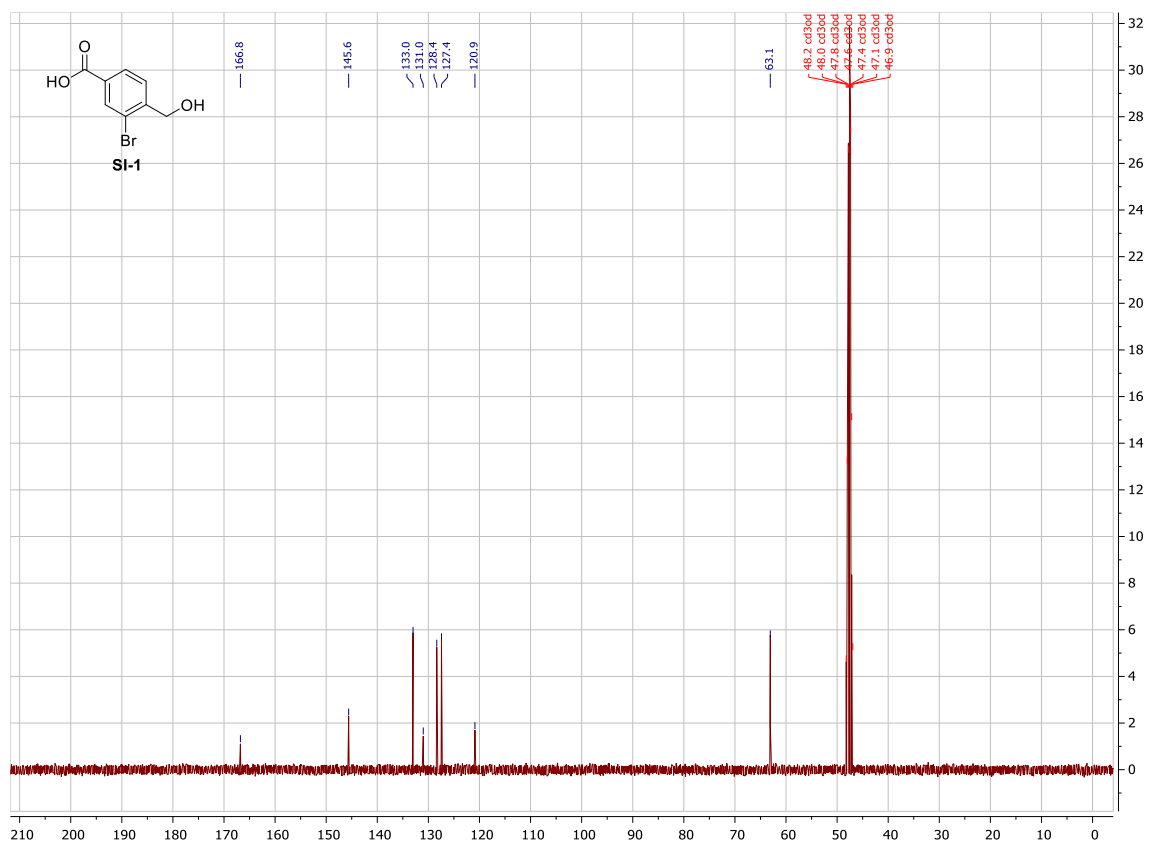
1. Bucevicius, J.; Keller-Findeisen, J.; Gilat, T.; Hell, S. W.; Lukinavicius, G., Rhodamine-Hoechst positional isomers for highly efficient staining of heterochromatin. *Chem Sci* **2019**, *10* (7), 1962-1970.
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NMR copies

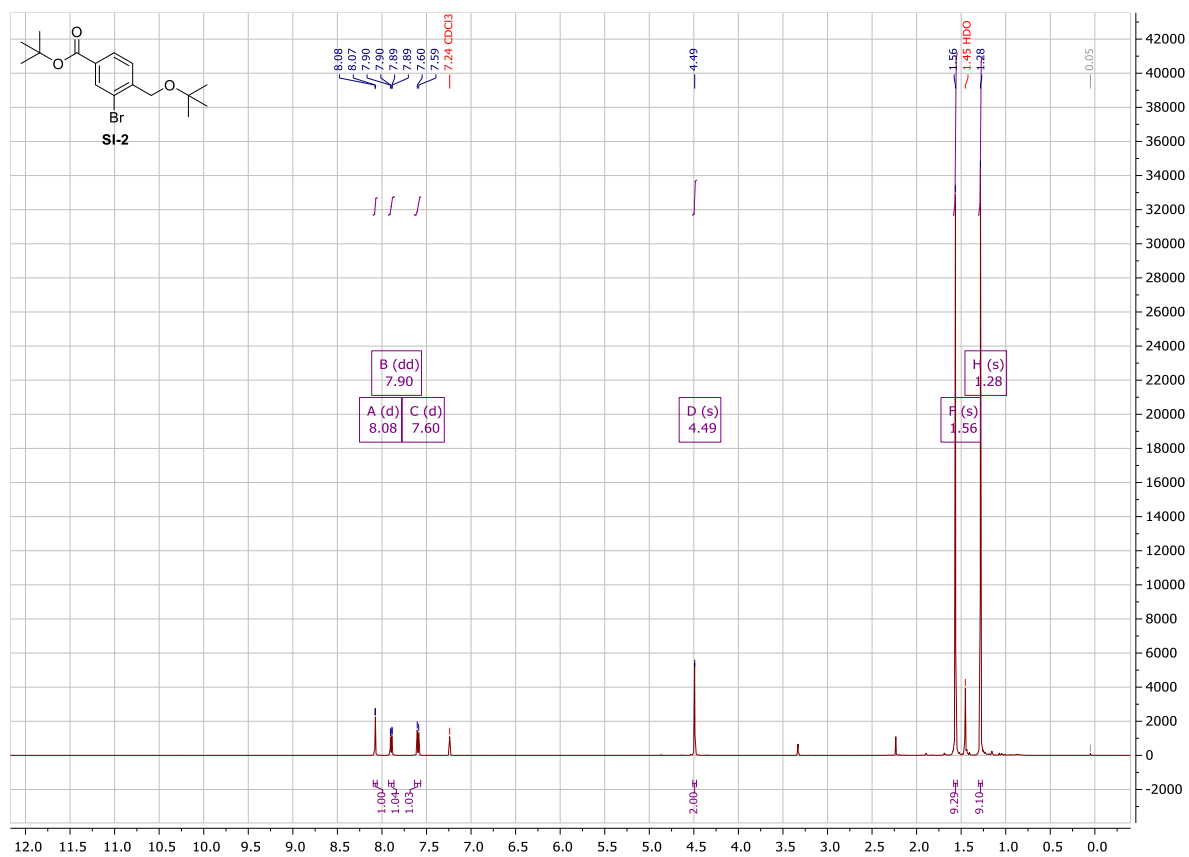
SI-1 ¹H NMR



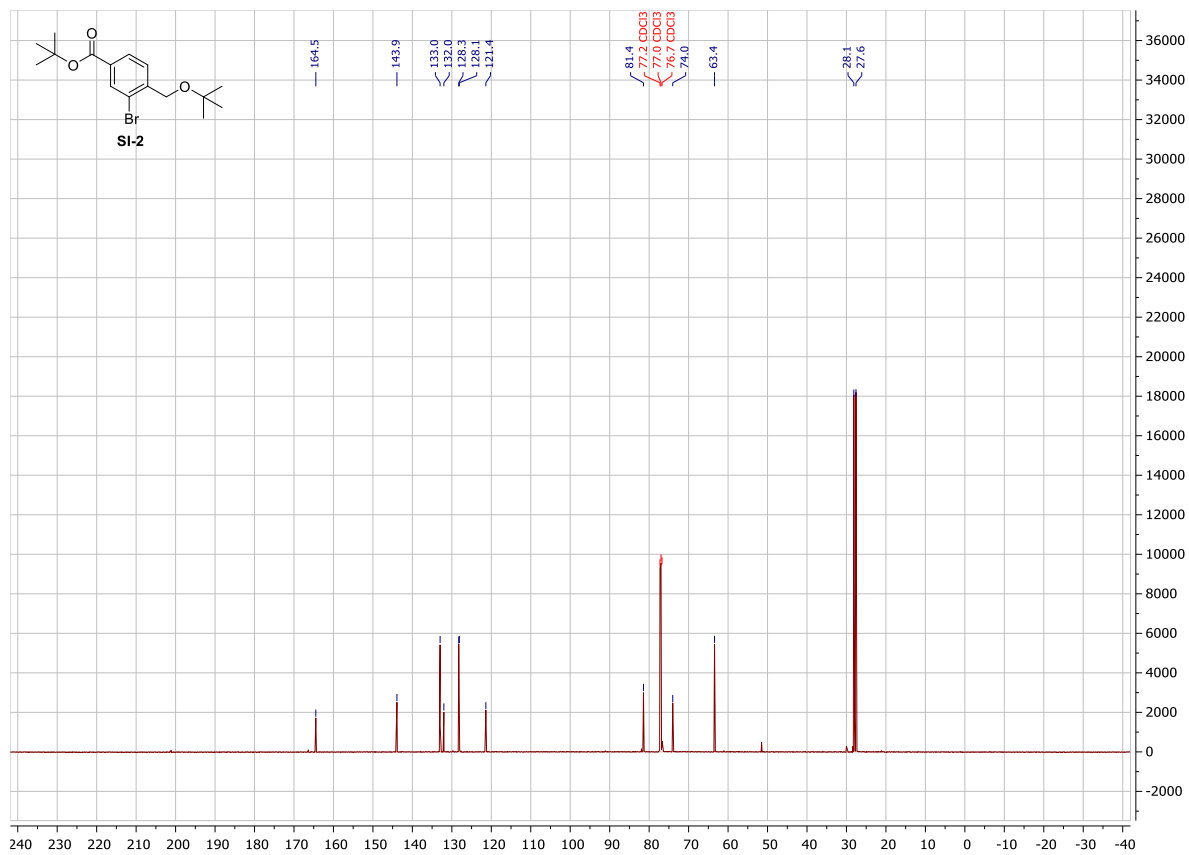
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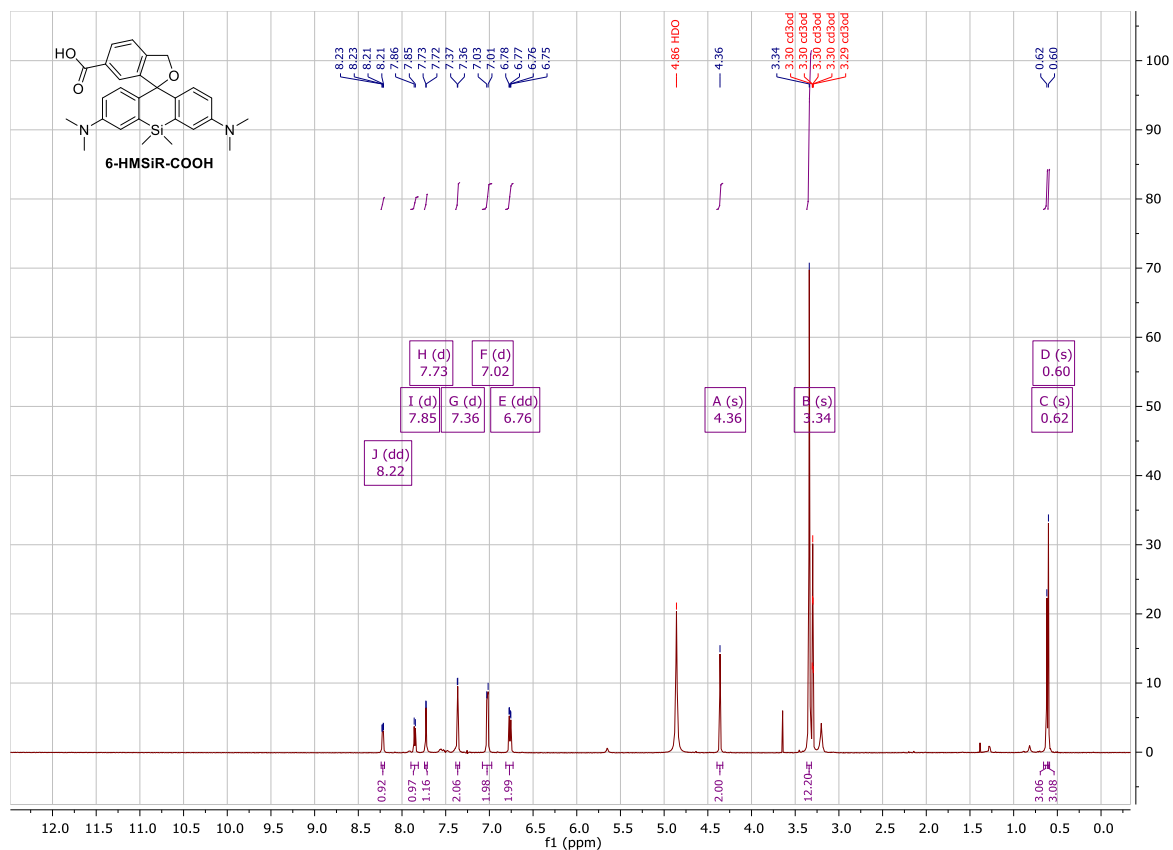
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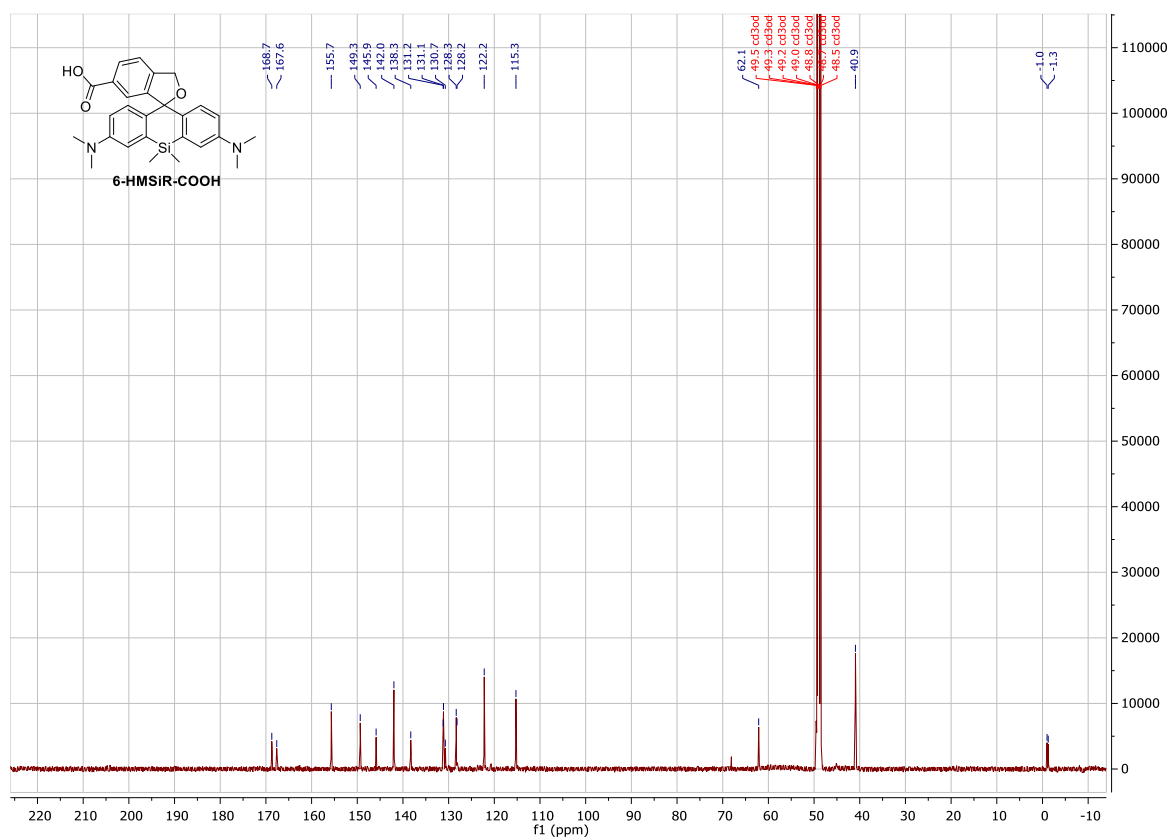
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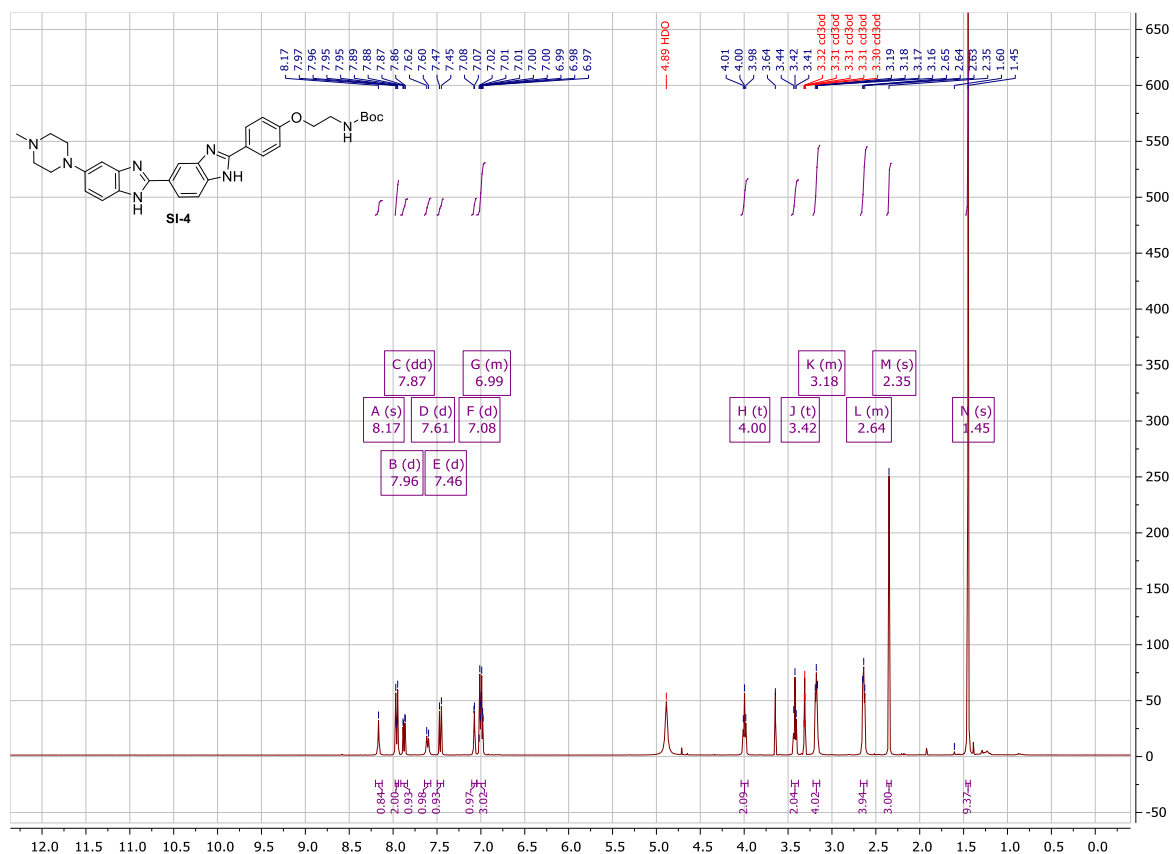
6-HMSiR-COOH ¹H NMR



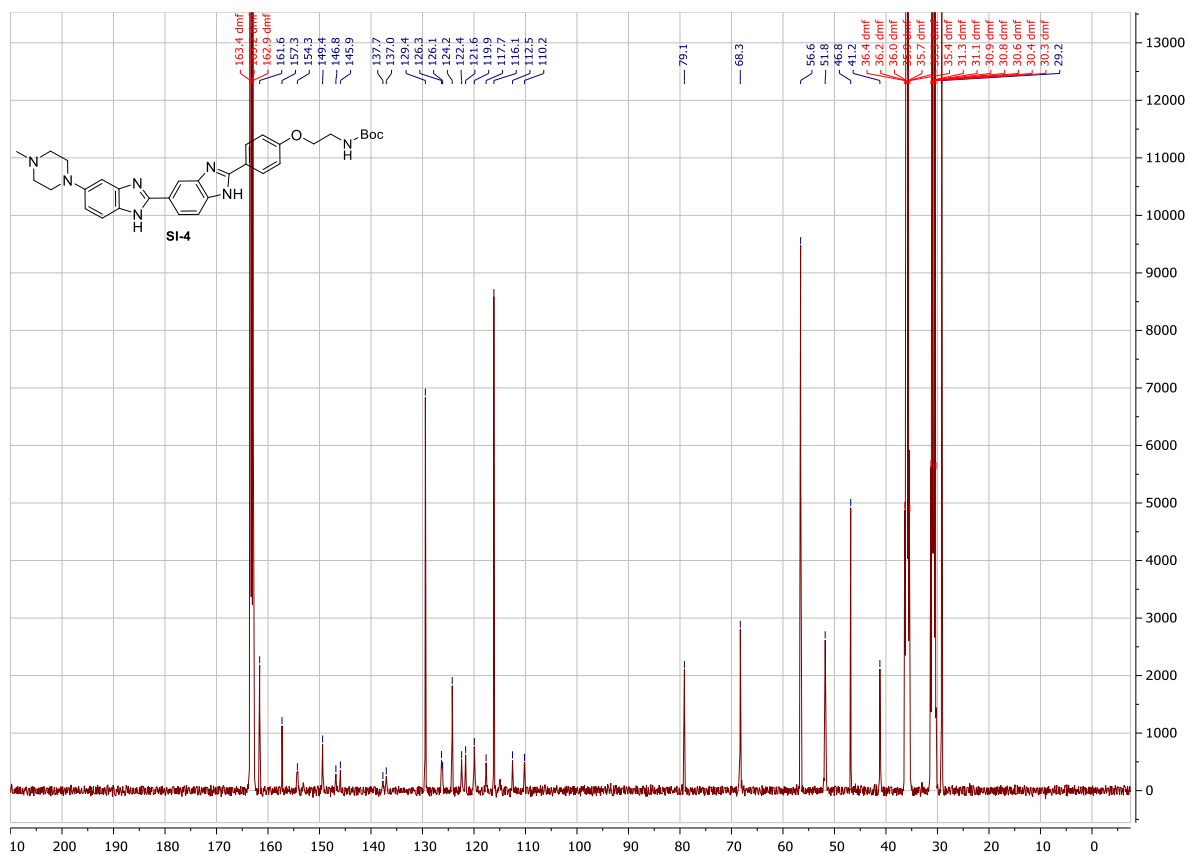
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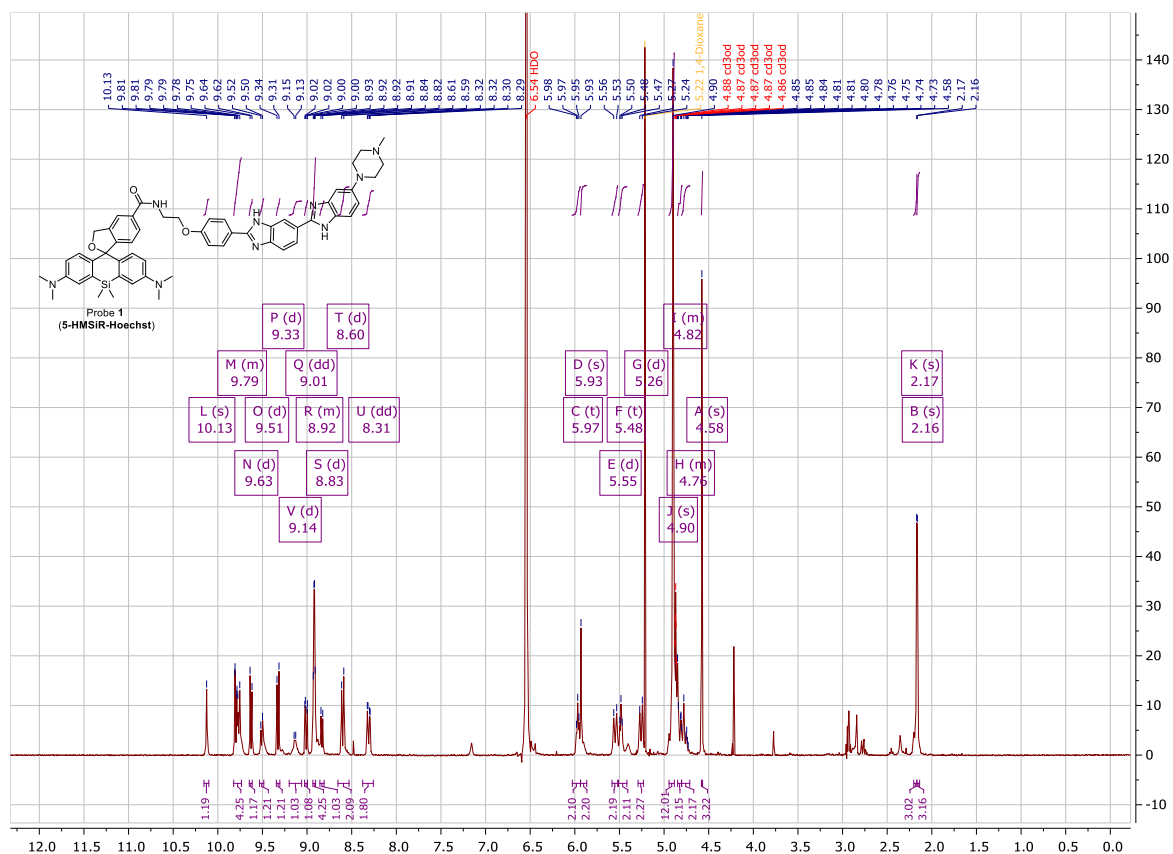
SI-4 ¹H NMR



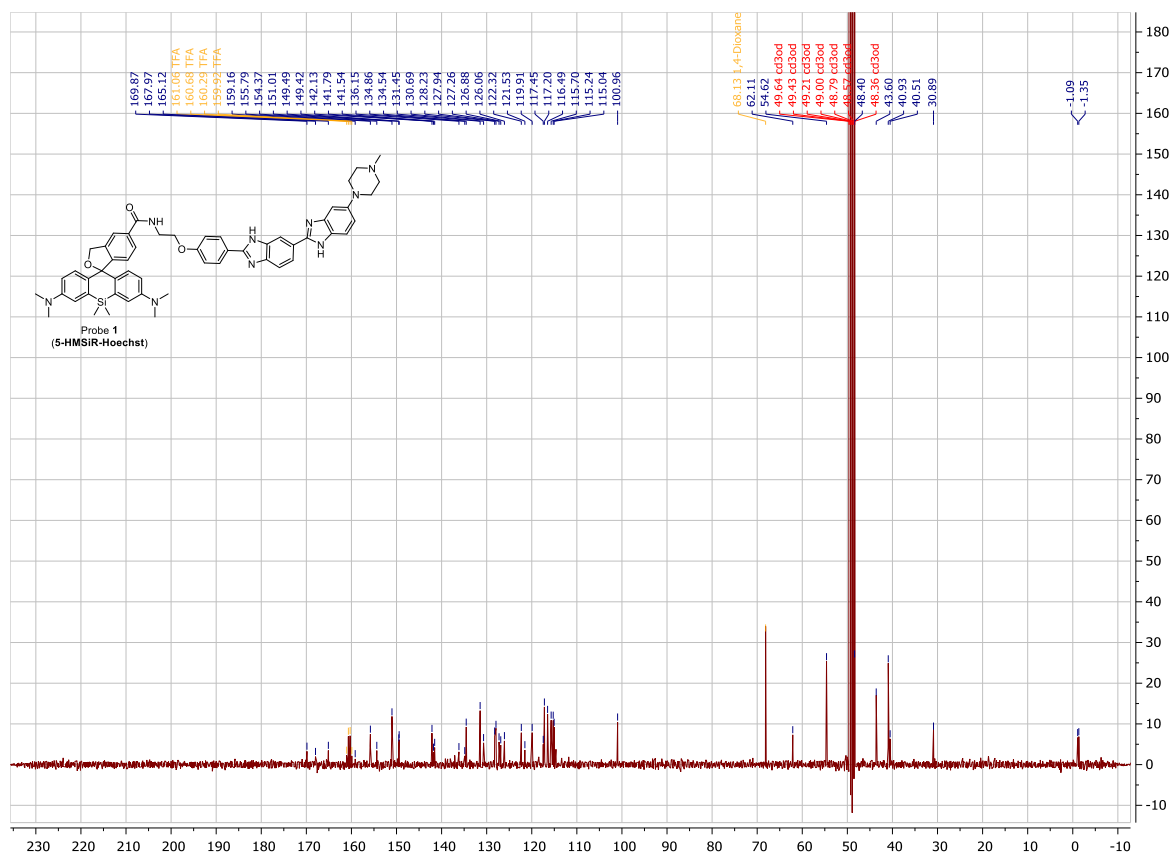
SI-4 ¹³C NMR



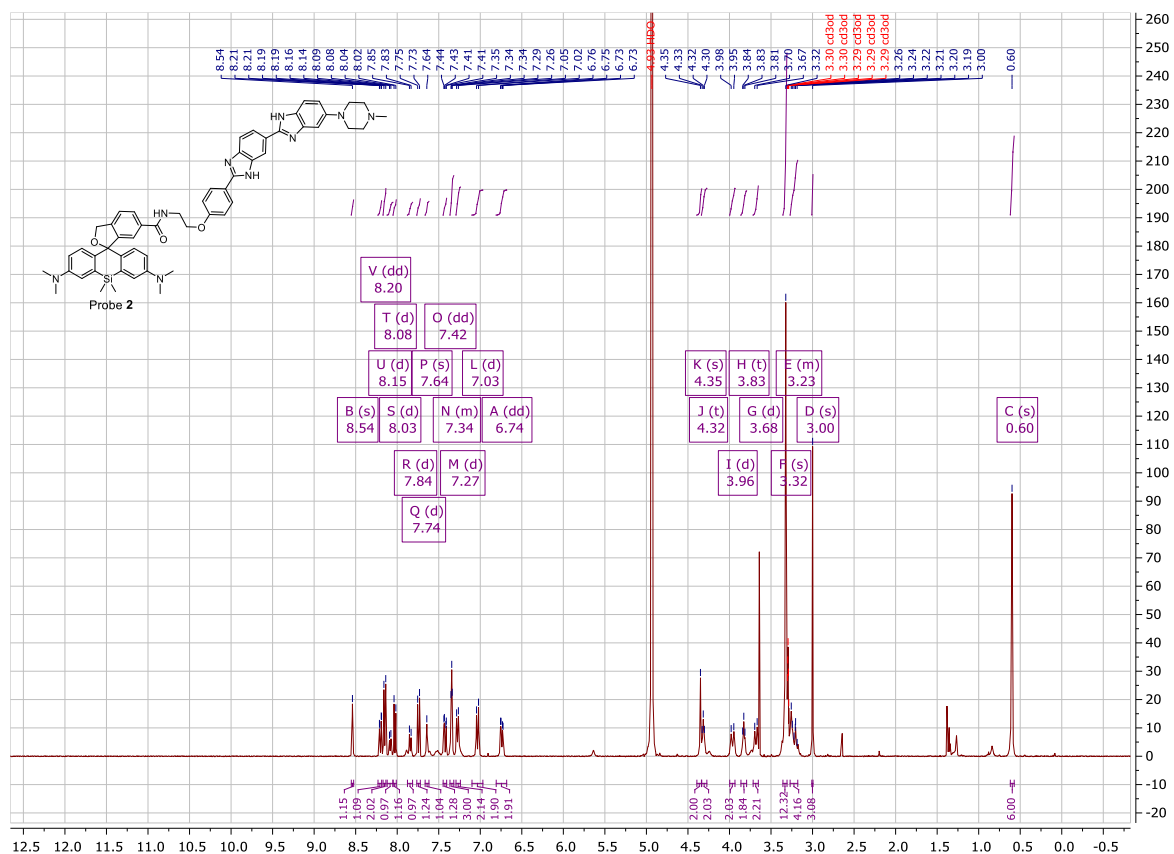
Probe 1 (5-HMSiR-Hoechst) ¹H NMR:



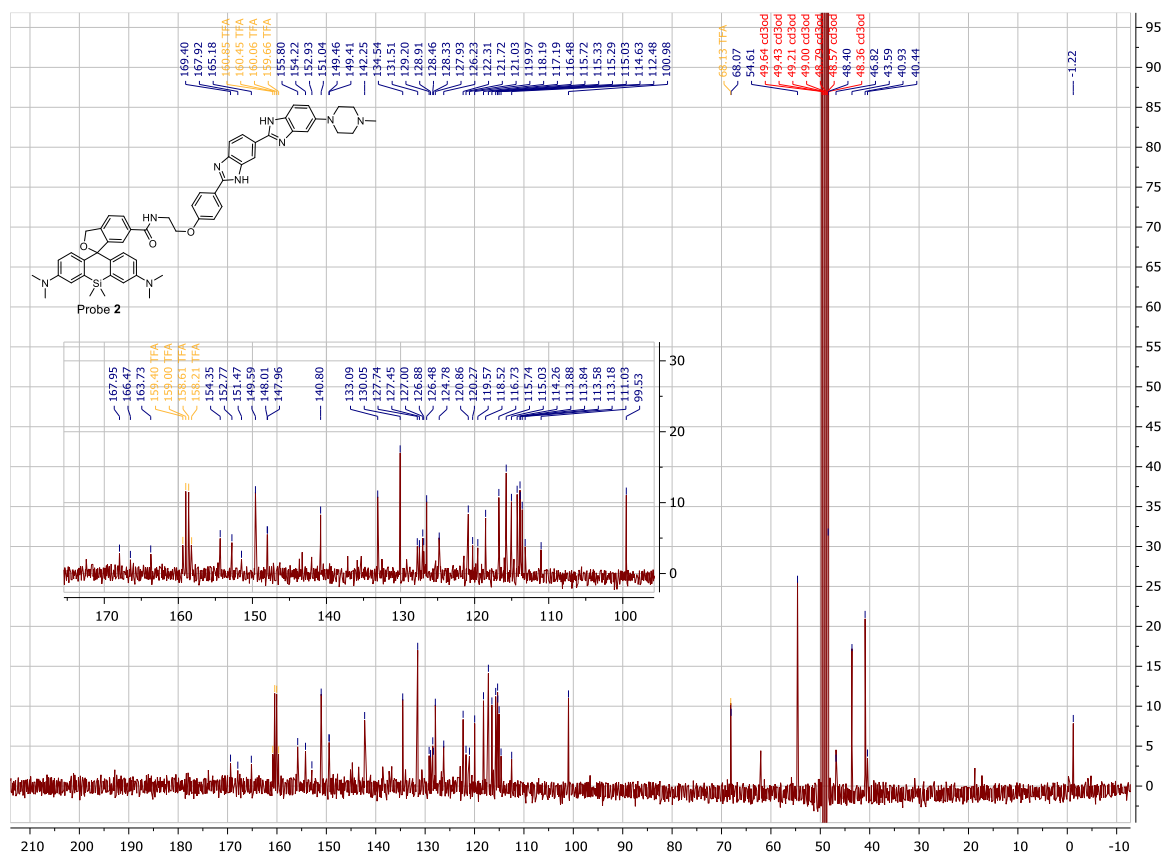
Probe 1 (5-HMSiR-Hoechst) ¹³C NMR:



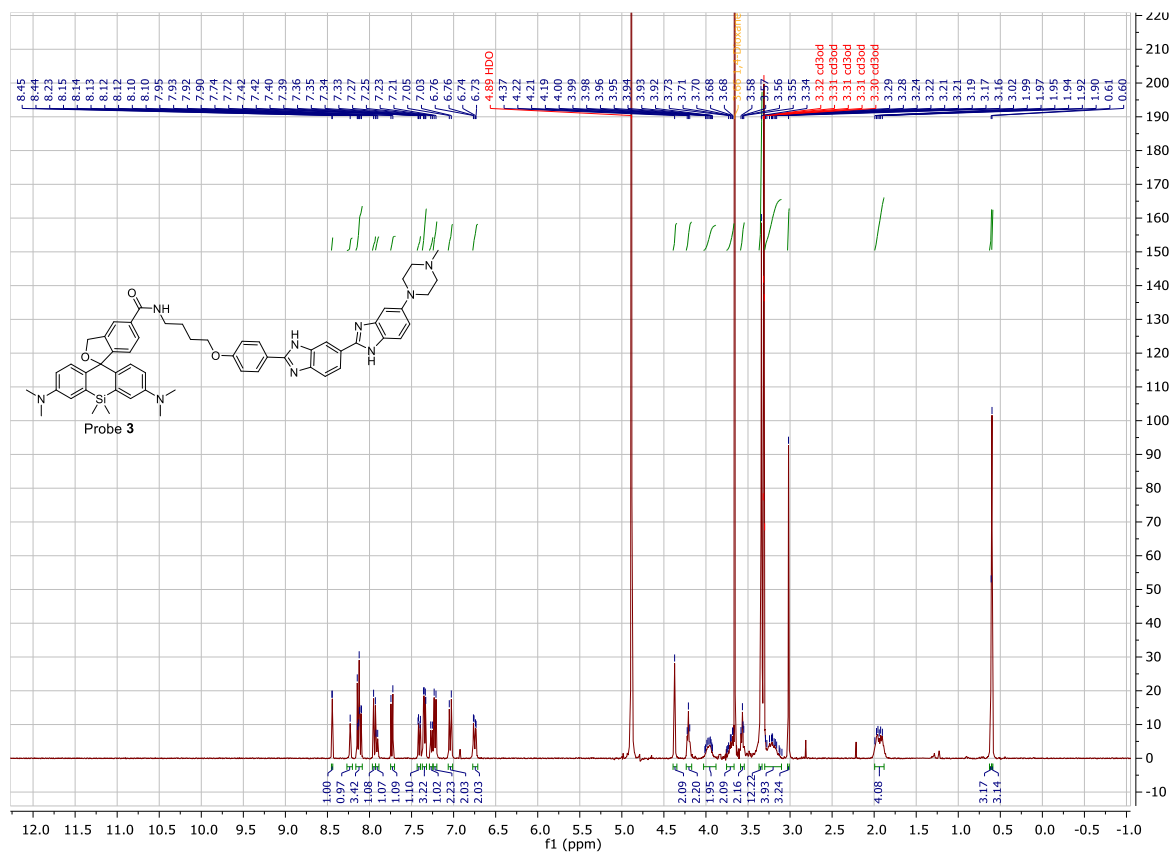
Probe 2 ¹H NMR:



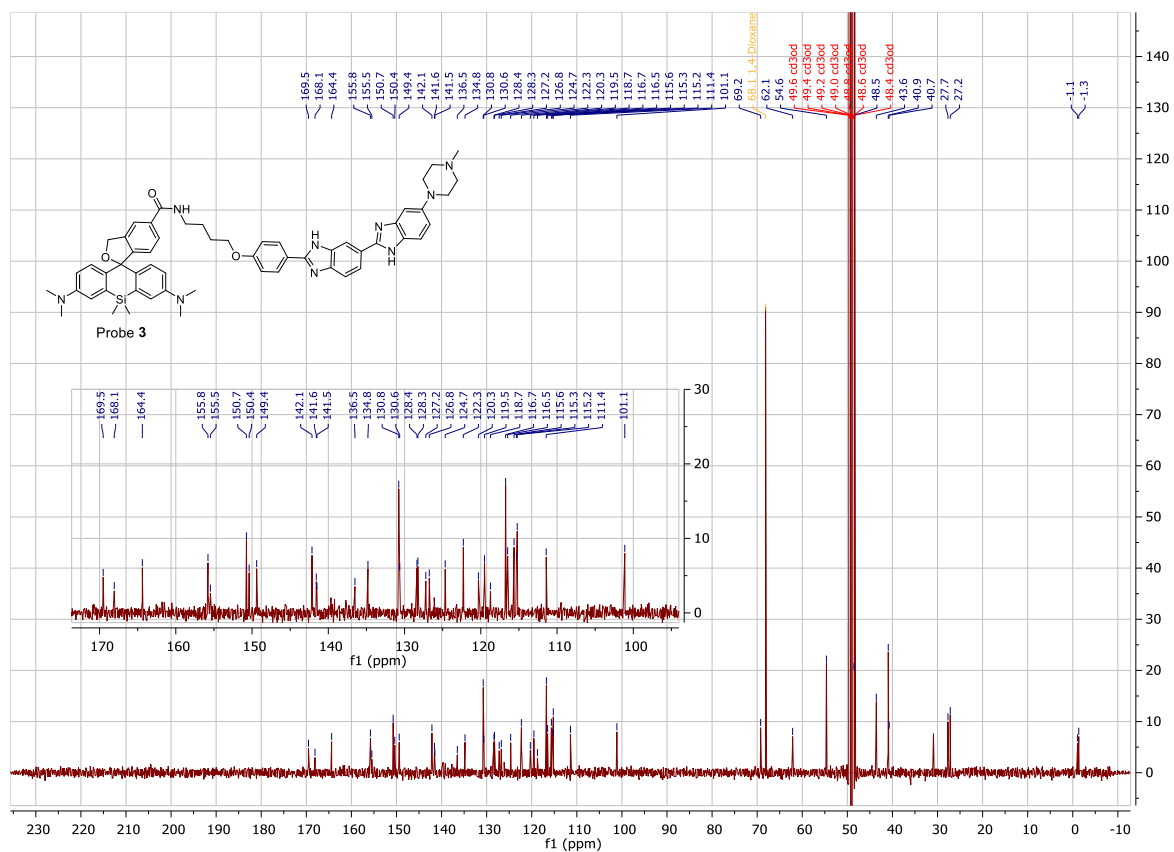
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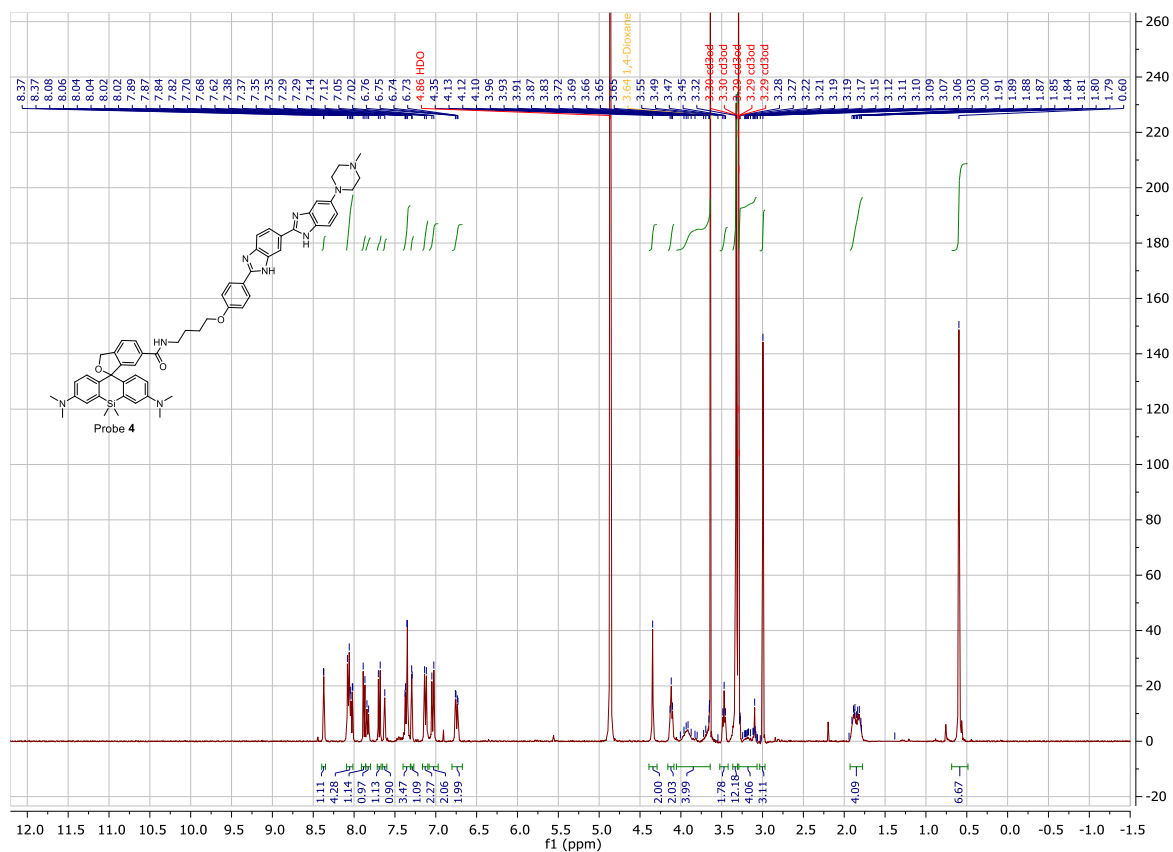
Probe 3 ¹H NMR:



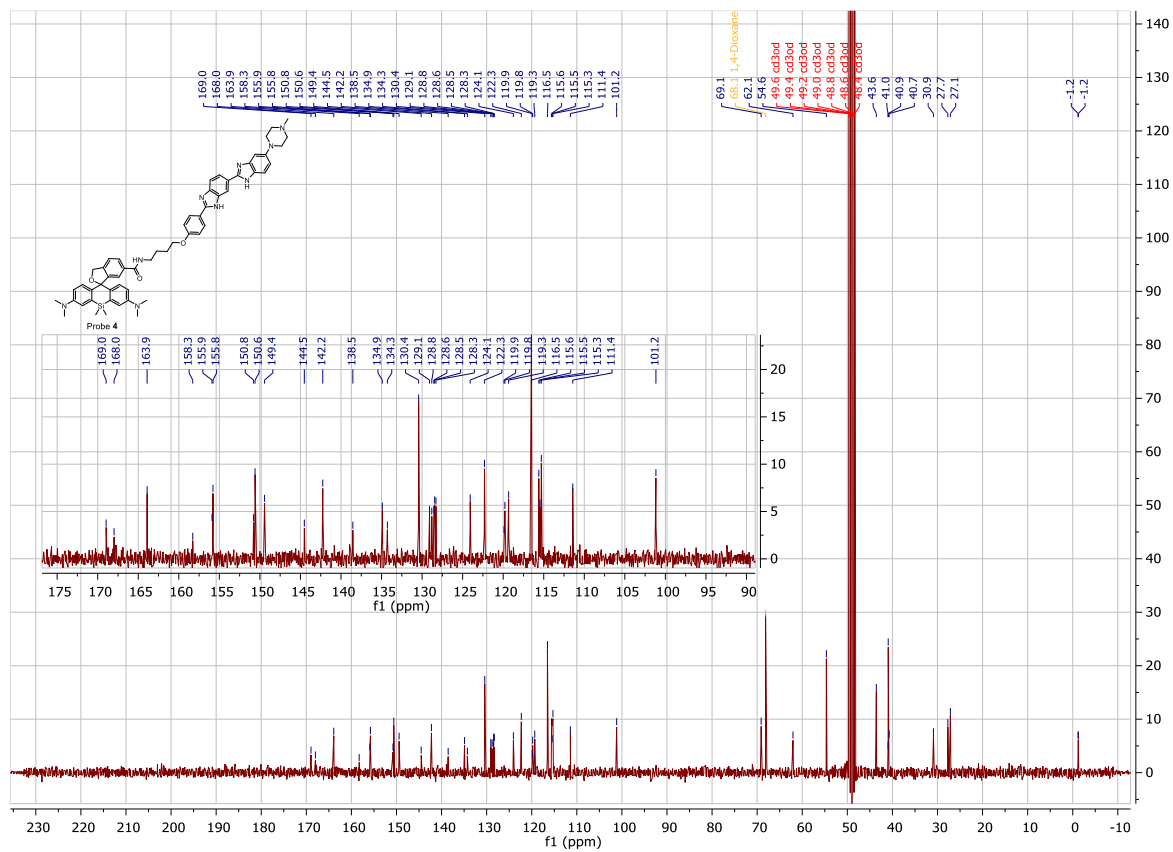
Probe 3 ¹³C NMR:



Probe 4 ¹H NMR:



Probe 4 ¹³C NMR:



Probe 5 ¹H NMR:



Probe 5 ¹³C NMR:

