

Supporting Information

Dynamic Host-Guest Interaction Enables Autonomous Single Molecule Blinking and Super-Resolution Imaging

Ranjan Sasmal¹, Nilanjana Das Saha^{1,2}, Florian Schueder^{3,4}, Divyesh Joshi⁵, Sheeba Vasu⁶, Ralf Jungmann^{3,4}, and Sarit S. Agasti^{1,2*}

¹New Chemistry Unit, Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bangalore, Karnataka 560064, India.

²Chemistry & Physics of Materials Unit, Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bangalore, Karnataka 560064, India.

³Max Planck Institute of Biochemistry, 82152 Martinsried near Munich, Germany.

⁴Department of Physics and Center for Nanoscience, Ludwig Maximilian University, 80539 Munich, Germany.

⁵Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bangalore, Karnataka 560064, India.

⁶Neuroscience Unit, Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bangalore, Karnataka 560064, India.

*Correspondence e-mail: sagasti@jncasr.ac.in

General

All the chemicals were purchased from either of the following company: Sigma Aldrich, Alfa Aesar, Thermo Fischer Scientific, TCI chemicals, Merck, SD fine chemicals and Spectrochem, unless mentioned specifically. TCO-PEG-NHS ester was purchased from Click Chemistry Tools (Cat. No. A137). Amino Phalloidin was purchased from American Peptide Company (Product No. 92-1-10). ZebaTM spin desalting columns were purchased from Thermo Fisher Scientific (Product No. 89883). Biotage[@] SNAP cartridge (KP-C18-SH, 12g) was purchased from Biotage (Part No. FSL0-1118-0012). Antibodies and dyes were purchased from commercial sources as listed below. Whenever necessary, solvents were dried by using standard solvent drying methods and then used for reactions. The yields of the compounds reported here refer to the yield of spectroscopically pure compounds after

purification. SH-TEG-ADA, CB[7] conjugated TCO, CB[7] conjugated antibodies and CB[7] conjugated phalloidin was prepared according to our previous report¹.

¹H NMR spectrum were recorded using Bruker AVANCE III 400 MHz and JEOL Delta 600 MHz instrument and data analysis was done using Spinworks_4.0. High Resolution Mass Spectrometry (HRMS) was carried out using Agilent 6538 Ultra High Definition (UHD) Accurate-Mass Q-TOF LC/MS. Liquid chromatography–mass spectrometry (LCMS) experiments were carried out using Waters LCMS system. High Performance Liquid Chromatography (HPLC) purification was carried out using Agilent 1260 infinity quaternary HPLC system equipped with analytical ZORBAX Eclipse plus C18 column (4.6 mm × 100 mm, 3.5 micron). The solvents used as eluent in HPLC purification were: solvent A (water containing 0.1% TFA) and solvent B (acetonitrile containing 0.1% TFA). A gradient elution of solvent B in solvent A from 5-100% was used for the purification. Purification of fluorophore-maleimide conjugates were carried out using a Biotage[®] SNAP cartridge (KP-C18-SH, 12 g) and water/acetonitrile was used as eluent. Absorbance measurement was carried out to check the concentration of fluorophores in a Eppendorf BioSpectrometer. The fluorescent imaging was carried out using an inverted Zeiss ELYRA PS1 microscope.

Supporting Table S1: List of primary antibodies used for immunostaining

| Target | Antibody commercial sources | Species |
|----------------------------------|---------------------------------------|---------|
| Microtubule (α -tubulin) | Thermo Fischer Scientific (MA1-80017) | Rat |
| Microtubule (β -tubulin) | DSHB (E7-s) | Mouse |
| Mitochondria (Tom20) | Santa Cruz (sc-11415) | Rabbit |

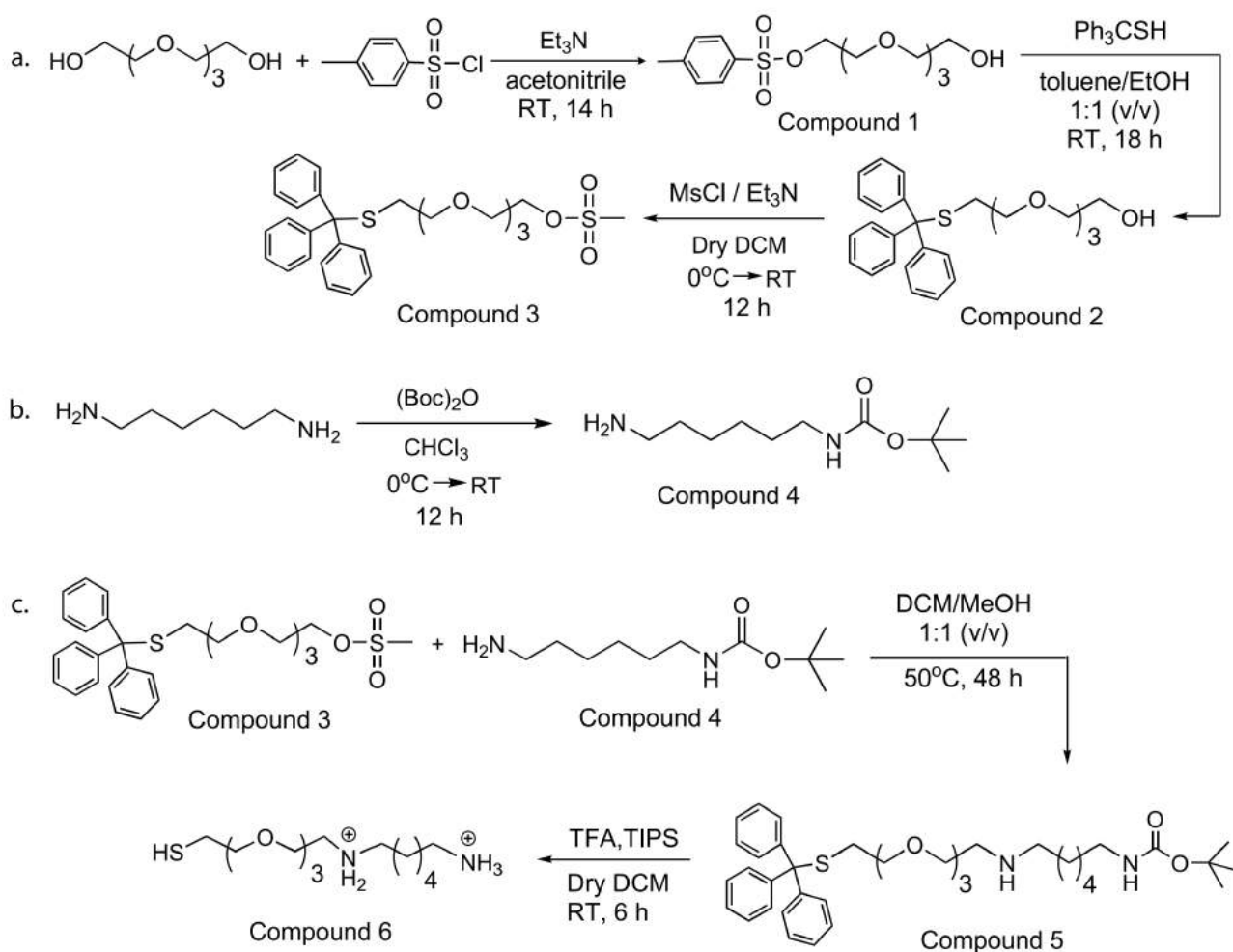
Supporting Table S2: List of secondary antibodies used for immunostaining

| Target | Host | Specification and commercial source |
|--------|--------|--|
| Mouse | Donkey | Donkey Anti-Mouse IgG (H+L) (min X Bov, Ck, Gt, GP, Sy Hms, Hrs, Hu, Rb, Rat, Shp SrProt) Jackson ImmunoResearch Laboratories (Cat. No. 715-005-151) |
| Rat | Donkey | Donkey Anti-Rat IgG (H+L) (min X Bov, Ck, Gt, GP, SyHms, Hrs, Hu, Ms, Rb, Shp Sr Prot) Jackson ImmunoResearch Laboratories (Cat. No. 712-005-153) |
| Mouse | Goat | Goat anti-Mouse IgG (H+L) |

| | | |
|--------|--------|---|
| | | (Secondary Antibody for Western Blot, IF, ICC and ELISA) Thermo Fischer scientific (Cat. No. A28174) |
| Rabbit | Donkey | Donkey Anti-Rabbit IgG (H+L) (min X Bov, Ck, Gt, GP, SyHms, Hrs, Hu, Ms, Rat, Shp SrProt) Jackson ImmunoResearch Laboratories (Cat. No. 711-005-152) |

Supporting Table S3: List of fluorophores used for host-guest mediated imaging.

| Fluorophores | Commercial source |
|-----------------------------------|-----------------------------------|
| Cy5 NHS ester | Lumiprobe (Cat. No. 23320) |
| Alexa Fluor 647 NHS ester | Thermo Fisher Scientific (A20006) |
| Silicon rhodamine (SiR) NHS ester | Spirochrome (SC003) |



Scheme S1| Synthetic scheme for the preparation of thiol modified hexamethylenediamine (HMD) guest.

Synthesis of compound 1: Tetra ethylene glycol (TEG, 10 g, 51.22 mmol) was taken in 250 ml RB Flask. 130 ml acetonitrile and triethyl amine (7.13 ml, 51.22 mmol) were added to it. Tosyl chloride (9.76 g, 51.22 mmol), dissolved in 20 ml acetonitrile was added drop wise from dropping funnel over 1 h keeping the reaction mixture at 0°C. After the addition, the reaction mixture was stirred at room temperature for 14 h. During the reaction, a white precipitate of triethyl ammonium hydrochloride was formed. After completion of reaction, the precipitate was filtered and washed with acetonitrile. The acetonitrile was evaporated and the compound was purified using flash silica (230-400 mesh) column (eluent: ethyl acetate(EtOAc)/hexane, gradient elution from 0 to 80%). Compound 1 was obtained as colourless oil (6.019 g, 35%). $R_f = 0.375$ (EtOAc/hexane: 7:3). Ditosylated derivative was formed as a side product (3.40 g, 20%). $R_f = 0.75$ (EtOAc/Hexane: 7:3). ^1H NMR (400 MHz, CDCl_3): δ 7.79(2H, d, ArH), 7.33(2H, d, ArH), 4.16 (2H, t, $-\text{CH}_2\text{OSO}_2-$), 3.69-3.59 (14H, m, $-\text{OCH}_2\text{CH}_2\text{O}-$), 2.44 (3H, s, $-\text{CH}_3$). ^1H NMR spectrum of compound 1 has been shown in Figure S1.

Synthesis of compound 2: A solution of NaOH (0.864 g, 21.6 mmol) in 8 ml water was added to a solution of triphenylmethanethiol (3.98 g, 14.402 mmol) in a mixture of toluene/ethanol (EtOH) (1:1

v/v, 50 ml). Compound 1 (5.019 g, 14.402 mmol) was dissolved in a second solution of toluene/EtOH (1:1 v/v, 50 ml), which was then added to the triphenylmethanethiol solution in one portion. The reaction mixture was stirred at room temperature for 18 h. After completion of reaction (monitored by TLC) the reaction mixture was poured into 20 ml saturated NaHCO₃ solution and extracted with diethyl ether (Et₂O) (3 × 40 ml). The combined organic layers were washed with brine (3 × 40 ml), dried over Na₂SO₄ and solvent was removed under vacuum to give a pale-yellow oil. The crude product was purified by silica column (60-120 mesh) chromatography (eluent: EtOAc/hexane, 1:2→3:1) to yield compound 2 as a pale-yellow oil (5.442 g, 85%). R_f = 0.42 (EtOAc/hexane: 3:1). ¹H NMR (400 MHz, CDCl₃): δ 7.41 (6H, d, ArH), 7.275 (6H, d, ArH), 7.20 (3H, m, ArH), 3.70 (2H, br, HOCH₂–), 3.63 (4H, t, –OCH₂CH₂O–), 3.58 (4H, t, –OCH₂CH₂O–), 3.45 (2H, t, –OCH₂CH₂OH), 3.30 (2H, t, –SCH₂CH₂O–), 2.43 (2H, t, –SCH₂–). ¹H NMR spectrum of compound 2 has been shown in Figure S2.

Synthesis of compound 3: A solution of compound 2 (5.43 g, 12.0 mmol) and triethylamine (2.5 ml, 18 mmol) in 60 ml dry dichloromethane (DCM) was stirred below 5°C under argon atmosphere. Methanesulfonyl chloride (1.68 ml, 24.0 mmol) was added in dropwise fashion while maintaining the temperature below 5°C. The reaction mixture was stirred for 30 min below 5°C and then allowed to stir at room temperature for another 12 h. After completion of reaction (monitored by TLC), the resulting residue was diluted to 100 ml with DCM and washed with 0.1 M HCl (2 × 25 ml), saturated NaHCO₃ solution (2 × 25 ml), and brine solution (1 × 25 ml). The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. The crude product was purified by flash silica column (eluent: EtOAc/hexane 1:1) to yield compound 3 as a pale-yellow oil (5.55 g, 88%). R_f = 0.45 (EtOAc/hexane: 1:1). ¹H NMR (400 MHz, CDCl₃): δ 7.41 (6H, d, ArH), 7.27 (6H, d, ArH), 7.21 (3H, m, ArH), 4.35 (2H, t, –CH₂OSO₂–), 3.73 (2H, br, –CH₂CH₂OSO₂–), 3.63 (4H, t, –OCH₂CH₂O–), 3.55 (2H, t, –OCH₂CH₂O–), 3.44 (2H, t, –OCH₂CH₂O–), 3.30 (2H, t, –SCH₂CH₂O–), 3.02 (3H, s, –OCH₃), 2.43 (2H, t, –SCH₂–). ¹H NMR spectrum of compound 3 has been shown in Figure S3.

Synthesis of compound 4: Compound 4 was synthesized by following a literature reported procedure². Hexamethylenediamine (HMD, 13.3 g, 114.5 mmol) was taken in 120 ml chloroform. Di-tert-butyl dicarbonate (2.5 g, 11.45 mmol) dissolved in 30 ml chloroform was added to the reaction mixture over two h using dropping funnel keeping the reaction mixture below 5°C. Then the reaction mixture was stirred at room temperature for 12 h. During the reaction a white precipitate was formed which was filtered. The filtrate was concentrated to 50 ml using rotary evaporator, washed with 0.1N HCl (2 × 50 ml), water (4 × 50 ml) and brine solution (2 × 30 ml). The organic layer was filtered through Na₂SO₄ to give compound 4. (2.1 g, 84%). ¹H NMR (400MHz, CDCl₃): δ 4.51 (1H, br, –HNBoc), 3.1

(2H, q, $-\text{CH}_2\text{NHCO}-$), 2.67(2H, t, $-\text{CH}_2\text{NH}_2$), 1.44(9H, s, $-\text{C}(\text{CH}_3)_3$), 1.43(8H, br, $-(\text{CH}_2)_4-$). ^1H NMR of compound 4 has been shown in Figure S4.

Synthesis of compound 5: Compound 4 (812.1 mg, 3.7545 mmol) was first dissolved in 2 ml dry DCM/methanol (MeOH) (1:1 v/v) and placed in a 10 ml round bottom (RB) flask. Compound 3 (400 mg, 0.7509 mmol) was dissolved in 2 ml of dry DCM/MeOH (1:1 v/v) in a vial and added to it. The reaction mixture was heated to 50°C and stirred for 48 h. After completion of reaction (monitored by TLC), the reaction mixture was concentrated under reduced pressure, diluted with DCM and directly charged on a flash silica column. (Eluent: gradient eluent of 0-5% MeOH in DCM v/v). Yield: 380 mg (0.5837 mmol, 78%, a viscous pale-yellow liquid). $R_f = 0.46$ (eluent DCM/MeOH: 19:1). ^1H NMR (400MHz, CDCl_3): δ 7.40 (6H, m, ArH), 7.27-7.24 (6H, m, ArH), 7.23-7.19 (3H, m, ArH), 4.50(1H, br, $-\text{NHCO}-$), 3.60-3.50(8H, m, $-\text{OCH}_2\text{CH}_2\text{O}-$), 3.44(2H, t, $-\text{OCH}_2\text{CH}_2\text{NH}-$), 3.30 (2H, t, $-\text{SCH}_2\text{CH}_2\text{O}-$) 3.09 (2H, m, $-\text{CH}_2\text{NHCO}-$), 2.79 (2H, t, $-\text{NHCH}_2\text{CH}_2\text{O}-$), 2.60 (2H, t, $-\text{NHCH}_2-$), 2.42 (2H, t, $-\text{SCH}_2-$), 1.46-1.15 (17H, m $-\text{C}(\text{CH}_3)_3 + -(\text{CH}_2)_2-$). HRMS: (ESI-MS): calculated m/z 651.3832 $[\text{M}+\text{H}]^+$, found 651.3833 $[\text{M}+\text{H}]^+$. ^1H NMR and HRMS spectra of compound 5 have been shown in Figure S5 and S6 respectively.

Synthesis of compound 6: Compound 5 (300 mg, 0.4613 mmol) was dissolved in 4.5 ml Dry DCM in a 10 ml RB flask. The solution was purged with nitrogen and an excess of trifluoro acetic acid (TFA, 710.8 μl , 9.226 mmol) was added. During the addition period of TFA, the color of the solution turned to yellow. Subsequently, triisopropylsilane (TIPS, 472.52 μl , 2.3065 mmol) was added to the reaction mixture and the color of mixture slowly changed to colorless. Then the reaction mixture was allowed to stir at room temperature for 6 h under nitrogen atmosphere. The volatile components (DCM, TFA and TIPS) were then removed under reduced pressure. The pale-yellow residue was purified by washing with hexane (3 x 10 ml) and diethyl ether (3 x 10 ml). A viscous colorless liquid was obtained after drying under high vacuum. Yield: 100 mg (0.3247 mmol, 70%). ^1H NMR (400MHz, CDCl_3): 3.76 (2H, t, $-\text{OCH}_2\text{CH}_2\text{NH}-$), 3.60-3.70 (10H, m, $-\text{CH}_2\text{O}- + -\text{OCH}_2-$), 3.18 (2H, br, $-\text{CH}_2\text{NH}_2$) 3.02 (4H, br, $-\text{CH}_2\text{NHCH}_2-$), 2.70 (2H, q, HSCH_2-), 1.78-1.66 (4H, m, $-\text{HNCH}_2\text{CH}_2-$), 1.43 (4H, br, $-\text{CH}_2\text{CH}_2-$). HRMS: (ESI-MS): calculated m/z 308.2212 $[\text{M}+\text{H}]^+$, Found 308.2211 $[\text{M}+\text{H}]^+$. ^1H NMR and HRMS spectra of compound 6 have been shown in Figure S7 and S8 respectively.

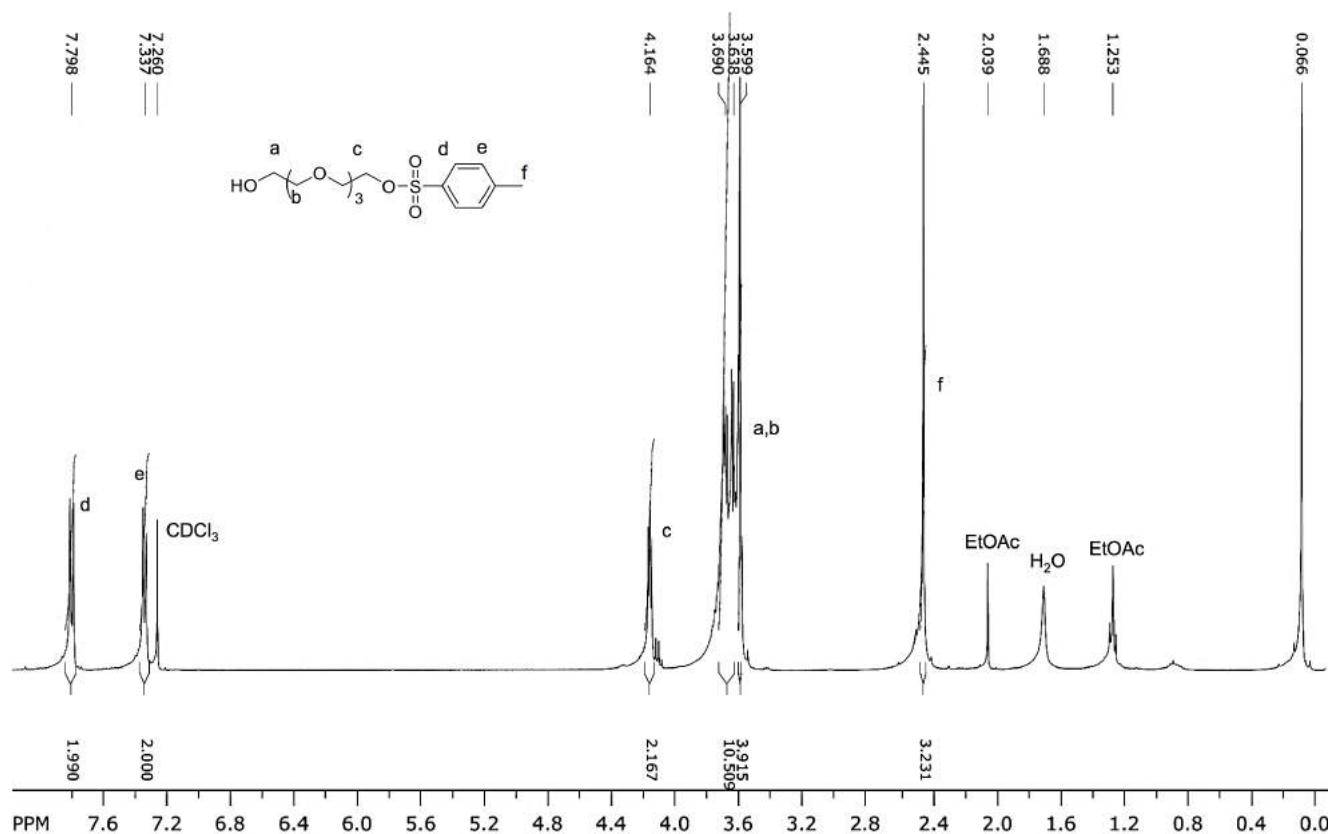


Figure S1 | ¹H NMR spectrum of Compound 1 (400 MHz, CDCl₃) in Bruker AVANCE III 400 MHz NMR instrument.

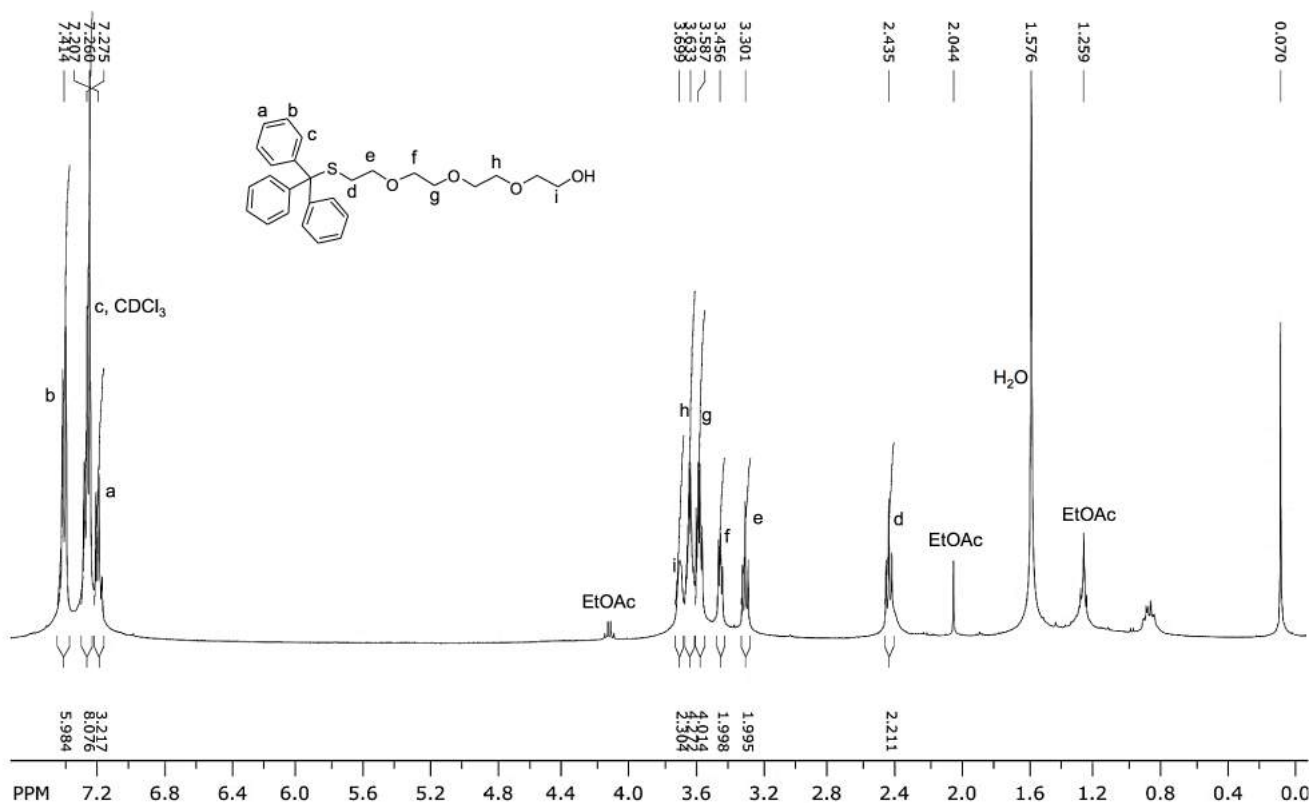


Figure S2 | ¹H NMR spectrum of Compound 2 (400 MHz, CDCl₃) in Bruker AVANCE III 400 MHz NMR instrument.

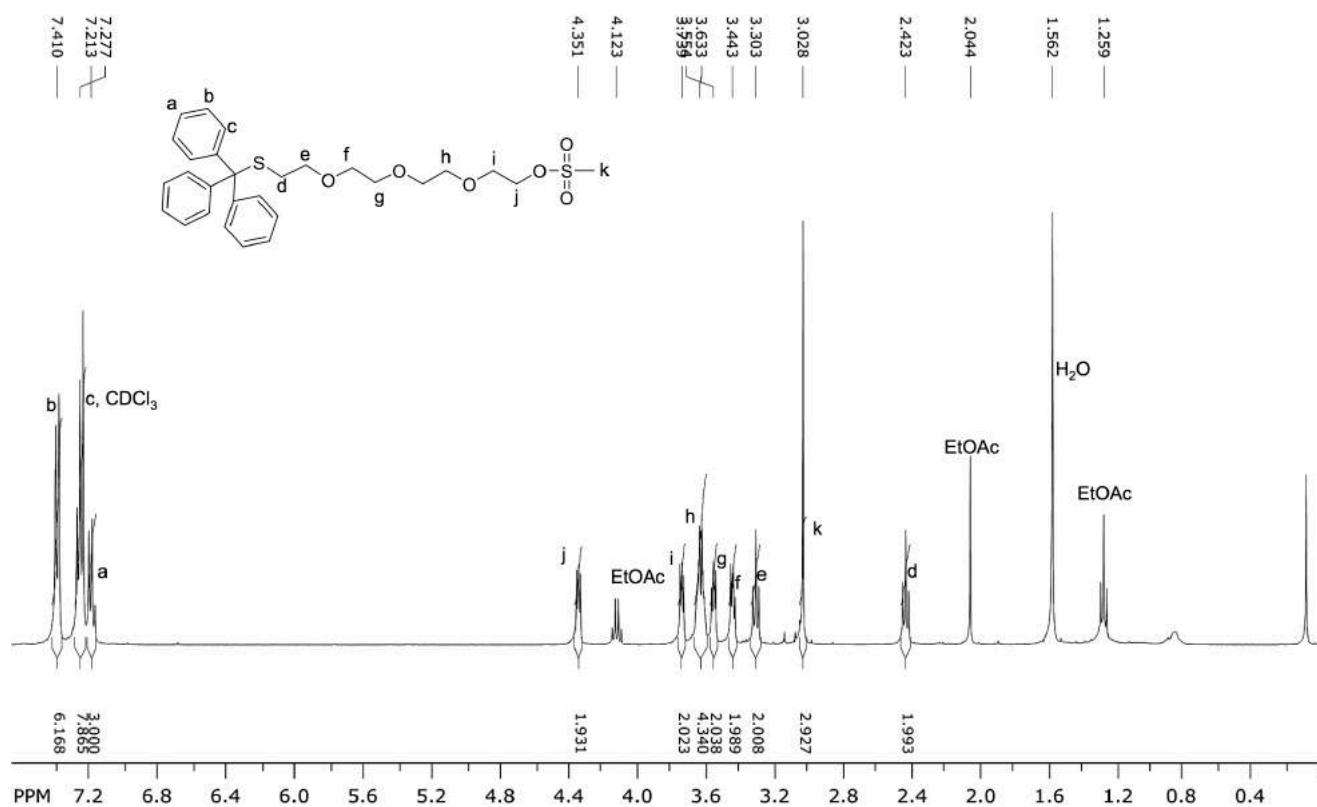


Figure S3 | ¹H NMR spectrum of Compound 3 (400 MHz, CDCl₃) in Bruker AVANCE III 400 MHz NMR instrument.

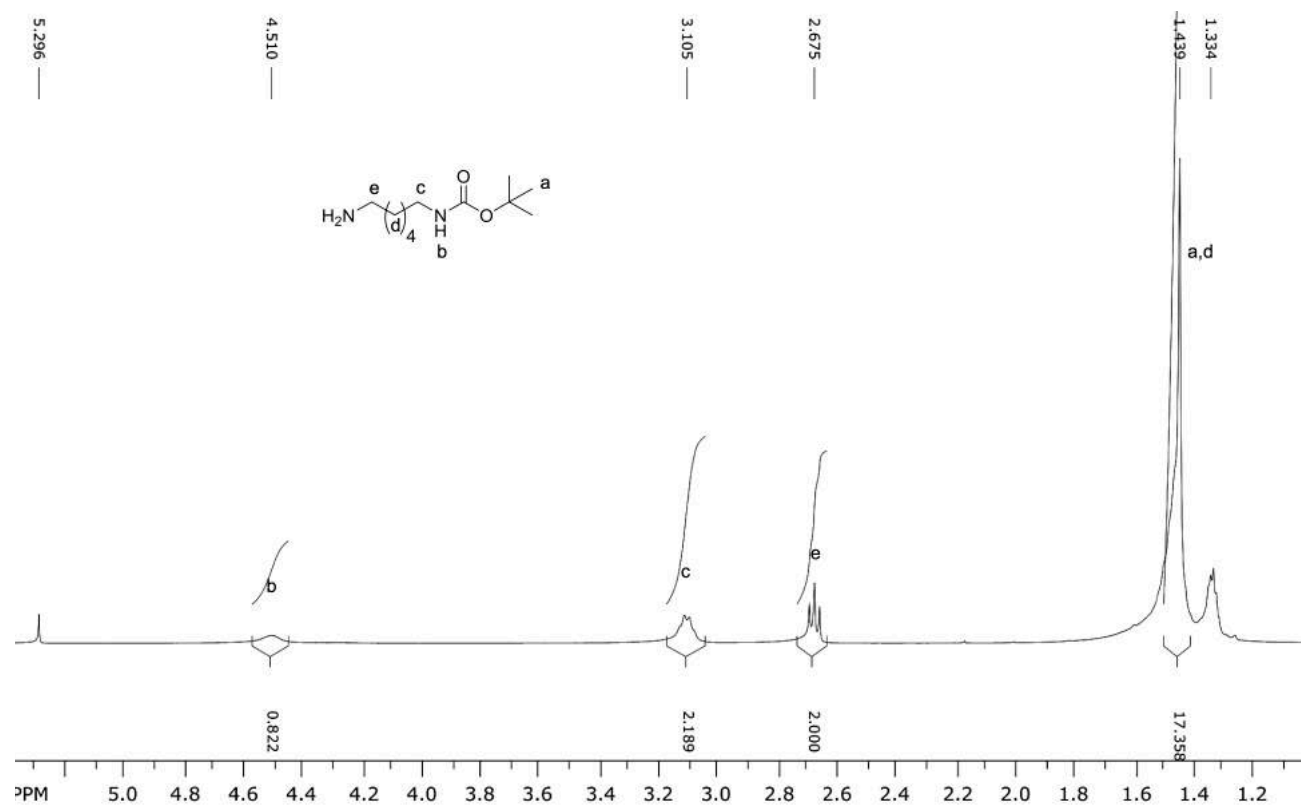


Figure S4 | ¹H NMR spectrum of Compound 4 (400 MHz, CDCl₃) in Bruker AVANCE III 400 MHz NMR instrument.

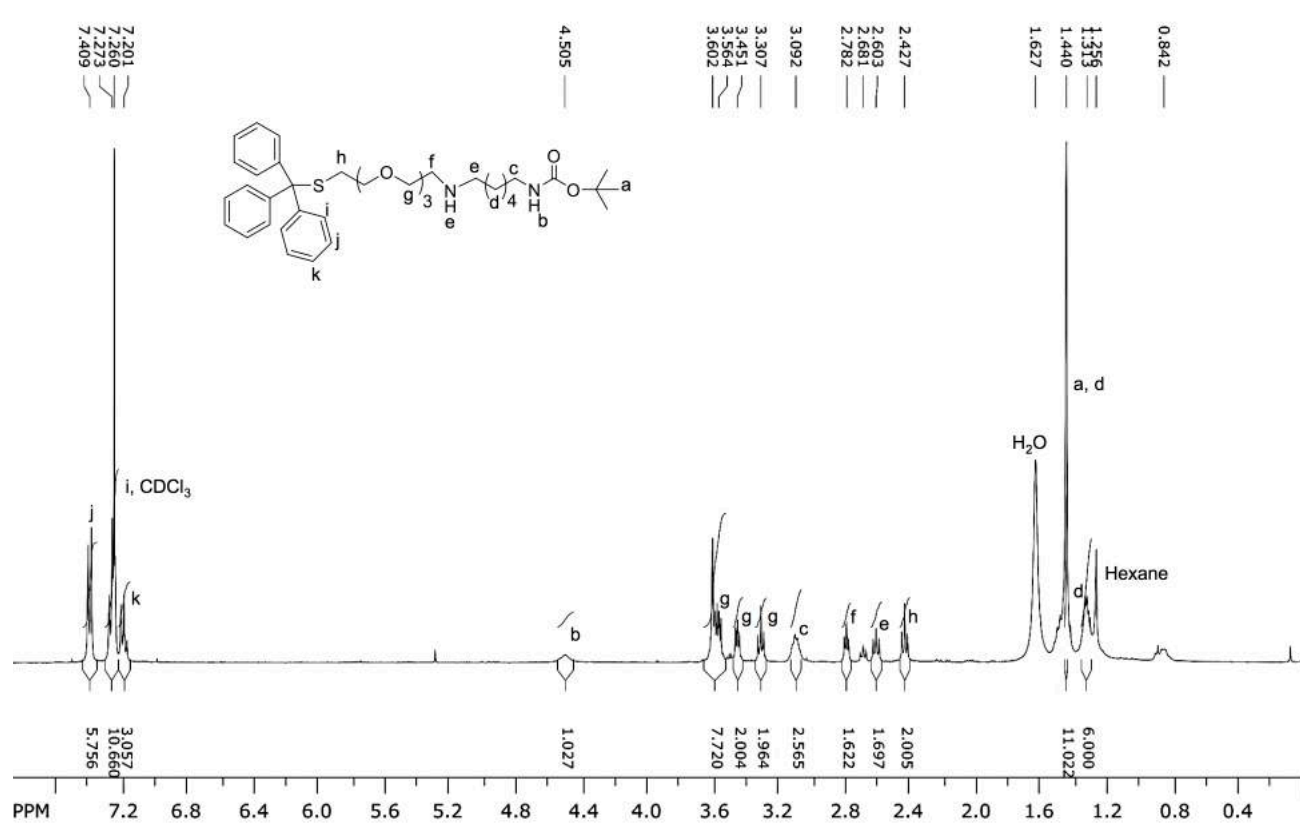


Figure S5 | ^1H NMR spectrum of Compound 5 (400 MHz, CDCl_3) in Bruker AVANCE III 400 MHz NMR instrument.

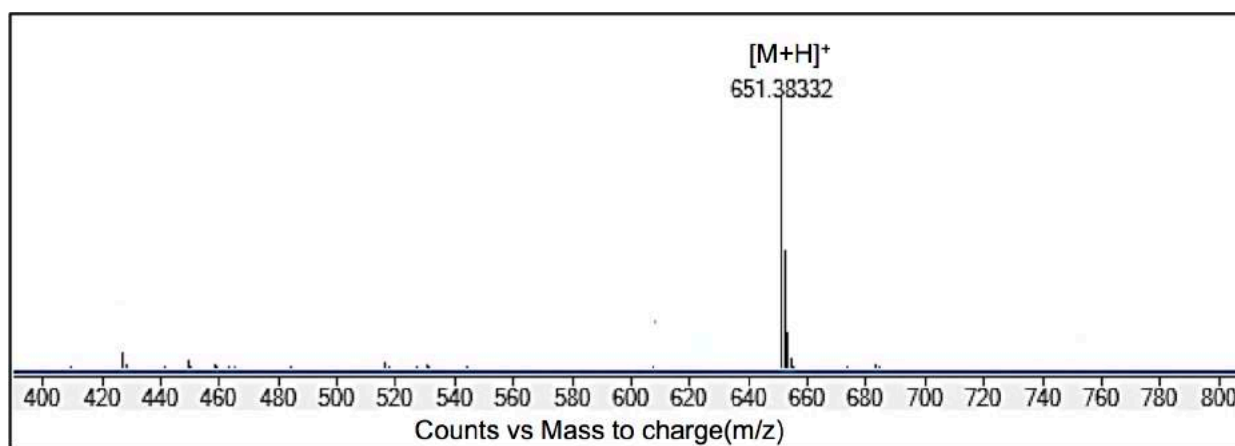


Figure S6 | HRMS spectrum of Compound 5.

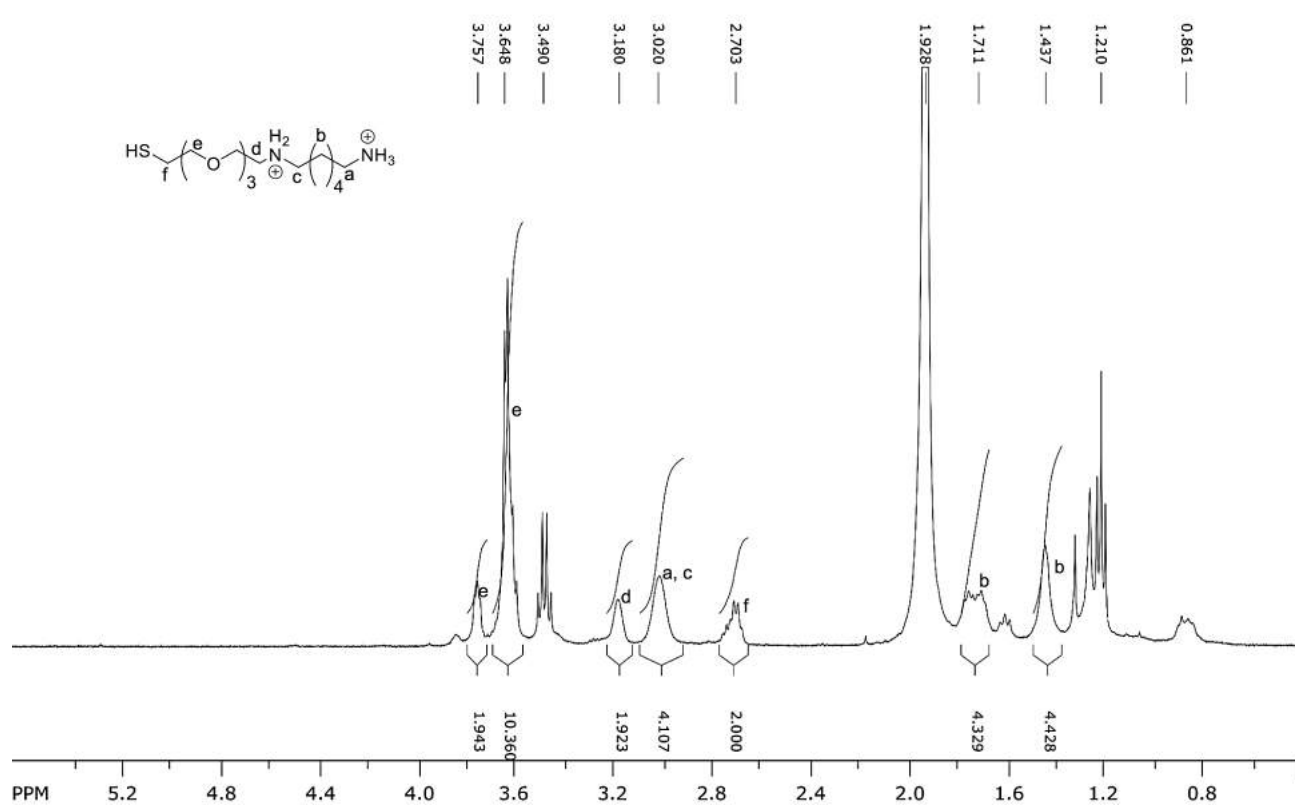


Figure S7 | ^1H NMR spectrum of Compound 6 (400 MHz, CDCl_3) in Bruker AVANCE III 400 MHz NMR instrument.

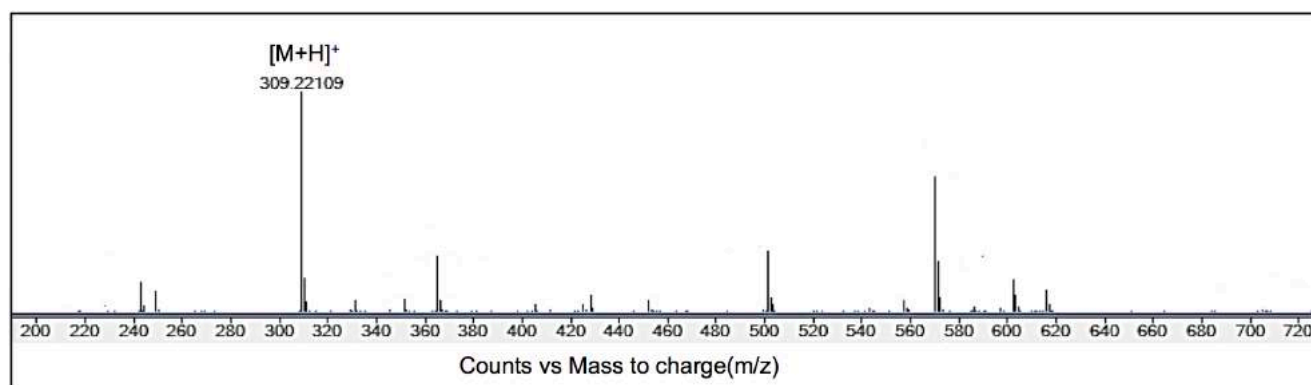
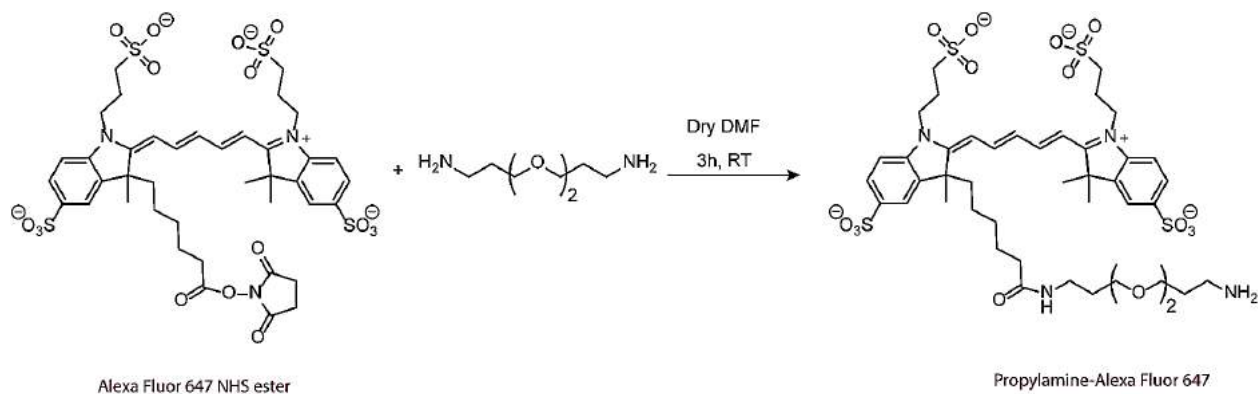


Figure S8 | HRMS spectrum of Compound 6.



Scheme S2 Synthetic scheme for the preparation of propylamine-Alexa Fluor 647 conjugate.

Synthesis of propylamine–Alexa Fluor 647: Diethylene glycol bis(3-aminopropyl) ether (TCI chemicals) (17.6 μg , 0.08 μmol) was taken in 1.5 ml microcentrifuge tube. Then Alexa Fluor 647 NHS ester (100 μg , 20 μl from 5 $\text{mg}\cdot\text{ml}^{-1}$ stock in DMF, 0.08 μmol) was added to it and the reaction mixture was incubated at room temperature for 3 h. After that the reaction mixture was diluted using water and injected to HPLC for purification. The blue colored eluent from the HPLC was collected and lyophilized to yield propylamine–Alexa Fluor 647 (86 μg , 91%). HRMS: calculated m/z 1059.3440 $[\text{M}+2\text{H}]^-$, 529.1684 $[\text{M}+\text{H}]^{2-}$, found 1059.4071 $[\text{M}+2\text{H}]^-$, 529.2307 $[\text{M}+\text{H}]^{2-}$. HPLC chromatogram and HRMS spectrum of propylamine–Alexa Fluor 647 have been shown in Figure S9 and S10 respectively.

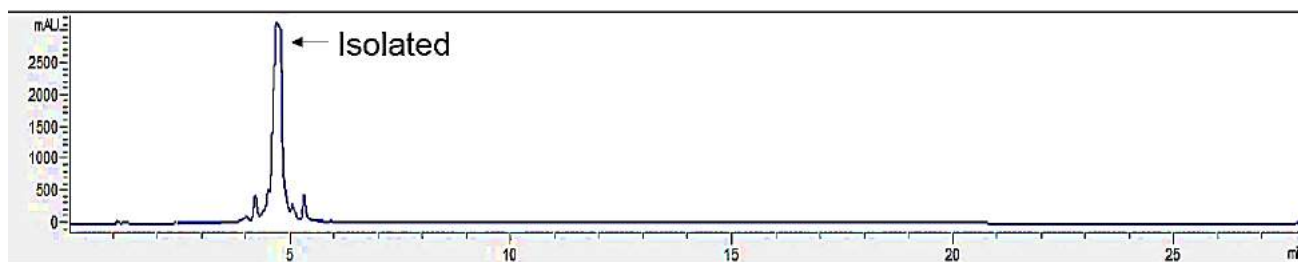


Figure S9 HPLC chromatogram of propylamine-Alexa 647 (crude mixture). The polarity of acetonitrile was changed from 5% to 100% over 25 min. The conjugated product was isolated at retention time 4.8 min. The absorbance was monitored at 647 nm.

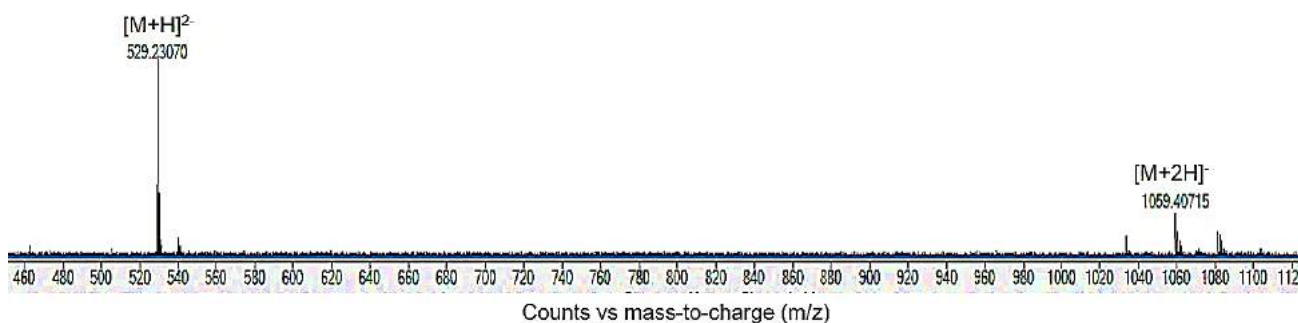
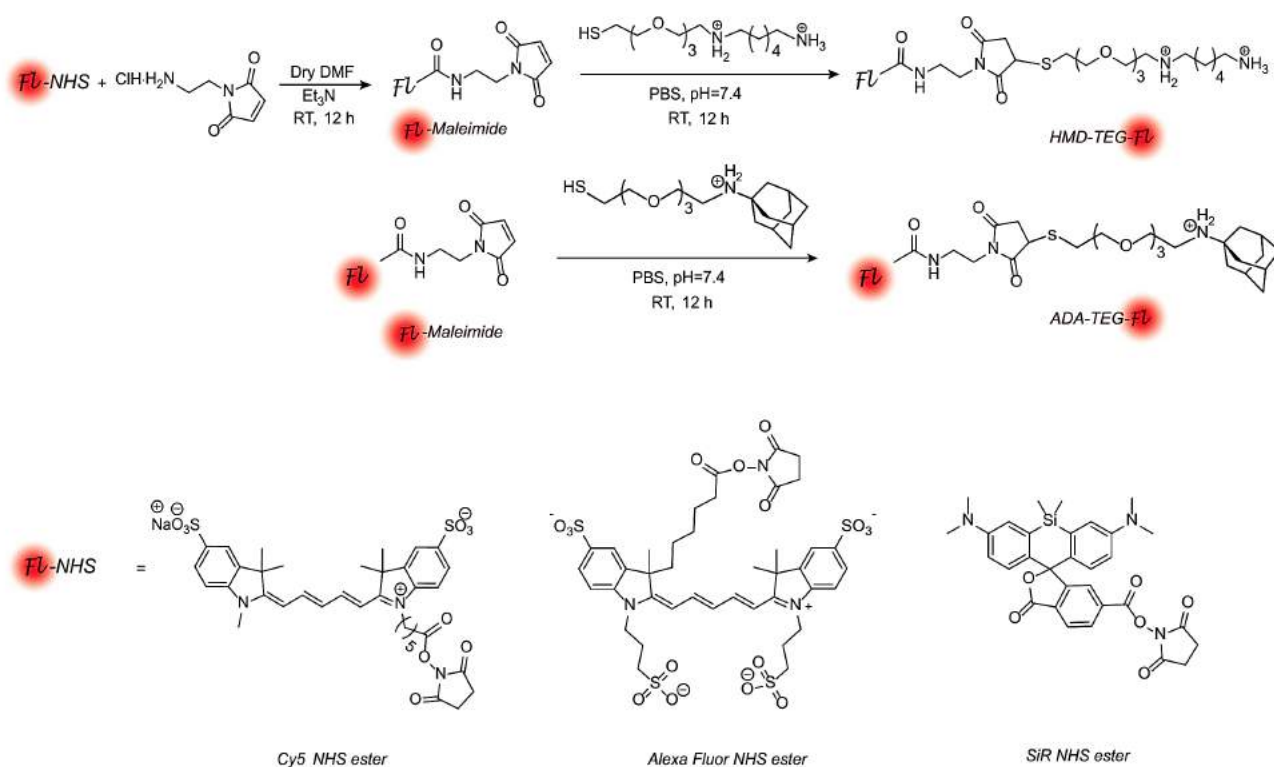


Figure S10 HRMS spectrum of propylamine–Alexa Fluor 647.



Scheme S3 | Synthetic scheme for the preparation of HMD imager and adamantylamine (ADA) imager with TEG spacer.

Synthesis of HMD-Cy5: Cy5 maleimide¹ (66.67 μg , 50 μl from 1.33 mg ml^{-1} stock solution in PBS (pH 7.4), 0.0876 μmol) was taken in 1.5 ml microcentrifuge tube. A stock solution of Compound 6 (16.4 μg , 50 μl from 0.82 mg ml^{-1} stock solution in PBS (pH 7.4), 0.1314 μmol) was added to the Cy5-maleimide solution and stirred for overnight at room temperature. After that the reaction mixture was directly injected to reverse phase HPLC for purification to yield HMD-Cy5 (90% from HPLC chromatogram). The purity of the isolated final compound was again analyzed by HPLC (see Figure S11). HRMS: (ESI-MS): calculated m/z 1073.4756 $[M+H]^+$, 537.2414 $[M+2H]^{2+}$; found 1073.4704 $[M+H]^+$, 537.2407 $[M+2H]^{2+}$. HPLC chromatogram and HRMS spectrum of HMD-Cy5 has been shown in Figure S11 and S12.

Synthesis of Alexa Fluor 647 maleimide: N-(2-aminoethyl) maleimide hydrochloride (20.186 μg , 2.0 μl from 10 mg ml^{-1} stock solution in DMF, 0.1143 μmol) was taken in 1.5 ml microcentrifuge tube. Triethyl amine (4 μL from 10 $\mu\text{l.ml}^{-1}$ stock in DMF (v/v), 0.2381 μmol) was added to it. Then Alexa Fluor 647 NHS ester (100 μg , 20 μl from 5 mg.ml^{-1} , 0.0953 μmol) was added to it and the reaction mixture was stirred at room temperature for 12 h. After that the reaction mixture was diluted using water and injected to HPLC for purification. The blue colored eluent from the HPLC was collected and lyophilized to yield Alexa Fluor 647 maleimide (56 μg , 69%). HRMS: ESI-MS:

calculated m/z 500.0993 $[M+Na]^{2-}$; found 500.1464 $[M+Na]^{2-}$. HPLC chromatogram and HRMS spectrum of Alexa Fluor 647 maleimide have been shown in Figure S13 and S14 respectively.

Synthesis of HMD-Alexa Fluor 647: Alexa Fluor 647 maleimide (5 μ g, 15.63 μ l from 0.315 mg ml^{-1} stock solution in PBS (pH 7.4), 5.1×10^{-3} μ mol) was taken in 1.5 ml microcentrifuge tube. A stock solution of SH-TEG-HMD (7.87 μ g, 7.87 μ l from 1 mg. ml^{-1} stock solution in PBS (pH 7.4), 0.026 μ mol) was added to the Alexa Fluor 647 maleimide solution and stirred for overnight at room temperature. After that the reaction mixture was directly injected to reverse phase HPLC for purification to yield HMD-Alexa Fluor 647 (90% from HPLC chromatogram). HRMS: (ESI-MS): calculated m/z 1287.4373 $[M+2H]^+$, 643.2150 $[M+H]^{2+}$; found 1287.4605 $[M+2H]^+$, 643.2602 $[M+H]^{2+}$. HPLC chromatogram and HRMS spectrum of HMD-Alexa Fluor 647 have been shown in Figure S15 and S16 respectively.

Synthesis of ADA-Alexa Fluor 647: Alexa Fluor 647 maleimide (5 μ g, 15.63 μ l from 0.315 mg ml^{-1} stock solution in PBS (pH 7.4), 5.1×10^{-3} μ mol)) was taken in 1.5 ml microcentrifuge tube. A stock solution of SH-TEG-ADA (8.93 μ g, 8.93 μ l from 1 mg. ml^{-1} stock solution in PBS (pH 7.4), 0.026 μ mol) was added to the solution and stirred for overnight at room temperature. After that the reaction mixture was directly injected in HPLC for purification to yield ADA-Alexa Fluor 647 (90% from HPLC chromatogram). HRMS: ESI-MS: calculated m/z 660.7174 $[M+H]^{2+}$, found 660.7213 $[M+H]^{2+}$. HPLC chromatogram and HRMS spectrum of ADA-Alexa Fluor 647 have been shown in Figure S17 and S18 respectively.

Synthesis of SiR maleimide: N-(2-aminoethyl) maleimide hydrochloride (62 μ g, 6.2 μ l from 10 mg ml^{-1} stock solution in DMF, 0.3510 μ mol) was taken in 1.5 ml microcentrifuge tube. Triethyl amine (1.22 μ L from 100 μ l. ml^{-1} stock in DMF (v/v), 0.8776 μ mol) was added to it. Then SiR NHS ester (100 μ g, 20 μ l from 5 mg. ml^{-1} 0.1756 μ mol) was added to it and the reaction mixture was incubated at room temperature for 12 h. After that the reaction mixture was diluted using water and injected to HPLC for purification. The blue colored eluent from the HPLC was collected and lyophilized to yield SiR maleimide (56 μ g, 69%). HRMS: calculated m/z 595.2371 $[M+H]^+$; found 595.2406 $[M+H]^+$. HPLC chromatogram and HRMS spectrum of SiR maleimide have been shown in Figure S19 and S20 respectively.

Synthesis of HMD-SiR: SiR maleimide (5 μ g, 8.33 μ l from 0.6 mg ml^{-1} stock solution in PBS (pH 7.4), 0.0084 μ mol) was taken in 1.5 ml microcentrifuge tube. A stock solution of SH-TEG-HMD (7.78 μ g, 7.78 μ l from 1 mg ml^{-1} stock solution in PBS (pH 7.4), 0.0252 μ mol) was added to the SiR-maleimide solution and stirred for overnight at room temperature. After that the reaction mixture was directly injected to reverse phase HPLC for purification to yield HMD-SiR (90% from HPLC

chromatogram). HRMS: (ESI-MS): calculated m/z 903.4505 $[M+H]^+$, 452.2289 $[M+2H]^{2+}$; found 903.4520 $[M+H]^+$, 452.2302 $[M+2H]^{2+}$. HPLC chromatogram and HRMS spectrum of HMD-SiR have been shown in Figure S21 and S22 respectively.

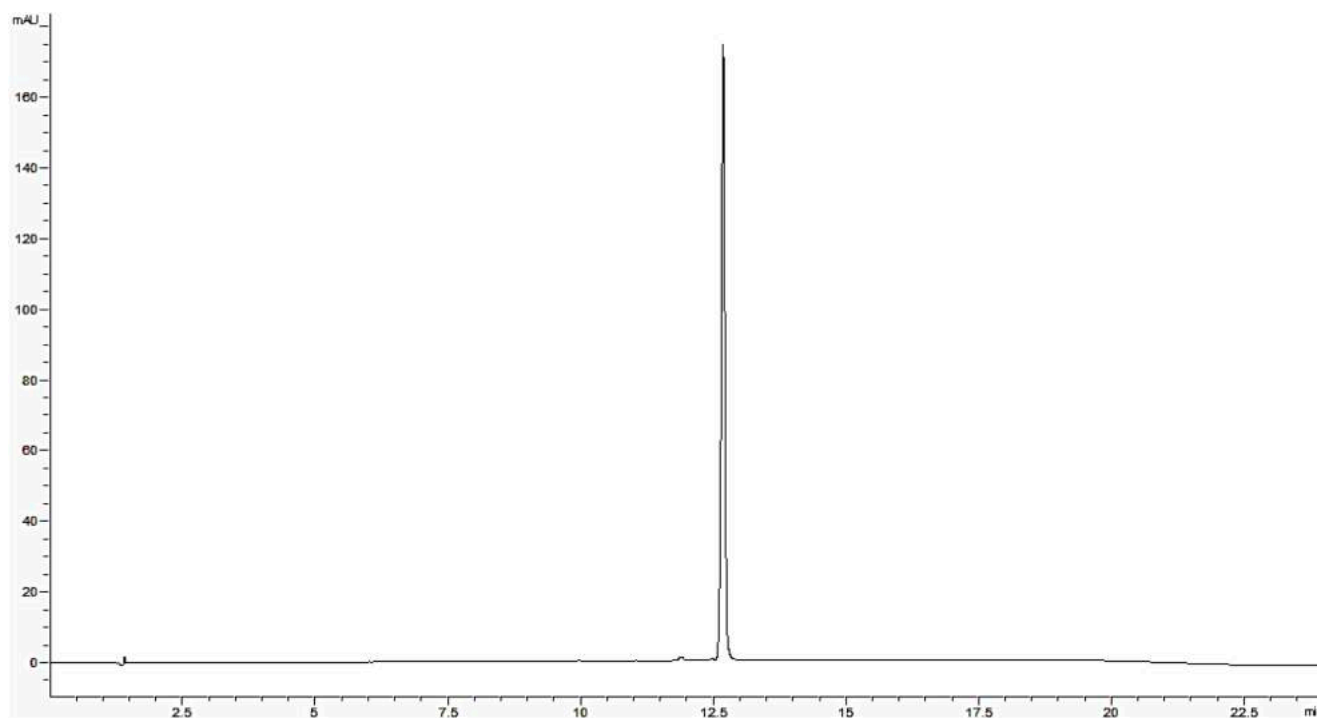


Figure S11 | HPLC chromatogram of HMD-Cy5 (purified product). The percentage of acetonitrile was changed from 5% to 35% over 20 min and HMD conjugated Cy5 was isolated at 12.8 min. The absorbance was monitored at 645 nm.

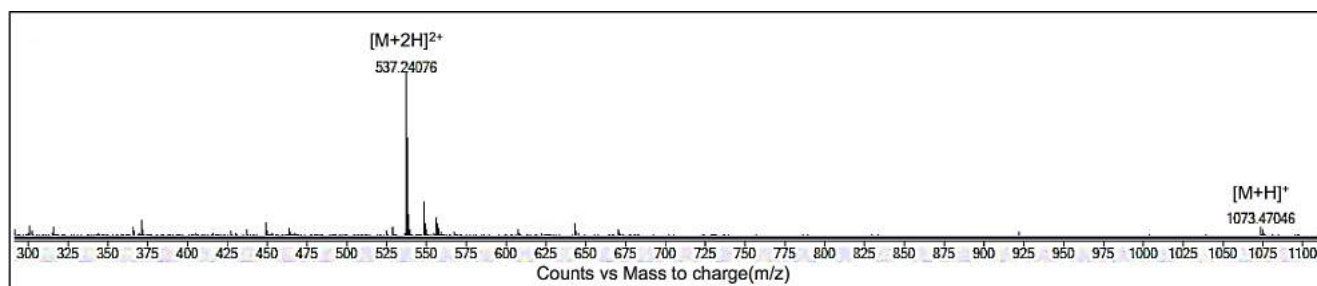


Figure S12 | HRMS spectrum of HMD-Cy5.

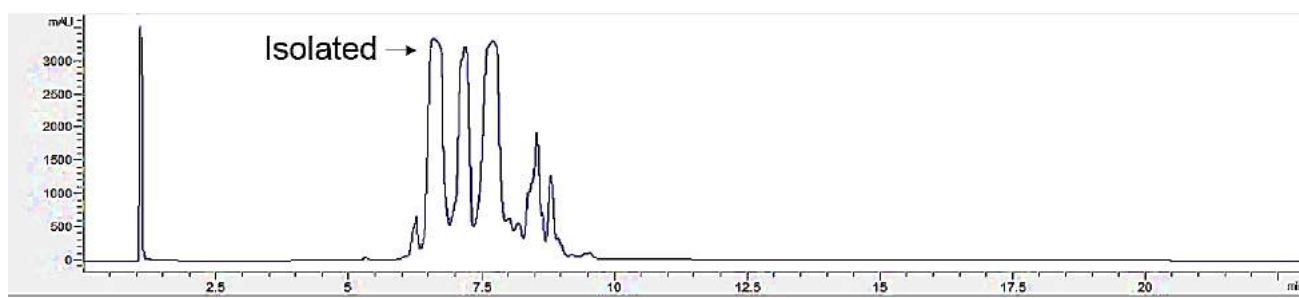


Figure S13| HPLC chromatogram of Alexa Fluor 647 maleimide (crude mixture). The percentage of acetonitrile was changed from 5% to 35% over 17 min and 100% at 25 min. The conjugated product was isolated at retention time 6.7 min. The absorbance was monitored at 647 nm.

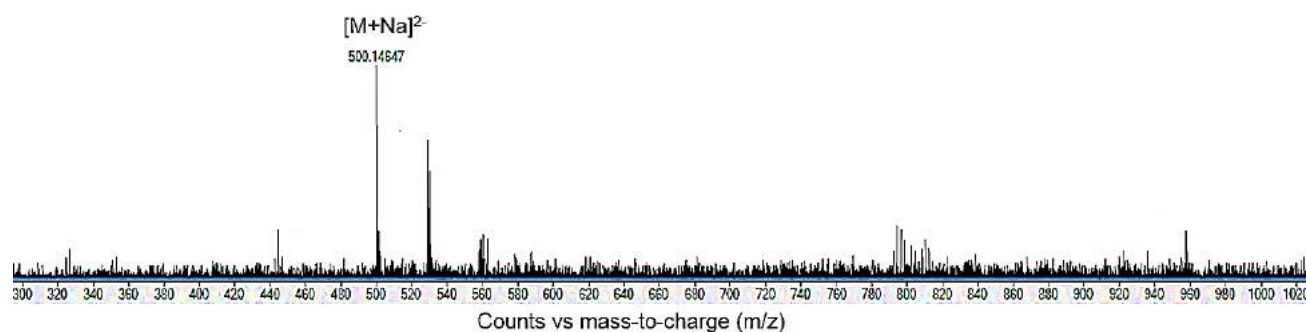


Figure S14| HRMS spectrum of Alexa Fluor 647 maleimide.

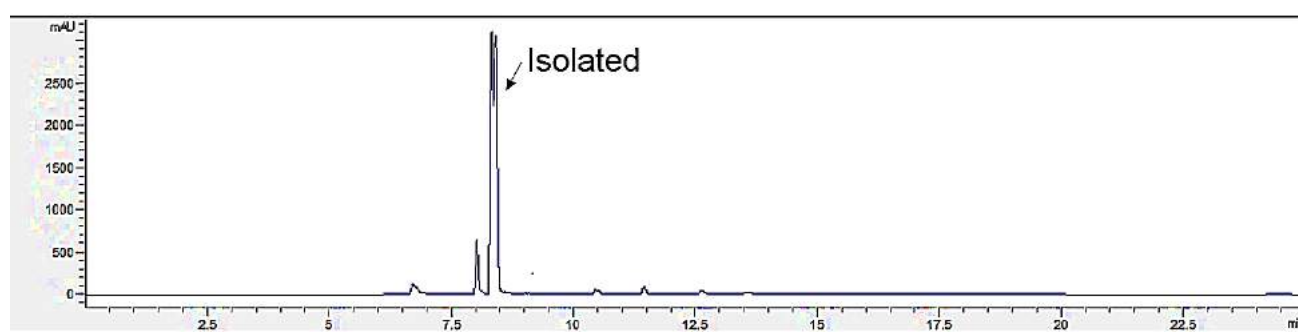


Figure S15| HPLC chromatogram of HMD-Alexa Fluor 647 (crude mixture). The percentage of acetonitrile was changed from 5% to 35% over 17 min and 100% at 25 min. The conjugated product was isolated at retention time 8.5 min. The absorbance was monitored at 647 nm.

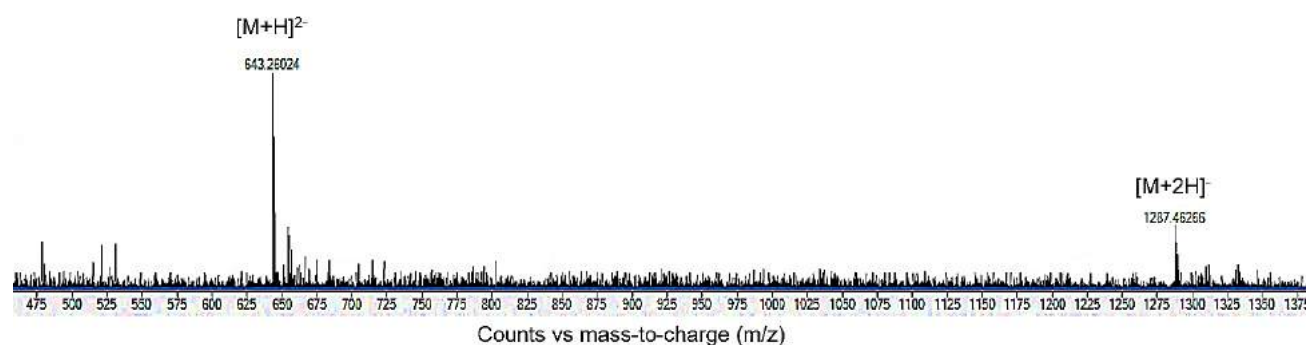


Figure S16| HRMS spectrum of HMD-Alexa Fluor 647.

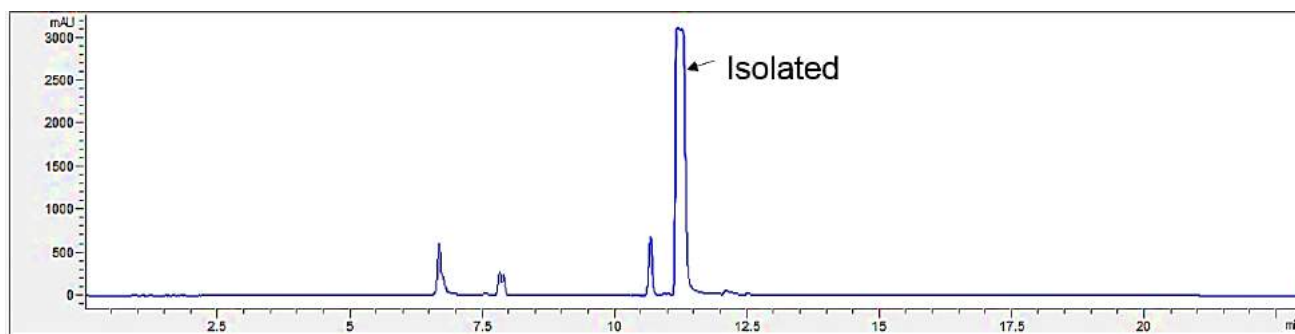


Figure S17 HPLC chromatogram of ADA-Alexa Fluor 647 (crude mixture). The percentage of acetonitrile was changed from 5% to 35% over 17 min and 100% at 25 min. The conjugated product was isolated at retention time 11.3 min. The absorbance was monitored at 647 nm.

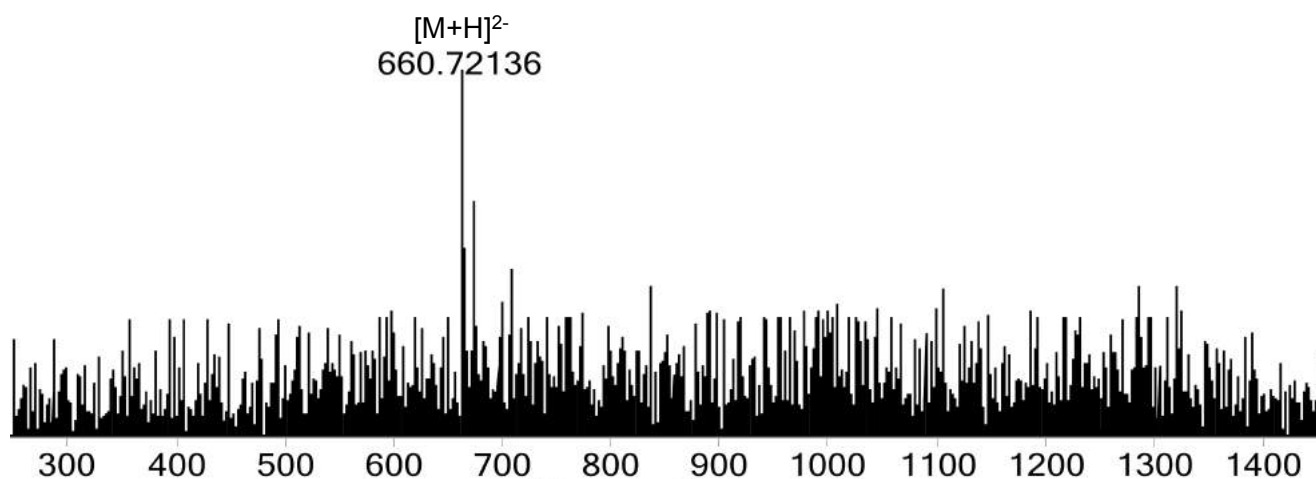


Figure S18 HRMS spectrum of ADA-Alexa Fluor 647.

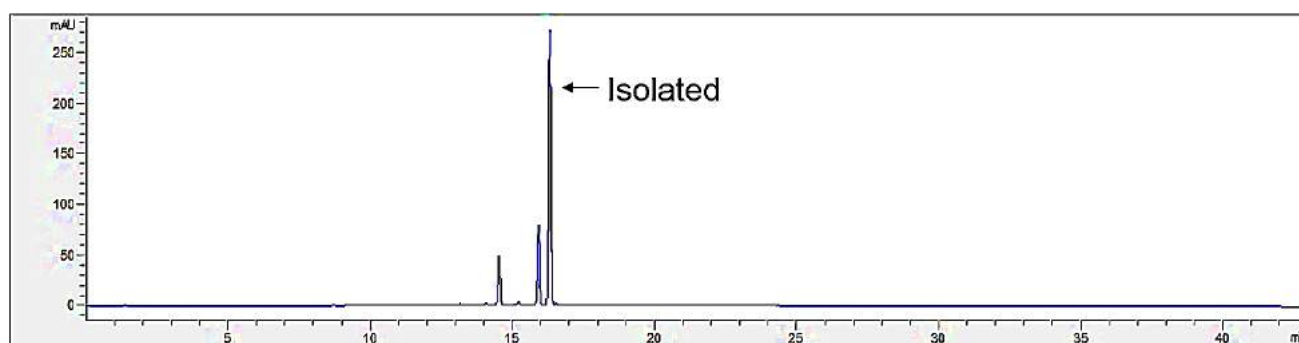


Figure S19 HPLC chromatogram of SiR maleimide (crude mixture). The percentage of acetonitrile was changed from 5% to 100% over 40 min. The conjugated product was isolated at retention time 16.3 min. The absorbance was monitored at 650 nm.

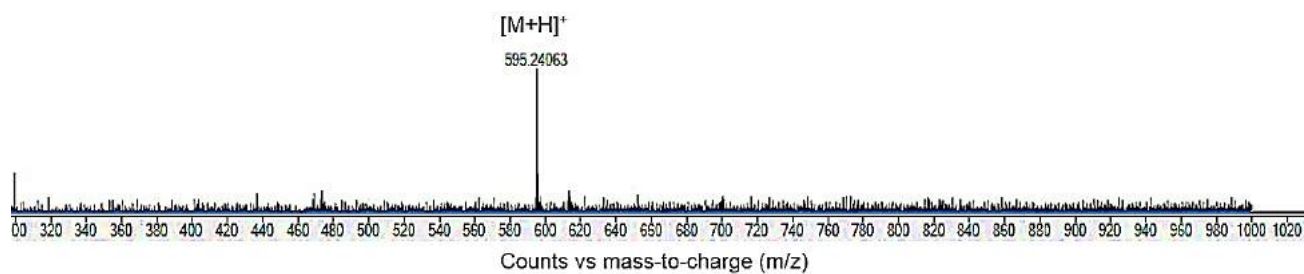


Figure S20 | HRMS spectrum of SiR maleimide.



Figure S21 | HPLC chromatogram of HMD-SiR (crude mixture). The percentage of acetonitrile was changed from 5% to 50% over 20 min and 100% at 22 min. The conjugated product was isolated at retention time 13.8 min. The absorbance was monitored at 650 nm.

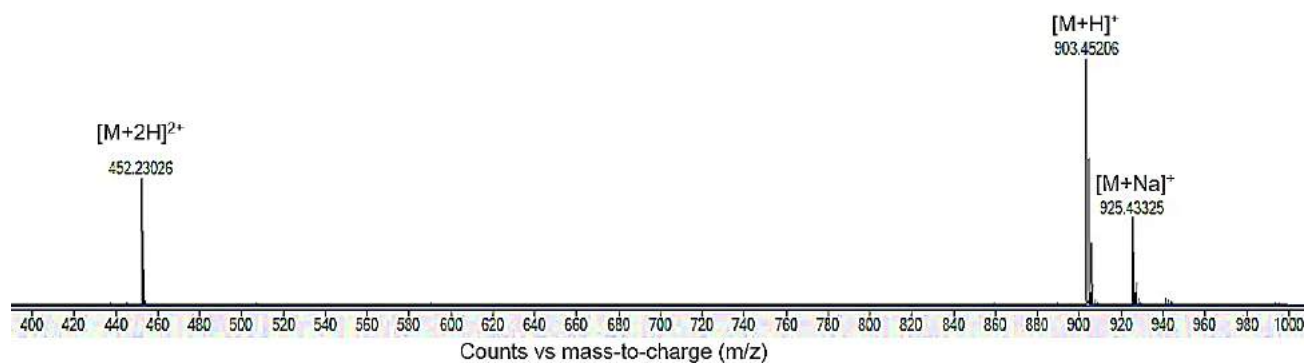
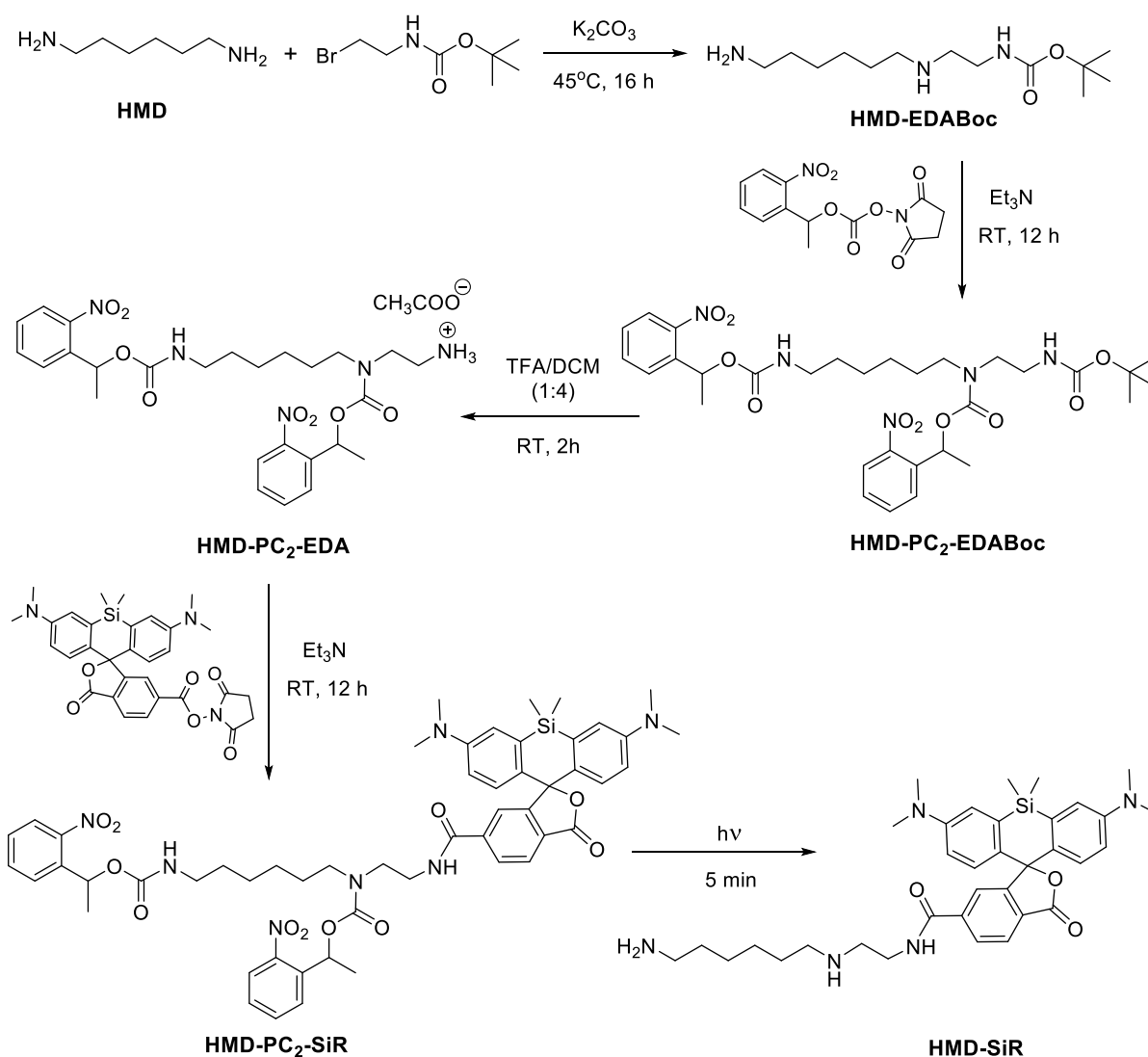


Figure S22 | HRMS spectrum of HMD-SiR with TEG spacer.

Synthesis of HMD conjugated SiR without TEG spacer:



Scheme S4 | Synthetic scheme for the preparation of HMD–SiR imager without TEG spacer.

Synthesis of HMD–EDABoc

To a stirred solution of HMD (500 mg, 4.31 mmol) in DMF, K_2CO_3 (1.79 g, 12.97 mmol) was added followed by slow addition of N–Boc bromo ethyl amine³ (90 mg, 0.403 mmol). The reaction mixture was stirred at $45^\circ C$ for 16 h. After that reaction was quenched using water and extracted with DCM. The crude reaction mixture was purified by column chromatography using DCM/MeOH as eluent. The purified product was characterized by 1H NMR. 1H NMR (600 MHz, $DMSO-d_6$): δ 1.35 (9H, s, –NH**Boc**), 1.51 (8H, m, –(**CH₂**)₄–), 2.60 (2H, t, –N**CH₂**–), 2.68 (4H, m, –N**CH₂**–), 2.70 (2H, m, –N**CH₂**–), 6.82 (1H, br, –NH–), 8.44 (1H, br, –NH–). 1H NMR spectrum was shown in Figure S23.

Synthesis of HMD-PC₂-EDABoc

HMD-EDABoc (100 mg, 0.3846 mmol) was dissolved in 1 ml dry DMF and triethyl amine (0.160 ml, 1.1538 mmol) was added into it. Disuccinimidyl bicarbonate (355.37 mg, 1.1538 mmol) was added to the reaction mixture and stirred for 10 h in argon atmosphere. The reaction was quenched using water and extracted with DCM. The crude reaction mixture was purified using DCM/MeOH as eluent. The purified product was characterized by using ¹H NMR and LCMS. ¹H NMR (600 MHz, CDCl₃): δ 1.25 (9H, s, -NH₂Boc), 1.40 (8H, m, -(CH₂)₄-), 1.64 (6H, d, -CH₃), 3.07 (8H, m, -NCH₂-), 3.65 (1H, br, -NH-), 6.22 (2H, br, -CH-), 7.40 (4H, t, -ArH-), 7.60 (2H, t, -ArH-), 7.91 (2H, t, -ArH-). LCMS (ESI-MS): calculated 668.29 [M+Na]⁺, 684.26 [M+K]⁺; found 668.74 [M+Na]⁺, 684.90 [M+K]⁺. ¹H NMR and LCMS spectra was shown in Figure S24 and Figure S26 respectively.

Synthesis of HMD-PC₂-EDA

HMD-PC₂-EDABoc (20 mg, mmol) was dissolved in 1 ml DCM/TFA (1:1) and allowed to stir at room temperature for 3 hours. Then the reaction mixture was evaporated to dryness and. Finally, the product was dissolved in ethyl acetate and washed twice with water and 2N NaOH solution. The ethyl acetate layer was evaporated and characterized by ¹H NMR and LCMS. ¹H NMR (CDCl₃, 600 MHz): δ 1.25–1.43 (8H, m, -(CH₂)₄-), 1.62 (6H, d, -CH₃), 2.07 (2H, br, -NHCH₂), 3.08 (6H, br, -NCH₂-), 4.76 (1H, br, -NH-), 6.21 (2H, br, -CH-), 7.40 (2H, t, -ArH-), 7.60 (4H, t, -ArH-), 7.91 (2H, br, -ArH-). LCMS (ESI-MS): Calculated 546.26 [M+H]⁺; found 546.11 [M+H]⁺. ¹H NMR and LCMS spectra was shown in Figure S25 and Figure S27 respectively. The characterized product was used for the next step without further purification.

Synthesis of HMD-PC₂-SiR

HMD-PC₂-EDA (144.0 μg, 0.2632 μmol, 14.4 μl from 10 mg.ml⁻¹ DMSO stock) was taken in a 0.5 ml microcentrifuge tube and Et₃N (26.6 μg, 0.2632 μmol, 3.65 μl from 1% Et₃N stock in DMSO) was added into it. Then SiR NHS ester (50 μg, 0.0877 μmol, 5 μl from 10 mg.ml⁻¹ DMSO stock) was added to it and stirred at room temperature for 6 h. After that the reaction mixture was diluted using water and injected to HPLC for purification. The purified product was characterized by LCMS. LCMS: ESI-MS: calculated m/z 1000.43 [M+H]⁺; found 1000.73 [M+H]⁺. HPLC chromatogram and LCMS spectrum of HMD-PC₂-SiR have been shown in Figure S28.

Synthesis of HMD-SiR

HMD-PC₂-SiR was dissolved in 50 μl acetonitrile/water (1:1, v/v) and subjected to photoirradiation using 365 nm UV light (50 mW.cm⁻²) for 5 min. The photo irradiated product was injected to HPLC to purify and characterized by HRMS. HRMS (ESI-MS): Calculated 614.3521 [M+H]⁺, 307.6797

$[M+2H]^{2+}$; found 614.3464 $[M+H]^+$, 307.6775 $[M+2H]^{2+}$; HPLC chromatogram and HRMS spectrum of HMD–SiR were shown in Figure S29.

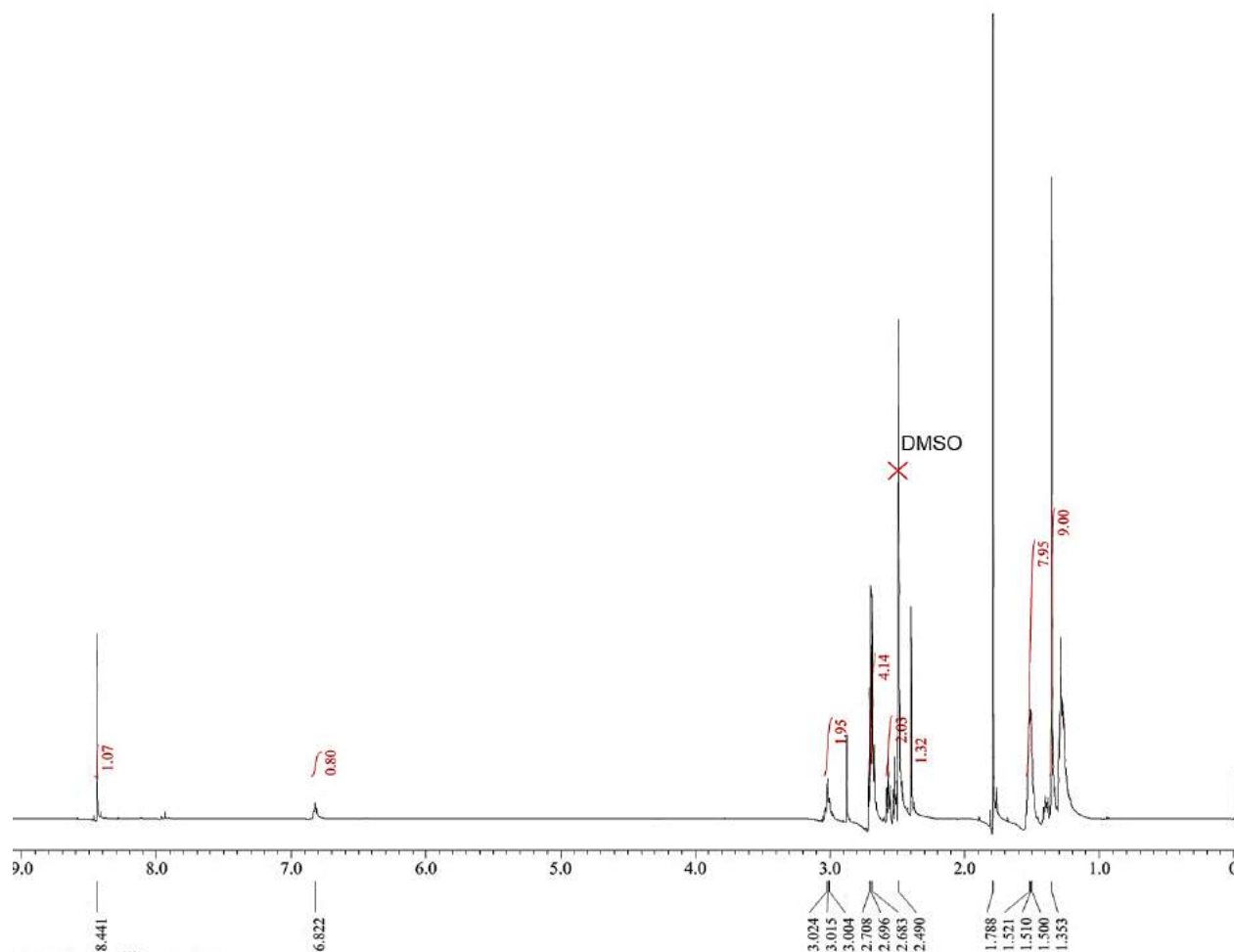


Figure S23 | ^1H NMR spectrum of HMD–EDABoc (600 MHz, DMSO-d_6) in JEOL Delta 600 MHz NMR instrument.

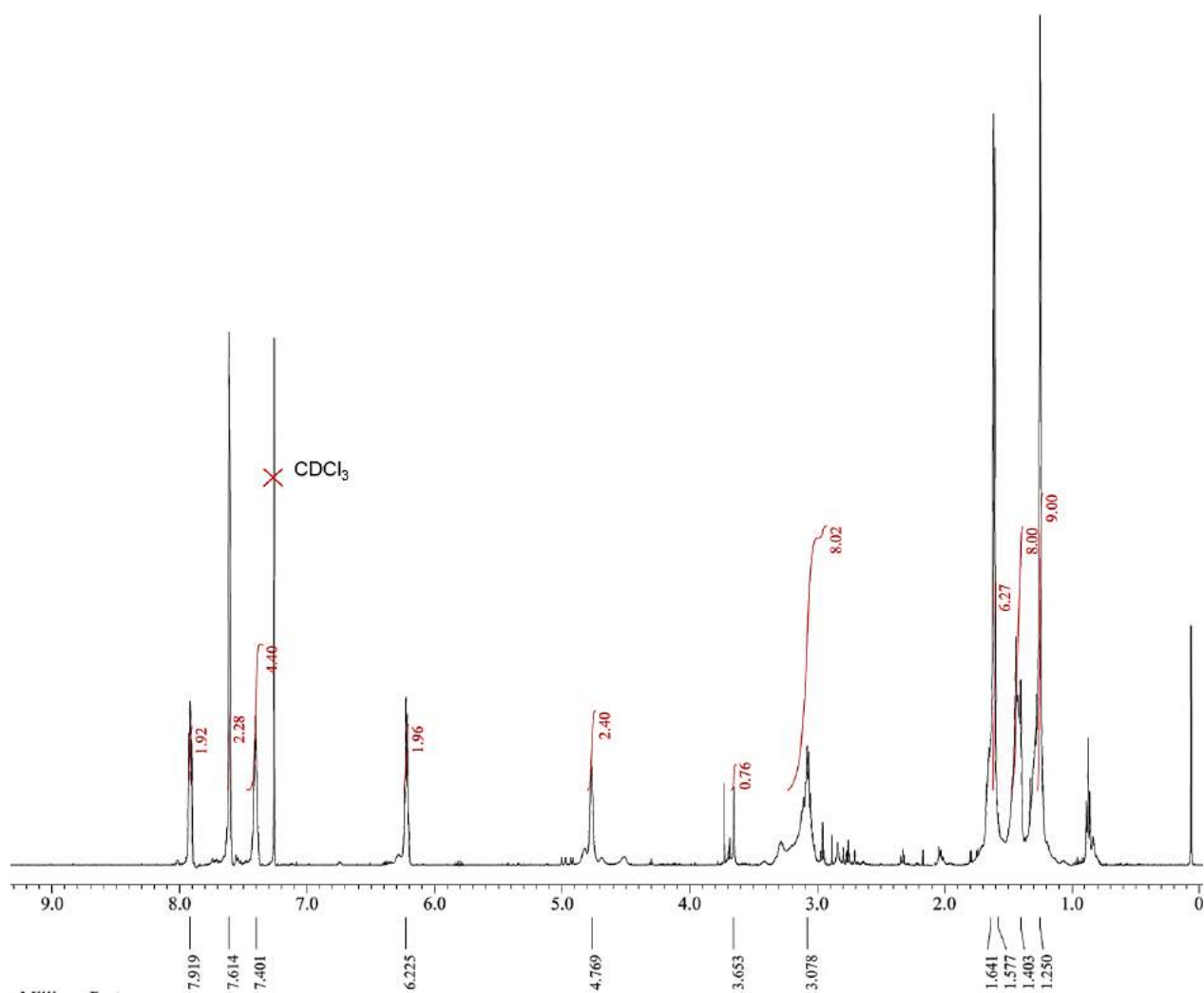


Figure S24 | ^1H NMR spectrum of HMD-PC₂-EDABoc (600 MHz, DMSO- d_6) in JEOL Delta 600 MHz NMR instrument.

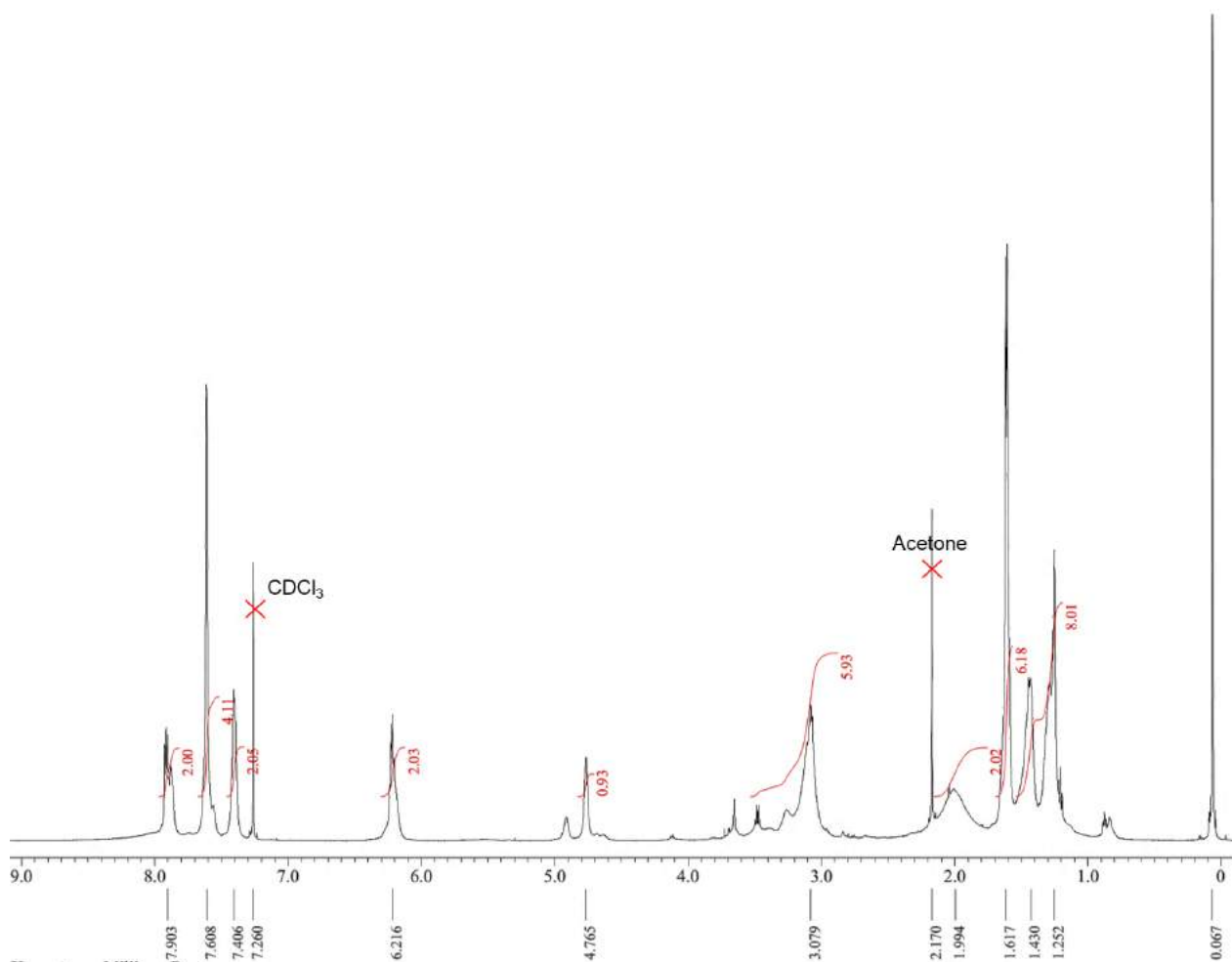


Figure S25 | ^1H NMR spectrum of HMD-PC₂-EDA (600 MHz, DMSO- d_6) in JEOL Delta 600 MHz NMR instrument.

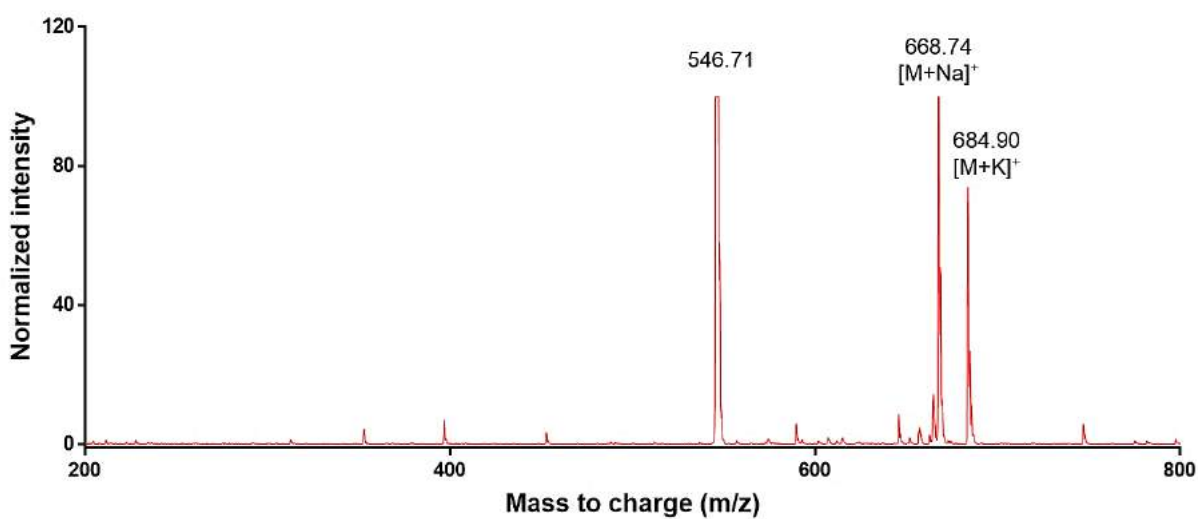


Figure S26 | LCMS spectrum of HMD-PC₂-EDABoc.;

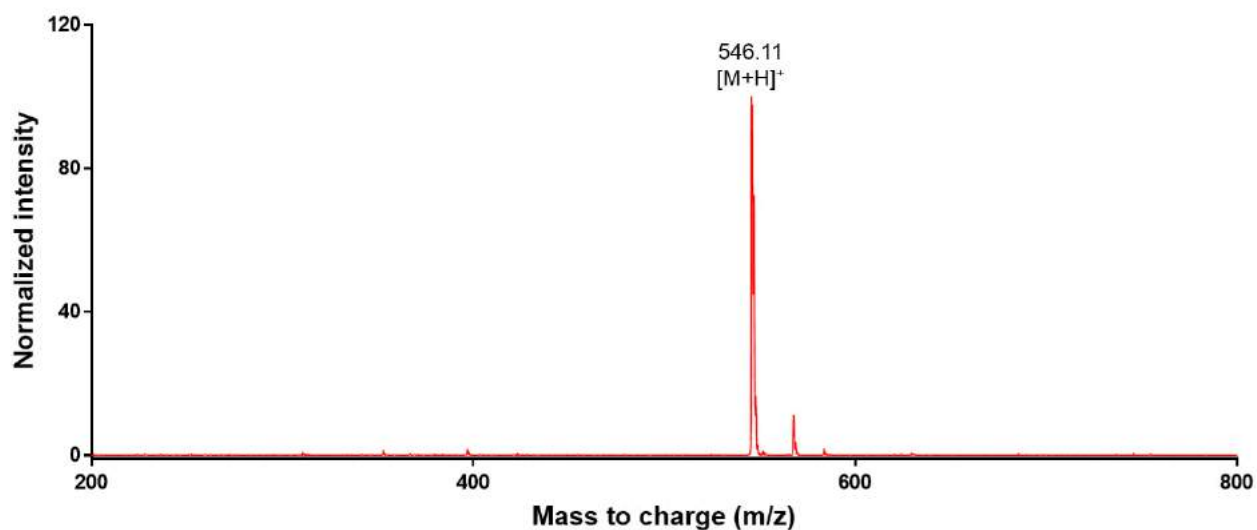


Figure S27 | LCMS spectrum of HMD-PC₂-EDA.

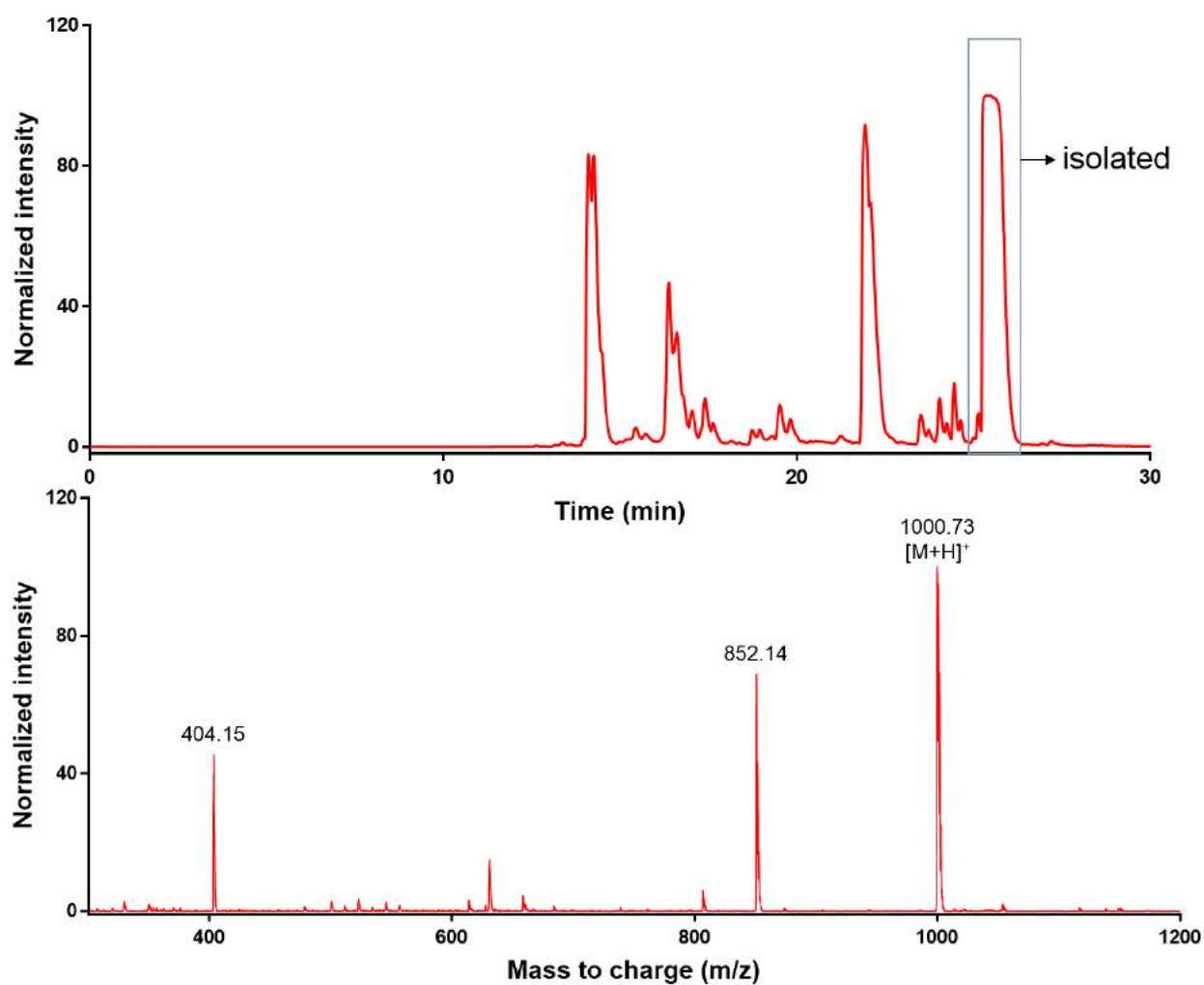


Figure S28 | HPLC chromatogram and LCMS spectrum of HMD-PC₂-SiR conjugate.

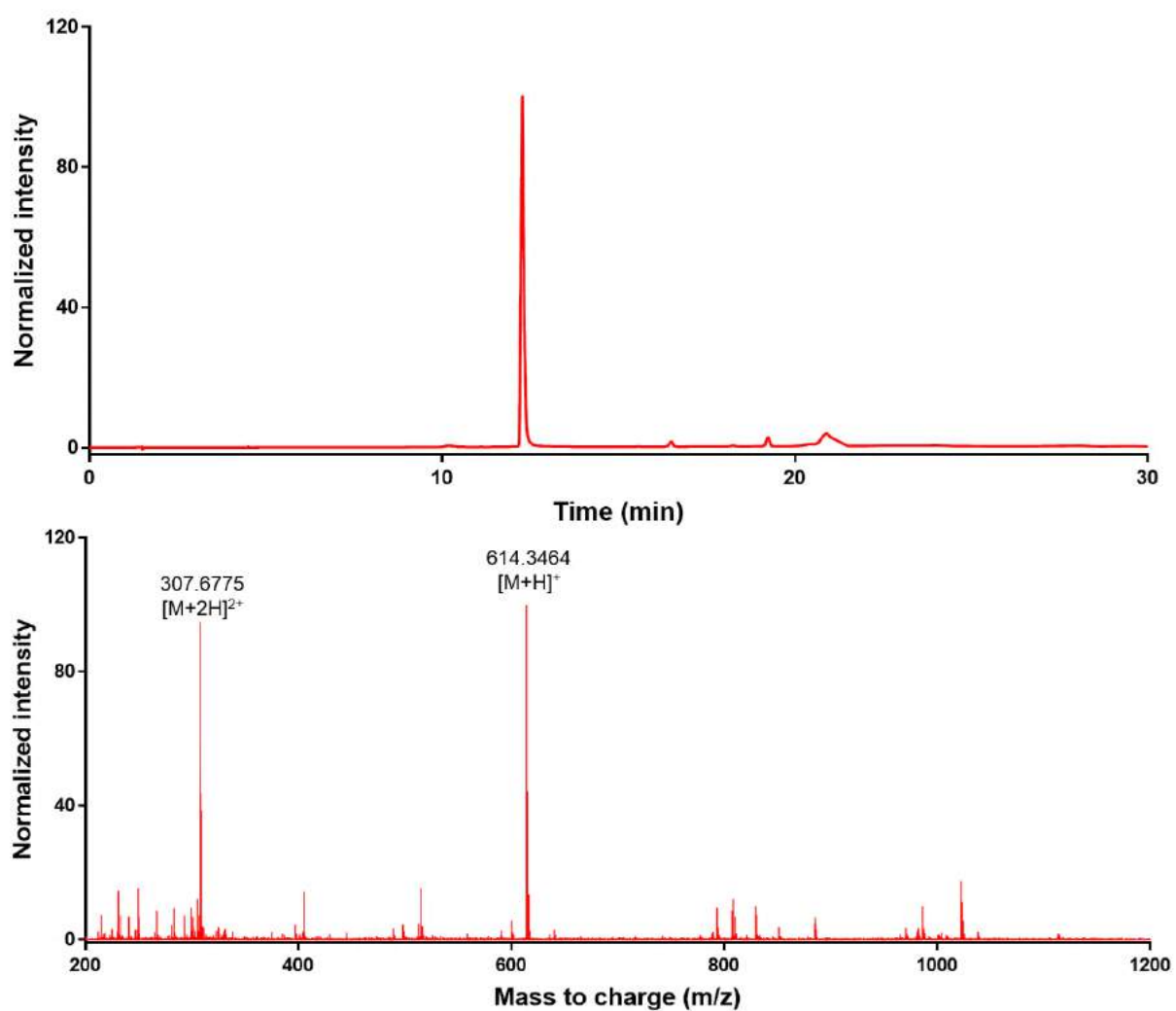
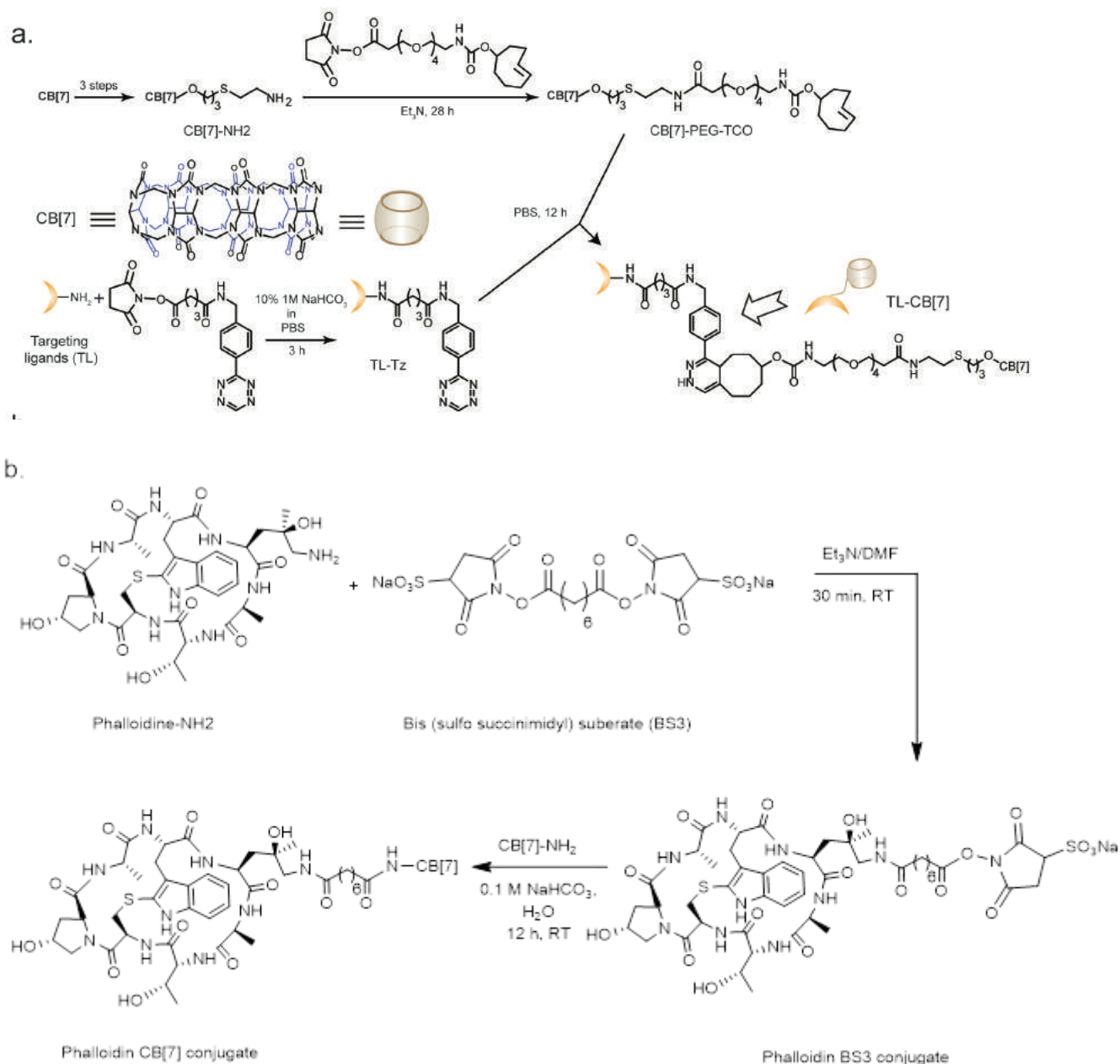


Figure S29 HPLC chromatogram and HRMS spectrum of HMD-SiR conjugate.

Synthesis of CB[7] conjugated targeting ligands (TLs):



Scheme S5| (a) Synthetic scheme for preparation of CB[7] conjugated antibodies and phalloidin through TCO–tetrazine ligation. Preparation of amine modified CB[7] and conjugation with antibodies and phalloidin were carried out in our reported manuscript¹. (b) Synthetic scheme for preparation of CB[7] conjugated phalloidin with minimal spacer.

Synthesis of CB[7] conjugated phalloidin with minimal spacer:

1. Amino phalloidin (80 μg , 16 μl from 5 mg ml^{-1} stock solution in dry DMF, 0.1016 μmol) was taken in 0.5 ml microcentrifuge tube.
2. 4.2 μl DMF solution containing 0.042 μl Et₃N was added to it.

3. Bis (sulfo succinimidyl) suberate (BS³) (1.5 mg, 2.62 μ mol, dissolved in 30 μ l DMF) was added to it and the reaction was stirred at room temperature for 30 min.
4. After the reaction, the reaction mixture was diluted using 50 μ l of miliQ water and injected to HPLC for purification using water/acetonitrile containing 0.1% TFA as eluent.
5. After purification it was characterized by LCMS. LCMS (ESI-MS): Calculated m/z 1121.39 [M+H]⁺, 561.20 [M+2H]²⁺; found 1121.69 [M+H]⁺, 561.70 [M+2H]²⁺ (see figure S30 and S31).
6. Phalloidin-BS³ conjugate (considering quantitative yield, 0.1016 μ mol) in 50 μ l H₂O was taken in 0.5 ml microcentrifuge tube and CB[7]-NH₂ (658.4 μ g, 50 μ l solution in H₂O, 0.5080 μ mol) was added to it and stirred at room temperature for 12 h to prepare CB[7] conjugated phalloidin.
7. CB[7] conjugated phalloidin was then purified by HPLC using water/acetonitrile containing 0.1% TFA as eluent.
8. The purified CB[7] conjugated phalloidin was characterized by LCMS. LCMS (ESI-MS): Calculated m/z 1187.93 [M + Cystamine dihydrochloride + 2H]²⁺; found 1188.10 [M + Cystamine dihydrochloride+2H]²⁺. (see figure S30 and S32).

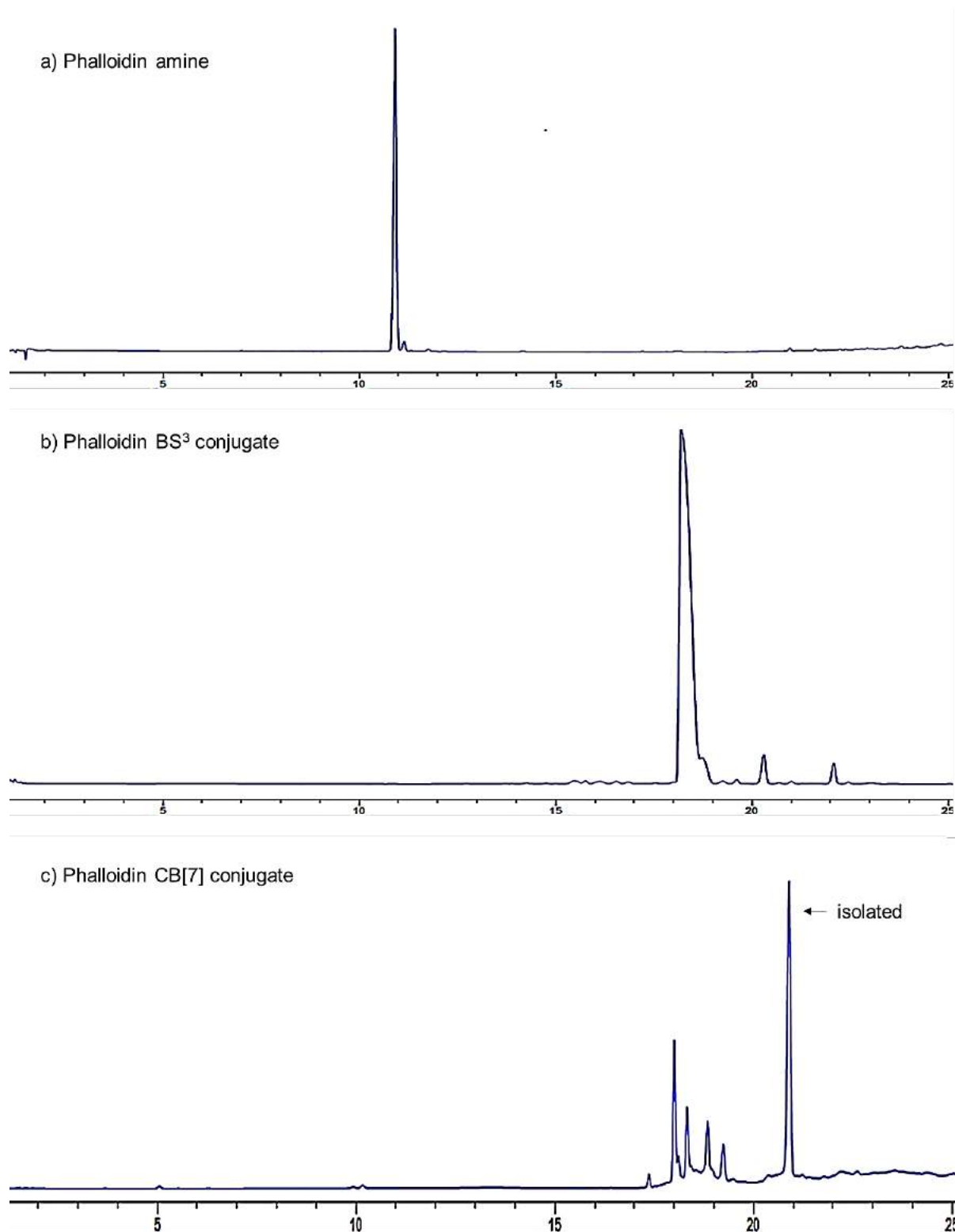


Figure S30 HPLC chromatogram of a) phalloidin amine, b) phalloidin-BS³ conjugate and c) phalloidin-CB[7] conjugate. HPLC purification was carried out by using water (0.1% TFA) and acetonitrile (0.1% TFA) as eluent. Acetonitrile polarity was changed from 5% to 35% over 25 min and upto 100% in 26 min. CB[7] conjugated phalloidin was isolated at Retention time 21 min.

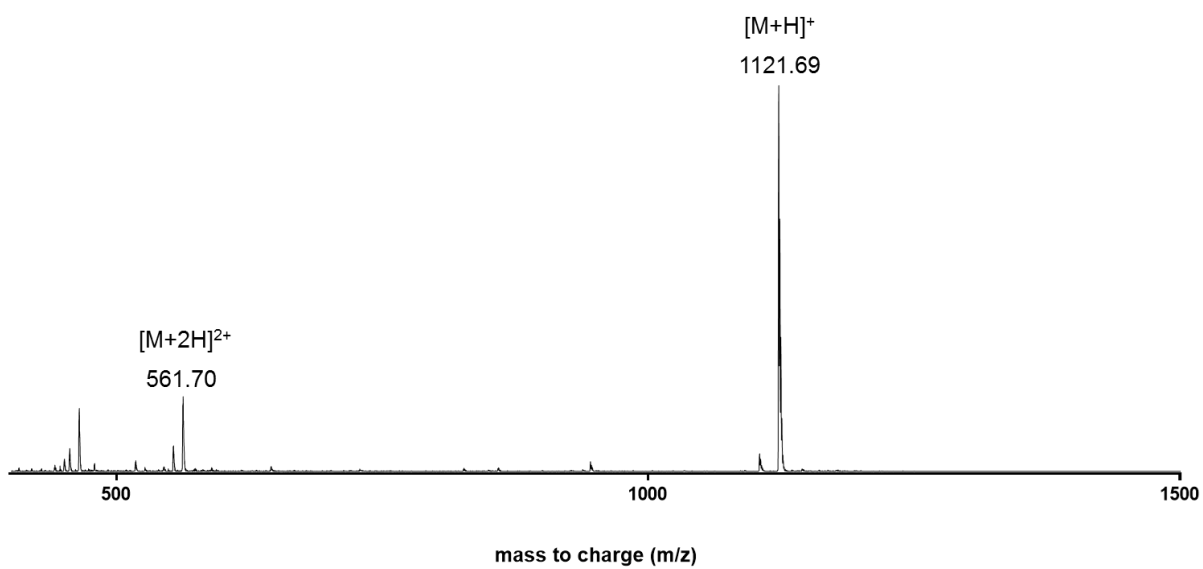


Figure S31 | LCMS of phalloidin-BS³ conjugate.

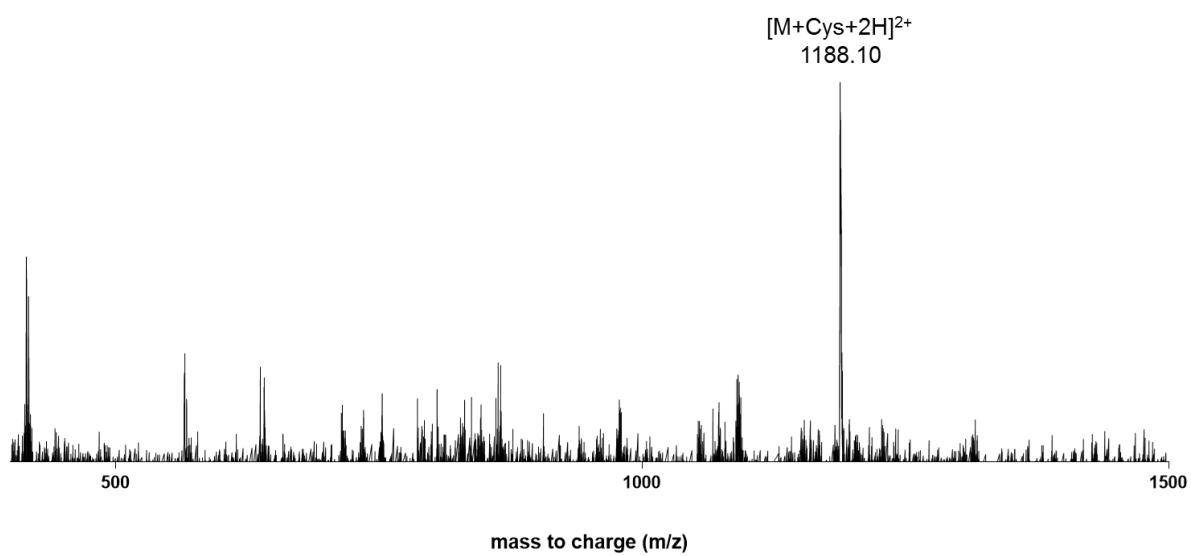
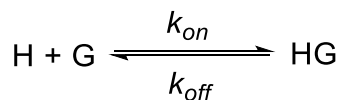


Figure S32 | LCMS of CB[7] conjugated phalloidin.

Estimation of residence time (i.e., binding time, τ_b) of the guest molecules:



For the above host-guest binding equilibria, the residence time (τ_b , bright time) is correlated to the dissociation rate constant (k_{off}) as, $\tau_b = 1/k_{\text{off}}$.⁴

As reported in the cited literatures and considering close to diffusion-controlled kinetics of host-guest binding, the association rate constant (k_{on}) for all the guest towards CB[7] can be considered on the order of $10^8 \text{ M}^{-1}\text{s}^{-1}$.^{5,6}

Now, for the propylamine guest with K_a (association constant) on the order of $10^3 - 10^4 \text{ M}^{-1}$, the k_{off} ($k_{\text{off}} = k_{\text{on}} / K_a$) can be estimated as $10^5 - 10^6 \text{ s}^{-1}$. The corresponding residence time τ_b ($\tau_b = 1/k_{\text{off}}$) will be around 0.01–0.001 ms.

For the HMD guest with K_a (association constant) on the order of $10^6 - 10^7 \text{ M}^{-1}$, the k_{off} ($k_{\text{off}} = k_{\text{on}} / K_a$) can be estimated as $10^2 - 10^1 \text{ s}^{-1}$. The corresponding residence time τ ($\tau = 1/k_{\text{off}}$) will be around 10–100 ms.

For the ADA guest with K_a (association constant) on the order of $10^{12} - 10^{13} \text{ M}^{-1}$, the k_{off} ($k_{\text{off}} = k_{\text{on}} / K_a$) can be estimated as $10^{-4} - 10^{-5} \text{ s}^{-1}$. The corresponding residence time τ ($\tau = 1/k_{\text{off}}$) will be around $10^7 - 10^8$ ms.

Comparison of non-specific binding behaviour of HMD-SiR fluor with and without TEG spacer:

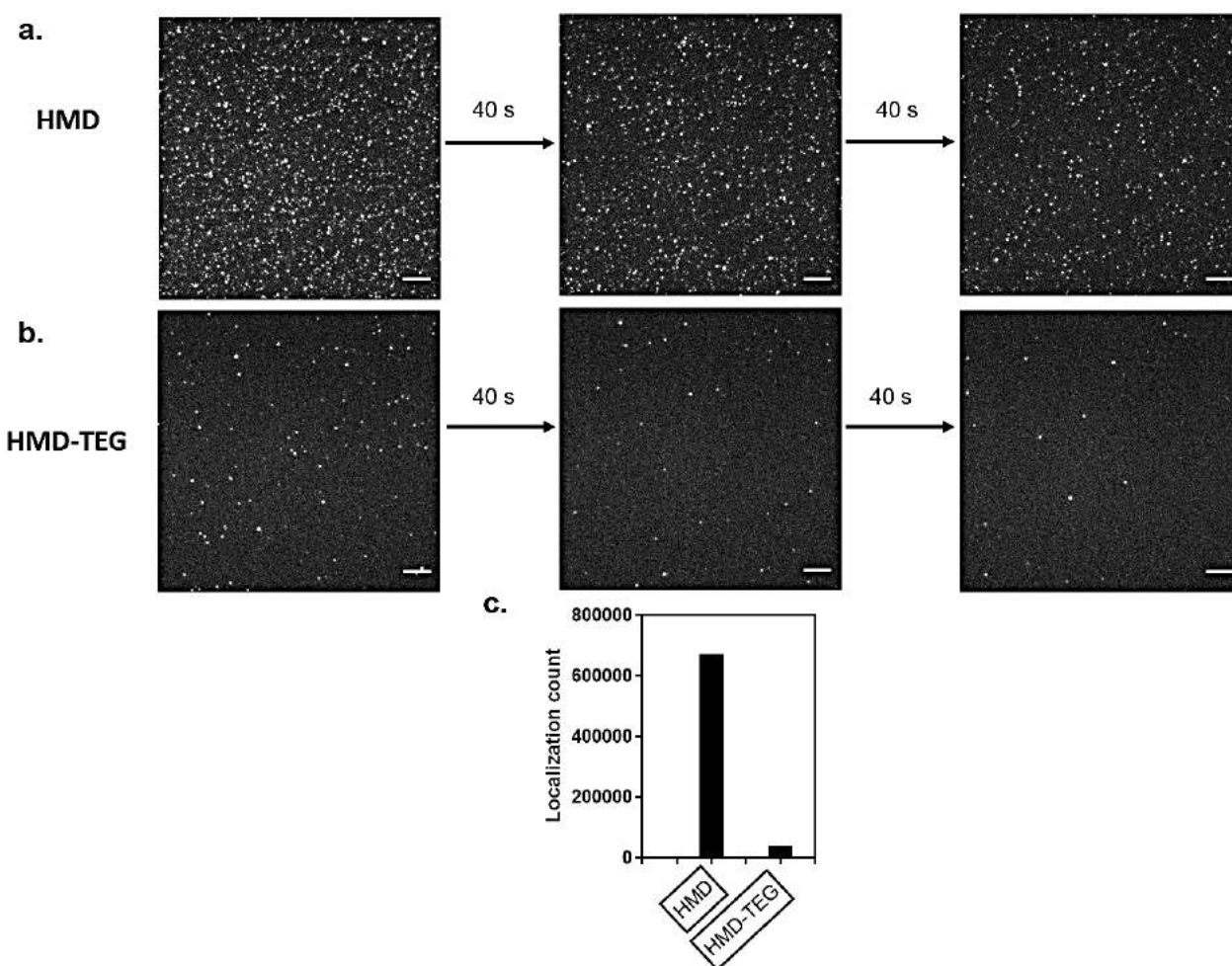


Figure S33 | Comparison of non-specific binding behavior of HMD-SiR fluor with and without TEG spacer. (a) HMD-SiR fluor (without TEG spacer) was allowed to absorb on glass surface for 30 min preincubated with BSA (30 min). Single molecule images were recorded over a time period of 80 s after quick wash with PBS. Three frames correspond to the images at 40 s interval (400 frames apart). TIRF images were recorded in $51.1 \mu\text{m} \times 51.1 \mu\text{m}$ dimension with 100 ms integration time maintaining laser power density of 245 W.Cm^{-2} . (b) Single molecule images for HMD-TEG-SiR fluor at 40 s interval (400 frames apart) shows very less non-specific binding compared to that of HMD-SiR fluor. (c) Total localization counts over 800 frames for HMD and HMD-TEG SiR also confirms the more non-specific binding (>18 fold) behavior of HMD-SiR fluor. Localization was counted using Picasso Render software. Scale bar $5 \mu\text{m}$ (a-b).

Synthesis of mono-substituted BSA-CB[7] conjugate:

1. Bovine serum albumin (BSA) was dissolved in PBS (pH 7.4) in a concentration of 4 mg.ml^{-1} .
2. $50 \mu\text{l}$ of the BSA stock solution ($200 \mu\text{g}$, $3 \times 10^{-3} \mu\text{mol}$) was taken in a microcentrifuge tube.

3. Tetrazine NHS ester ($1.19\ \mu\text{g}$, $1.19\ \mu\text{l}$ from $1\ \text{mg}\cdot\text{ml}^{-1}$ stock in DMF, $3\times 10^{-3}\ \mu\text{mol}$) was diluted in $50\ \mu\text{l}$ of PBS (pH 7.4) and added immediately to the BSA solution. The mixture was incubated at room temperature for 2 h.
4. The conjugated product was purified by Zeba spin column (pre-equilibrated with PBS).
5. The purified product was again incubated with CB[7]-PEG-TCO¹ ($2.5\ \mu\text{g}$, $1.53\ \mu\text{l}$ stock solution $1.66\ \text{mg}\ \text{ml}^{-1}$ in PBS, $1.5\times 10^{-3}\ \mu\text{mol}$).
6. After 3 h incubation at room temperature, CB[7] conjugated BSA was purified by using Zeba spin column (pre-equilibrated with PBS).
7. Purified product was used for the single molecule imaging experiments.

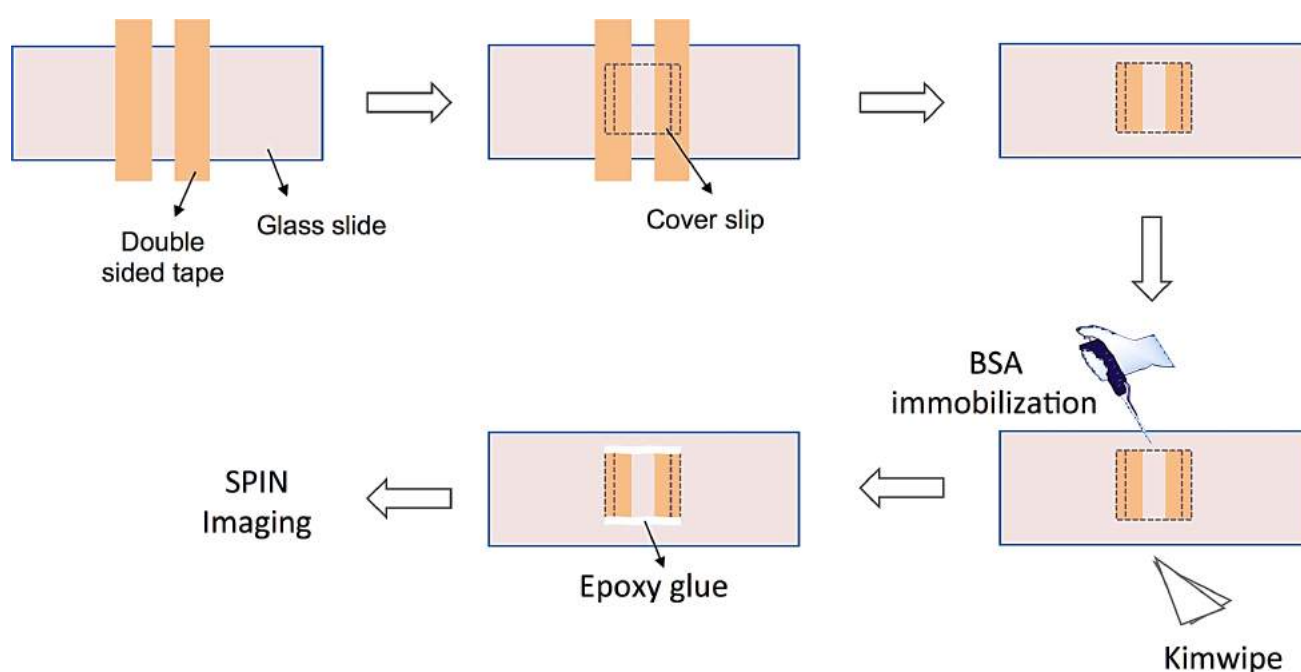


Figure S34 Schematic showing the strategy to prepare fluidic channel for single molecule imaging.

Sample preparation for single molecule imaging experiment:

1. Single molecule imaging experiment was carried out by immobilizing CB[7] conjugated BSA on a cover slip. For this purpose, a custom-made flow chamber was made.
2. Before making the flow chamber, both cover slip and microscope slide were cleaned by sonication using water, acetone and ethanol for 15 min each.
3. To make flow channel setup, initially two stripes of sticky double-sided tape were placed around 5 mm apart on $75\times 25\ \text{mm}$ microscope slide.
4. The cleaned cover slip was placed on the top of two stripes of sticky double-sided tape.

5. After pressing the coverslip thoroughly against the tape, extra uncovered tape was carefully removed.
6. The formed channel in between the cover slip and microscope slide was cleaned by flowing water and PBS.
7. 2.5 μ l of 7.5 μ M BSA–CB[7], diluted in 17.5 μ l of 1 mg.ml⁻¹ of unmodified BSA, was flown to the channel and incubated for 30 min to allow BSA to interact with the coverslip non-specifically.
8. After 30 min, the channel was washed twice with PBS.
9. 1 nM solution of guest conjugated Alexa Fluor 647 in PBS was flown through the channel and two sides of the channel were sealed.
10. The host-guest mediated imaging was carried out by facing the cover slip towards the objective.
11. A control experiment was done by incubating the flow chamber with unmodified BSA (1 mg.ml⁻¹) only, keeping the other imaging conditions same.

Protocol for single molecule imaging experiment: The host-guest mediated images for single molecule binding kinetics were captured using an inverted Zeiss ELYRA PS1 microscope, capable to illuminating the sample with TIRF illumination mode. The microscope was fitted with a definite focus system to eliminate *z* drift. 642 nm (150 mW) laser has been used for excitation of Alexa 647 dye. Imaging was performed using a Zeiss oil-immersion TIRF objective (alpha Plan-apochromat DIC 100 \times /1.46 Oil DIC M27, numerical aperture (NA) 1.46 oil). Fluorescence light was spectrally filtered with emission filter (MBS-642+EF LP 655 for laser line 642) and imaged on an electron-multiplying charge-coupled device (EMCCD) camera (Andor iXon DU897, quantum yield >90%, 512 x 512 pixels). Camera readout rate was 1 MHz at 16 bit. The recorded image dimension was 25.50 μ m \times 25.50 μ m. EMCCD camera gain was kept at 100 during image acquisition. Laser power density of \sim 138 W.Cm⁻² was used to excite the fluorophore molecules. Power density was estimated from the field of view and the laser power at the back focal plane of the objective. A total of 10,000 images were recorded with an integration time of 50 ms. Images were analysed by using a custom-programmed software package named 'Picasso'.

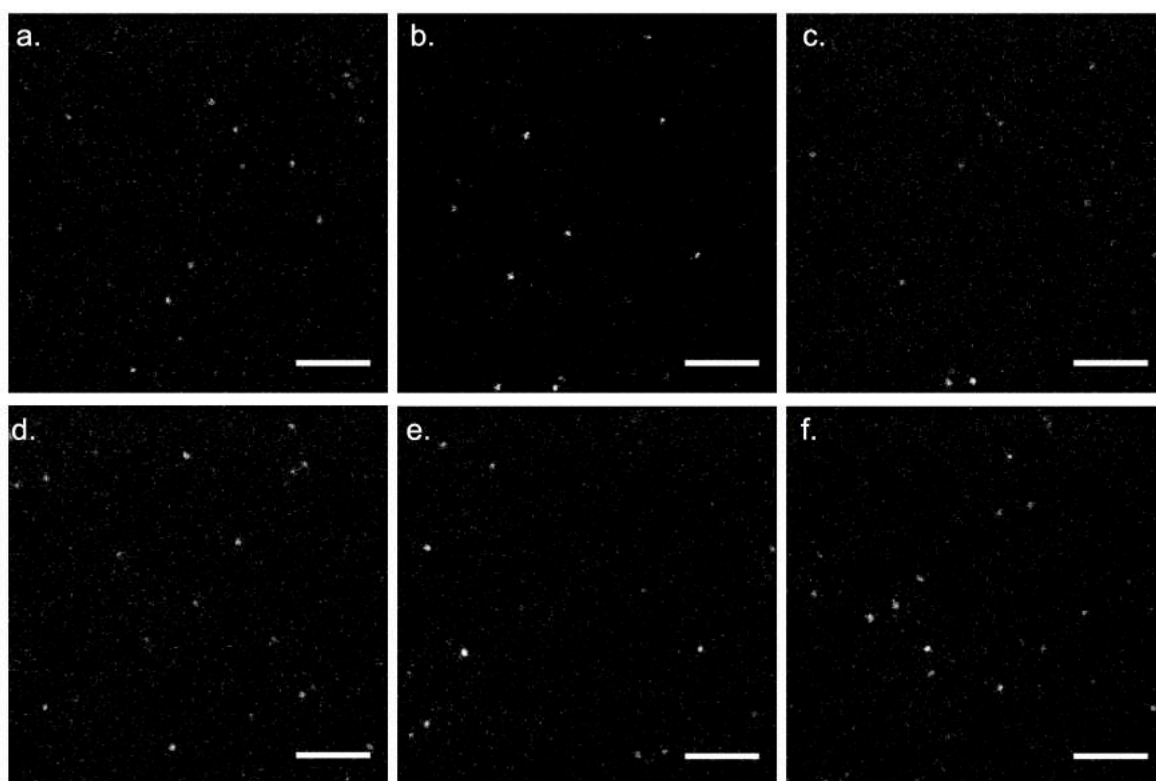


Figure S35| Single-molecule imaging of BSA-CB[7] immobilized surface using Alexa Flour 647 conjugated HMD guest (1 nM). Six frames (a-f) correspond to the images at 1 min interval (i.e. 1200 frames apart). Scale bar: 5 μm (a-f).

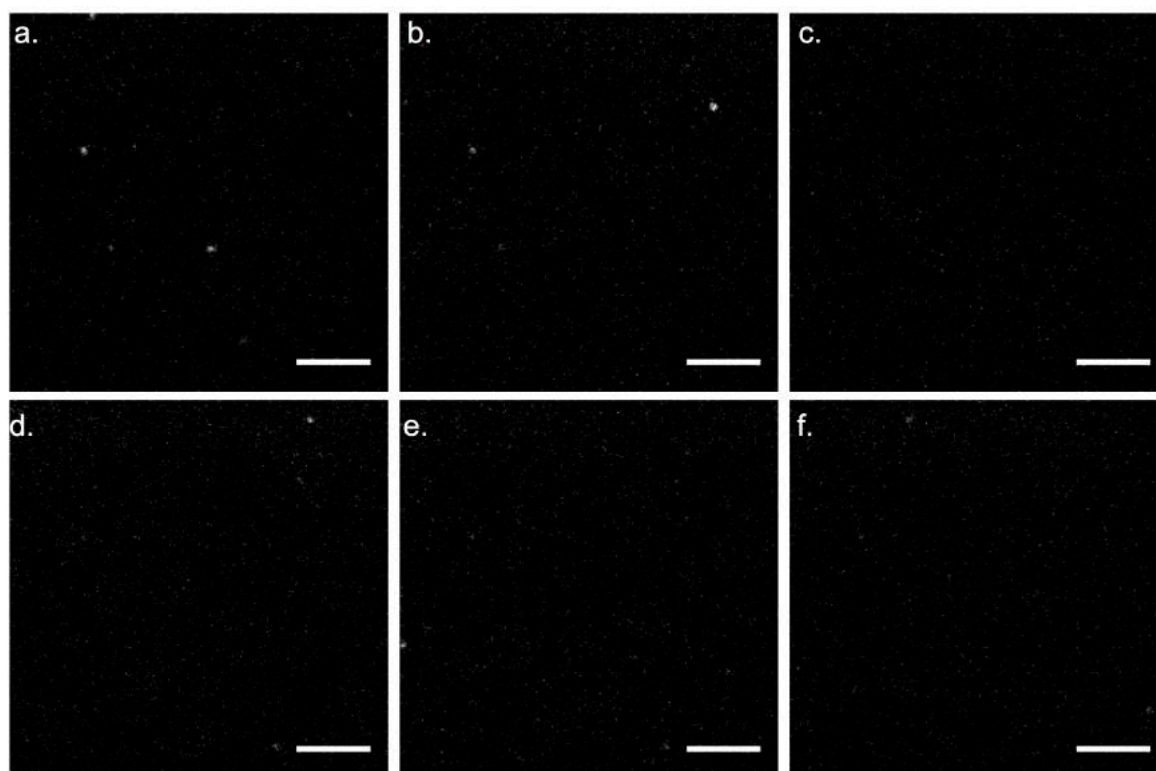


Figure S36| Single-molecule imaging of unmodified BSA immobilized surface using Alexa Flour 647 conjugated HMD guest (1 nM). Six frames (a-f) correspond to the images at 1 min interval (i.e. 1200 frames apart). Scale bar: 5 μm (a-f).

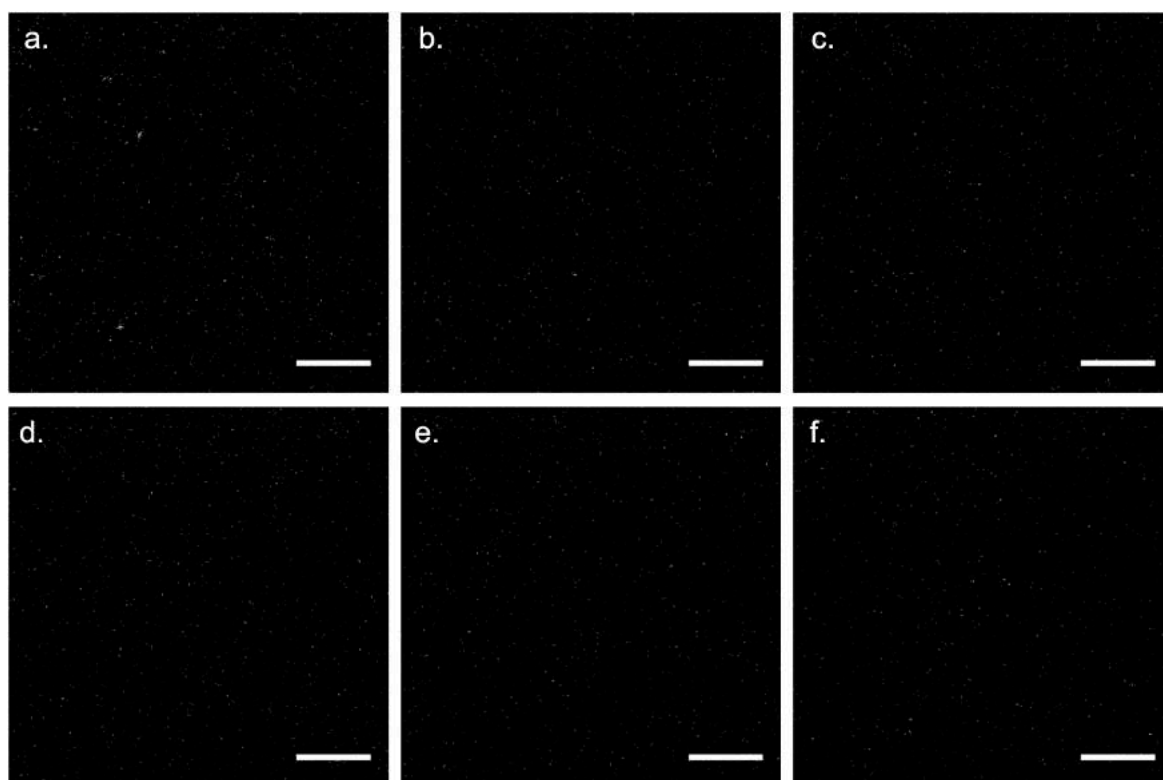


Figure S37 | Single-molecule imaging of BSA-CB[7] immobilized surface using Alexa Fluor 647 conjugated propylamine guest (1 nM). Six frames (a-f) correspond to the images at 1 min interval (i.e. 1200 frames apart). Scale bar: 5 μm (a-f).

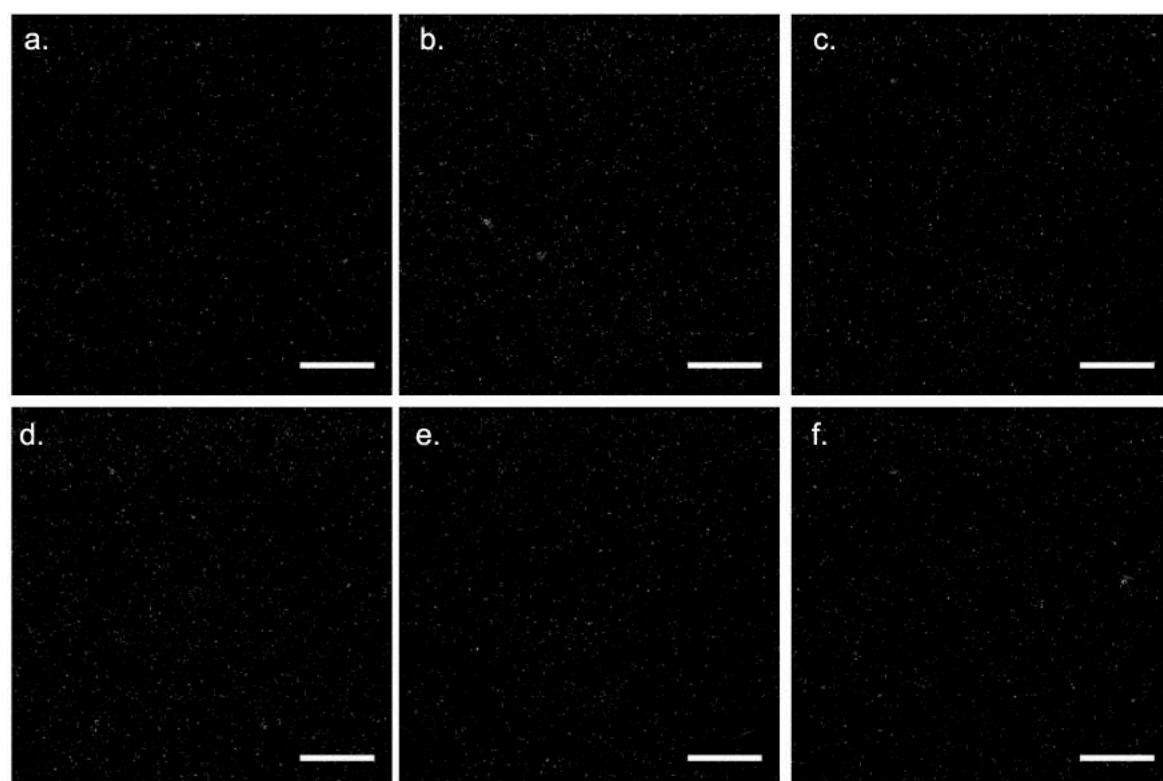


Figure S38 | Single-molecule imaging of unmodified BSA immobilized surface using Alexa Fluor 647 conjugated propylamine guest (1 nM). Six frames (a-f) correspond to the images at 1 min interval (i.e. 1200 frames apart). Scale bar: 5 μm (a-f).

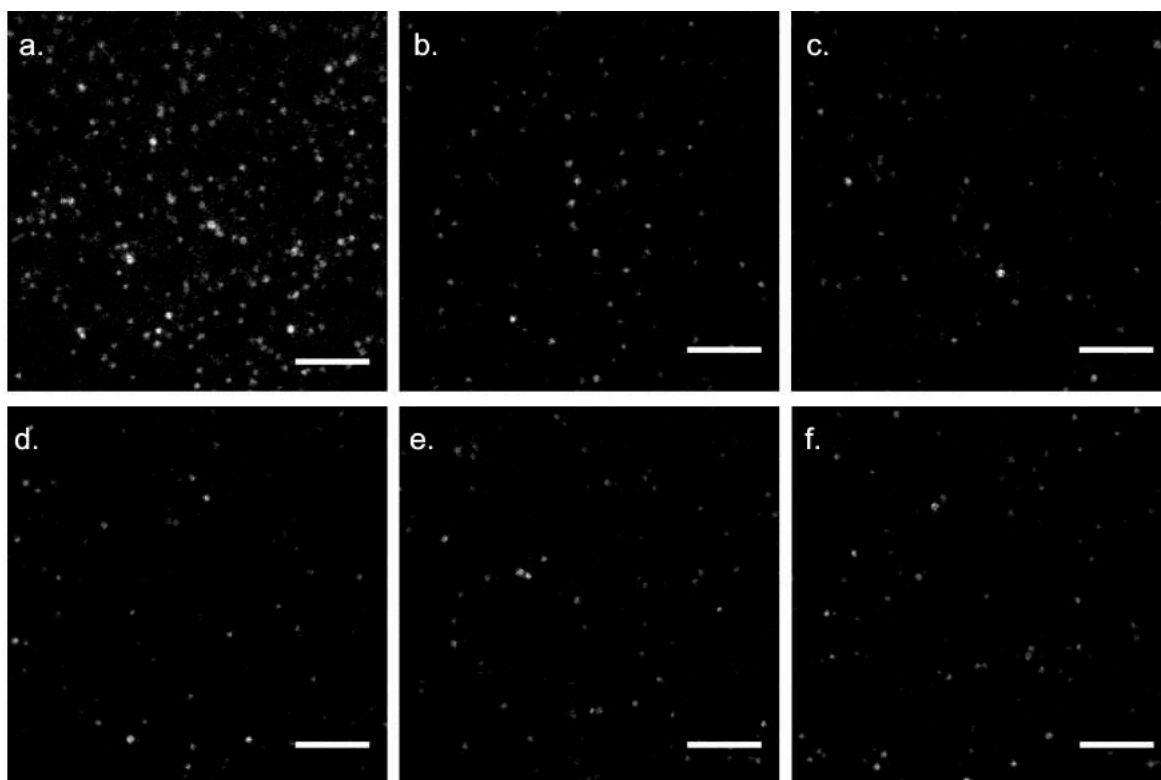


Figure S39 | Single-molecule imaging of BSA-CB[7] immobilized surface using Alexa Flour 647 conjugated ADA guest (1 nM). Six frames (a-f) correspond to the images at 1 min interval (i.e. 1200 frames apart). Scale bar: 5 μm (a-f).

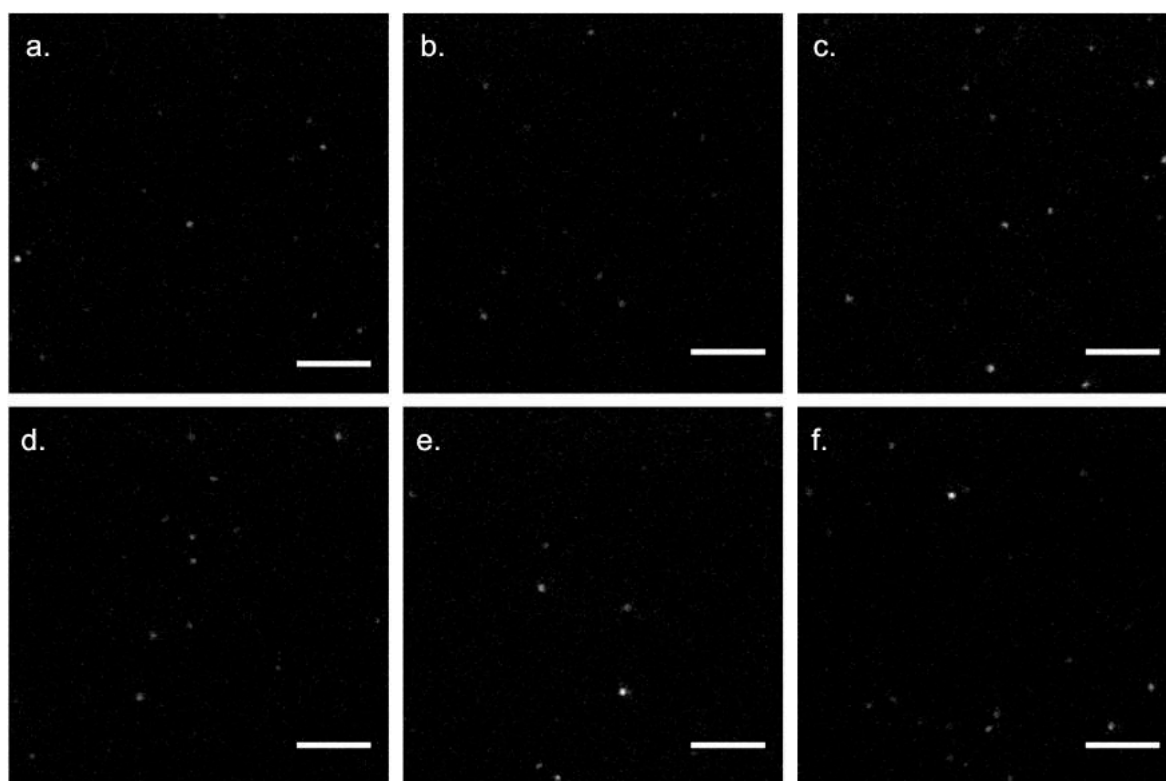


Figure S40 | Single-molecule imaging of unmodified BSA immobilized surface using Alexa Flour 647 conjugated ADA guest (1 nM). Six frames (a-f) correspond to the images at 1 min interval (i.e. 1200 frames apart). Scale bar: 5 μm (a-f).

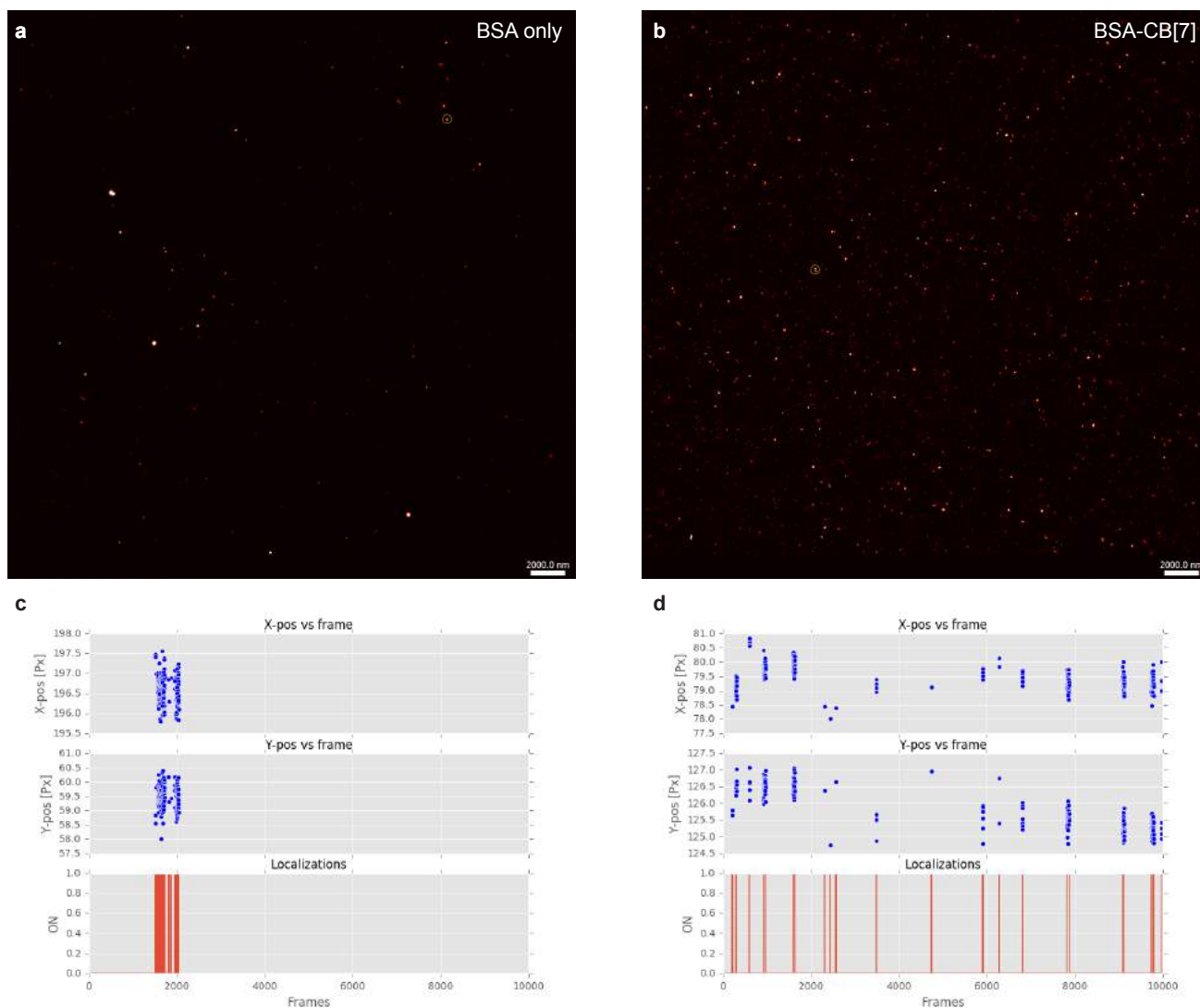


Figure S41 | Reconstructed images from single molecule imaging experiment with Alexa Flour 647 conjugated HMD guest. A total of 10,000 frames were recorded with an exposure time of 50 ms for reconstructing the super-resolved image. (a) Reconstructed image of unmodified BSA surface shows only the presence of very few fluorescent spots, corresponding to the non-specific binding of the imager with the surface. (b) Reconstructed image of BSA–CB[7] surface shows a large number of fluorescence spots. These spots appeared due the specific interaction between surface immobilized CB[7] with Alexa Flour 647 conjugated HMD guest. (c) Fluorescence trace analysis of a localized spot from unmodified BSA surface shows non-specifically appeared event. (d) Fluorescence trace analysis of a localized spot from BSA–CB[7] surface shows transient and repetitive binding events throughout the recording time. These transient and repetitive binding events appear due to the specific and dynamic interaction between surface immobilized CB[7] host with Alexa Flour 647 conjugated HMD guest .Scale bar: 2 μ m (a-b).

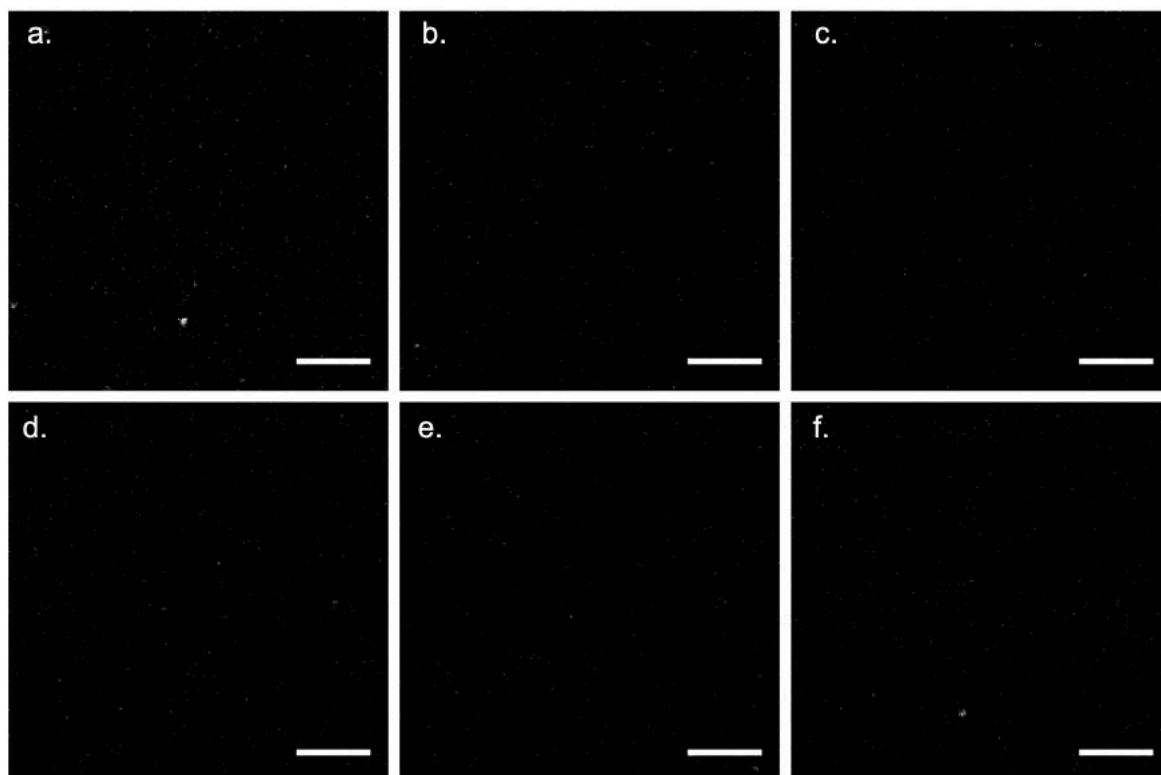


Figure S42 | Imaging of BSA-CB[7] immobilized surface after washing of Alexa Fluor 647 conjugated HMD guest. Six frames (a-f) correspond to the images at 1 min interval (i.e. 1200 frames apart). Scale bar: 5 μm (a-f).

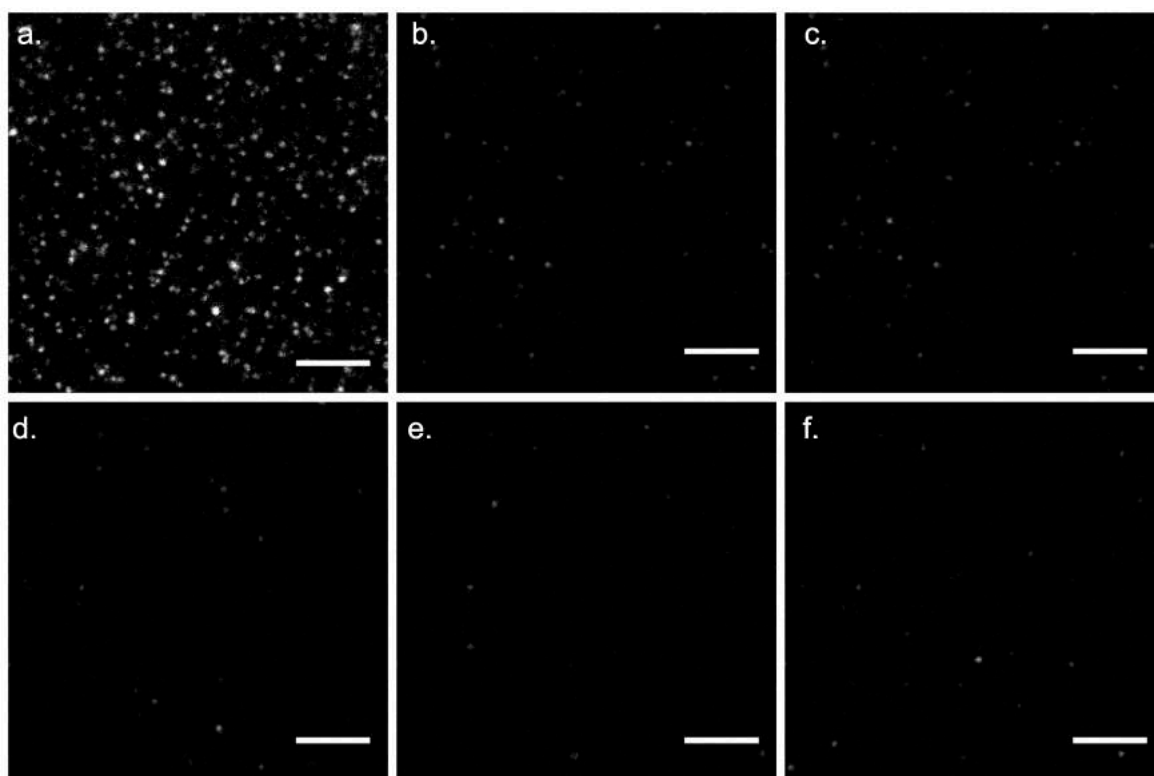


Figure S43 | Imaging of BSA-CB[7] immobilized surface after washing of Alexa Fluor 647 conjugated ADA guest. Six frames (a-f) correspond to the images at 1 min interval (i.e. 1200 frames apart). Scale bar: 5 μm (a-f).

Cell culture

Mouse embryonic fibroblasts (MEFs) and Saphenous vein endothelial cell line (SVEC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco-BRL, USA) and 2 mM Glutamax (Invitrogen, Carlsbad, USA). MEFs were isolated from E14.5 mouse embryos by routine procedure. At confluence, before plating, cells were washed, trypsinized and suspended in culture medium. Cells were counted and then in a typical experiment, ~25,000 cells/well were plated in 8-well chamber slide system (Eppendorf). Cells were then maintained at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h to 60% confluence. Thereafter, cells were used for imaging experiment.

Immunostaining protocol for fixed cells:

1. Culture media was removed from chamber wells and cells were washed twice with PBS (pH 7.4).
 2. Cells were fixed for 7 min with chilled methanol at -20°C or, 15 min with 4% PFA at RT or, 10 min with 3% Paraformaldehyde and 0.1% Glutaraldehyde in PBS.
 3. followed by washing for three times with PBS.
 4. Cells were permeabilized with 0.25% v/v Triton X-100 in PBS for 10 min (This step was skipped for methanol fixation method).
 5. Cells were then washed three times with PBS.
 6. Cells were blocked for 2 h with 3% bovine serum albumin in PBS at room temperature.
 7. Cells were incubated for 24 h at 4°C with primary antibody (10 µg ml⁻¹) diluted in PBS containing 3% bovine serum albumin.
 8. Excess antibody was removed by three times washing with PBS (with 5 min incubation each time).
 9. Cells were incubated with secondary antibodies (10 µg ml⁻¹) diluted in PBS containing 3% bovine serum albumin for 2 h.
 10. Excess secondary antibody was removed by three times washing with PBS (with 5 min incubation each time).
 11. Drift marker solution (gold nanoparticle of diameter ~100 nm) in PBS was added to each well and the chamber slide was centrifuged at 450 rcf for 5 min.
9. Appropriate concentration of imager was added after removing the PBS for host-guest mediated imaging.

Labeling protocol for CB[7] conjugated phalloidin:

1. Culture media was removed from chamber wells and cells were washed twice in PBS (pH 7.4).
2. Cell were fixed for 15 min with 4% paraformaldehyde in PBS.
3. Excess PFA was removed by washing for three times washing with PBS.
4. Cells were permeabilized with 0.25% v/v Triton X-100 in PBS for 10 min.

5. Cells were washed three times with PBS.
6. 3% bovine serum albumin and 0.1% v/v Triton X-100 in PBS was used for blocking. Blocking continued for 2 h at room temperature.
7. Cells were Stained for overnight at 4°C with CB[7] conjugated phalloidin (2 μ M) diluted in PBS (pH 7.4).
8. Cells were washed once before fixing the drift marker.
9. Drift marker solution (gold nanoparticle of diameter \sim 100 nm) in PBS was added to each well and the chamber slide was centrifuged at 450 rcf for 5 min.
10. Appropriate concentration of imager was added after removing the PBS for host-guest mediated imaging.

Host–guest mediated imaging of thoracic muscle tissue

Dissection of thoracic muscle tissue: Adult wild type drosophila flies were collected which were maintained in 12 h light and 12 h dark at 25°C. Once collected, flies were kept in ice for around 15 minutes for anesthetizing.

Thoracic muscle dissection

1. After flies were anesthetized, they were submerged in PBS, placed dorsally on the dissection plate and were pierced with insect pins on the abdomen region.
2. Using forceps, top layer of thorax was dissected slowly, peeled out gently and bunch of clustered thoracic muscles were taken out.
3. Dissected muscle tissues were transferred in chilled PBS in labelled wells of the glass dish kept on ice and were allowed to settle.

Immunohistochemistry for thoracic muscle tissue

1. After dissections were carried out, tissues were fixed with 4% paraformaldehyde (PFA) at room temperature for 30 minutes with gentle shaking.
2. Tissues were washed with at least thrice with 5 minutes incubation for each time using PBS containing 0.5% Triton X–100 (0.5% PBT).
3. Samples were then blocked using 10% horse serum in 0.5% PBT for 1 h at room temperature.
4. Phalloidin–CB[7] (2 μ M) was added to each chamber and incubated overnight at 4°C.
5. Afterwards, samples were washed three times with 0.5% PBT for 5 min incubation each time.
6. Appropriate concentration of HMD–Cy5 was added to the phalloidin labeled tissue and proceed for host–guest mediated super resolution imaging.

Live cell delivery of phalloidin-CB[7] conjugate:

1. Culture media was removed from the chamber wells and live cells were washed twice with DPBS.
2. 2 μ M concentration of Phalloidin-CB[7] conjugate was assembled with NP-HMD at a concentration ratio of 10:1 and added to the cells.
3. Cells were then maintained at 37°C with 5% CO₂ in a humidified atmosphere for 3 h.
4. After 3 h incubation, the cells were washed twice with DPBS.
5. Cells were incubated with 10 nM PBS solution of silicon rhodamine conjugated HMD imager.

The imaging was started immediately after the imager incubation.

Protocol for 2D and 3D host-guest mediated imaging of cells and tissues: The Host-guest mediated single molecule blinking images for were captured using an inverted Zeiss ELYRA PS1 microscope, capable to illuminating the sample with TIRF illumination mode. The microscope was fitted with a definite focus system to eliminate z drift. 642 nm (150 mW) laser has been used for excitation of Alexa 647, Cy5 and SiR dye. Imaging was performed using a Zeiss oil-immersion TIRF objective (alpha Plan-apochromat DIC 100 \times /1.46 Oil DIC M27, numerical aperture (NA) 1.46 oil). Fluorescence light was spectrally filtered with emission filter (MBS-642+EF LP 655 for laser line 642) and imaged on an electron-multiplying charge-coupled device (EMCCD) camera (Andor iXon DU897, quantum yield >90%, 512 x 512 pixels). Camera readout rate was 1 MHz at 16 bit. Power density was estimated from the field of view and the laser power at the back focal plane of the objective. For 3D imaging, a phase ramp was introduced over one-half of the detection beam path with a glass wedge. For live cell imaging, SiR imager incubated cells were maintained at 37°C with 5% CO₂ in a humidified atmosphere fitted with the microscope. A detailed information on imaging parameters have been mentioned in supplementary table 4.

Supplementary table 4: Microscopy parameters for host-guest based imaging in cells and tissues.

| Fig. no. | Imaging targets | Fixation method | Labeling method | Imagers | Laser power (W.Cm ⁻²) | Integration time (ms) | EMCCD gain |
|-----------------|----------------------------|-----------------------------|------------------------------------|------------------|---|------------------------------|-------------------|
| Fig.3 | Microtubule in fixed cell | Methanol | Primary + Secondary antibody–CB[7] | HMD–Cy5 (1 nM) | 138 | 50 | 100 |
| Fig. S39 | 3D mitochondria | 3% PFA+ 0.1% Glutaraldehyde | Primary + Secondary antibody–CB[7] | HMD–Cy5 (2.5 nM) | 138 | 50 | 100 |
| Fig. 4b | Actin in fixed cell | 4% PFA | Phalloidin–CB[7] | HMD–Cy5 (1 nM) | 392 | 50 | 100 |
| Fig. 4e | Actin in fixed tissue | 4% PFA | Phalloidin–TEG–CB[7] | HMD–Cy5 (100 nM) | 303 | 50 | 100 |
| Fig. 5b | Actin in live cell | NA | Phalloidin–TEG–CB[7] | HMD–SiR (10 nM) | 173 | 30 | 200 |
| Fig. S45 | Additional live cell actin | NA | Phalloidin–TEG–CB[7] | HMD–SiR (10 nM) | 173 | 30 | 200 |

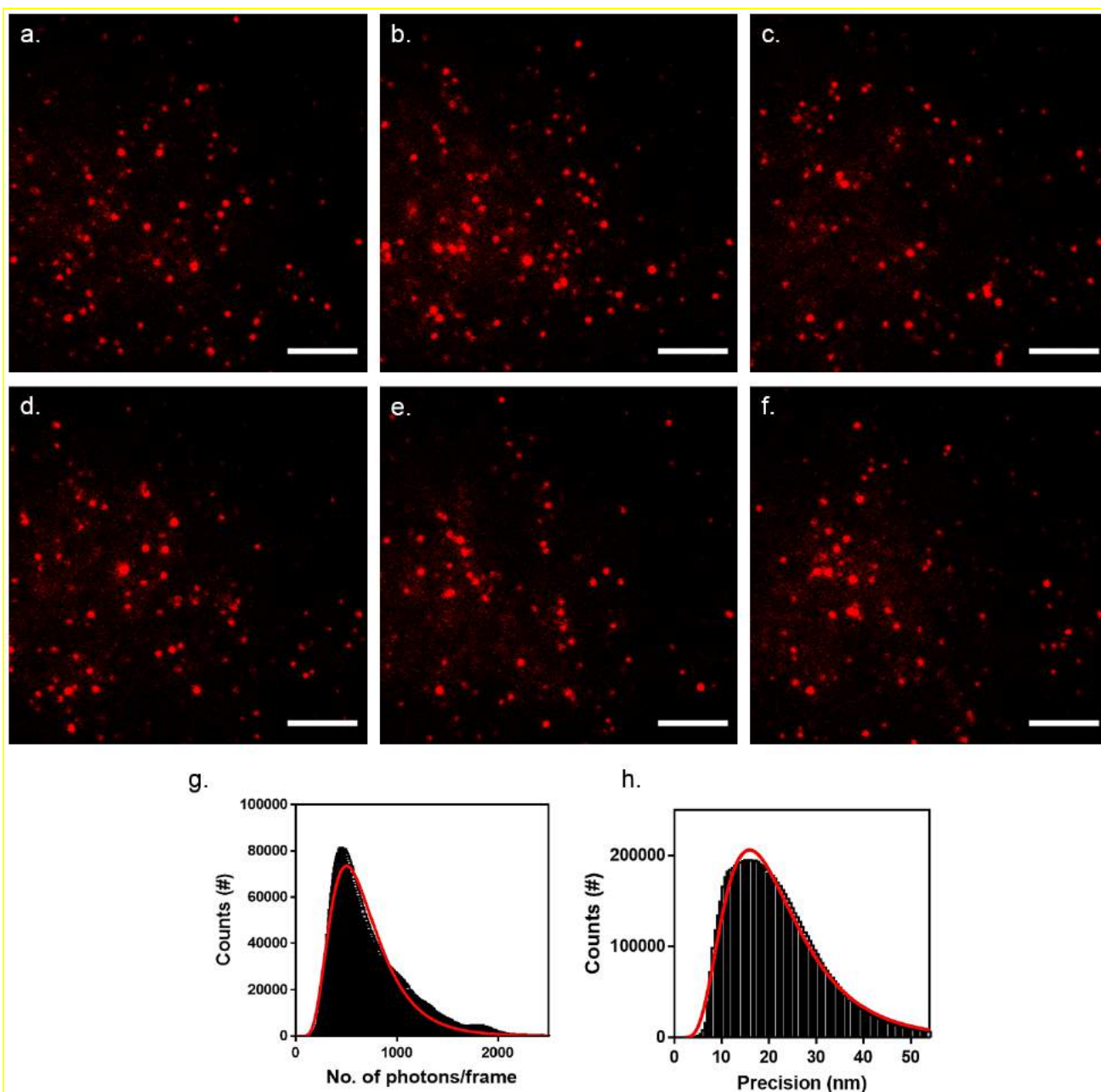


Figure S44 (a–f) Single molecule blinking frames from CB[7]–HMD mediated super resolution imaging from cells on microtubule targets. Six frames (a–f) correspond to the images at 1 min interval (i.e. 1200 frames apart). Scale bar: 5 μ m (a–f). (g) Number of photons per frame and (h) localization precision data for the super resolution imaging of microtubule from figure 3.

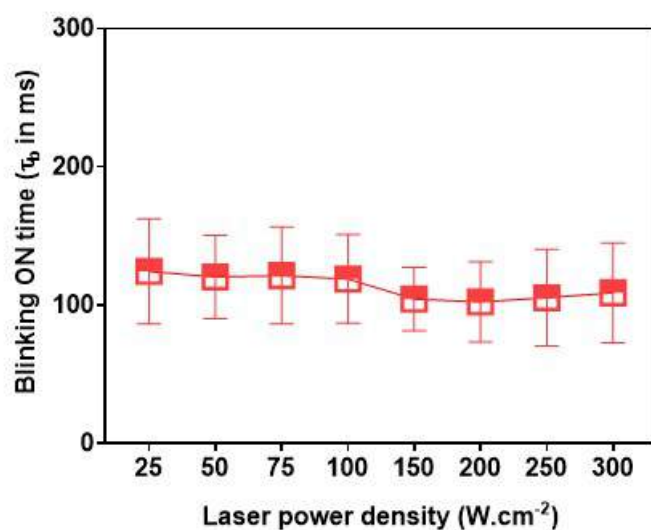


Figure S45 Plot of Fluorescence ON time (number of frames) vs laser power density. Integration time was kept 50 ms for performing the experiment.

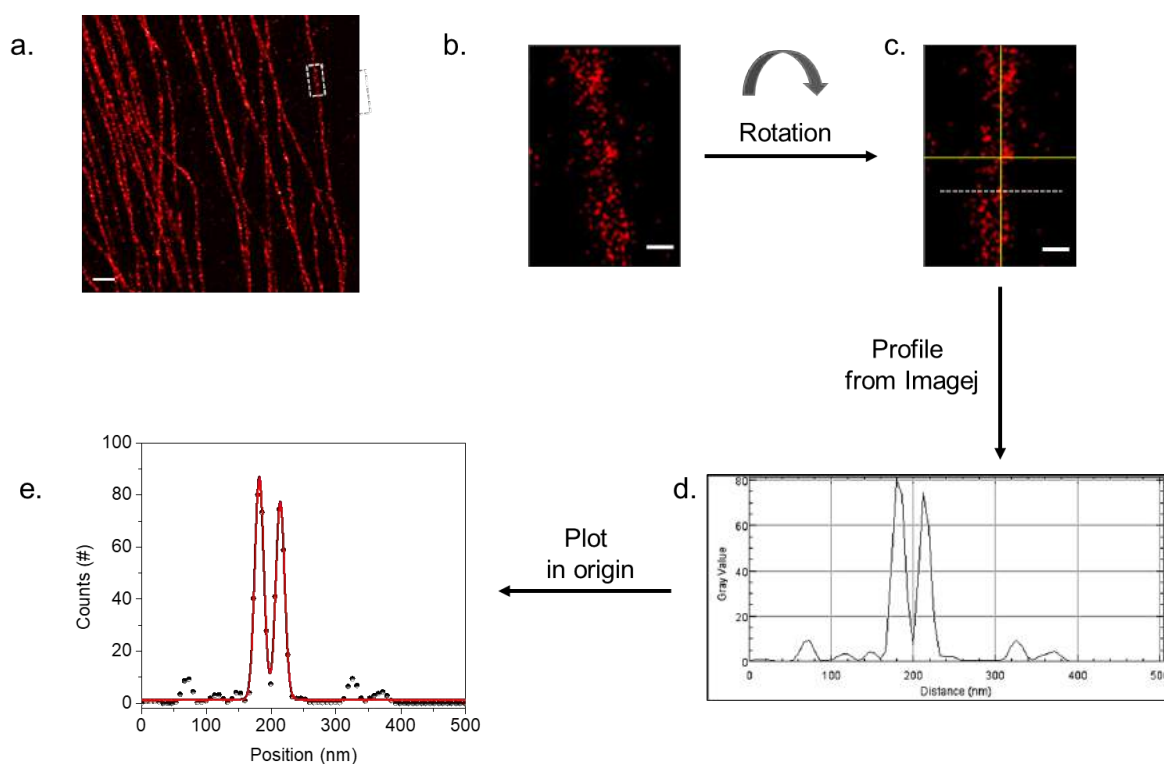
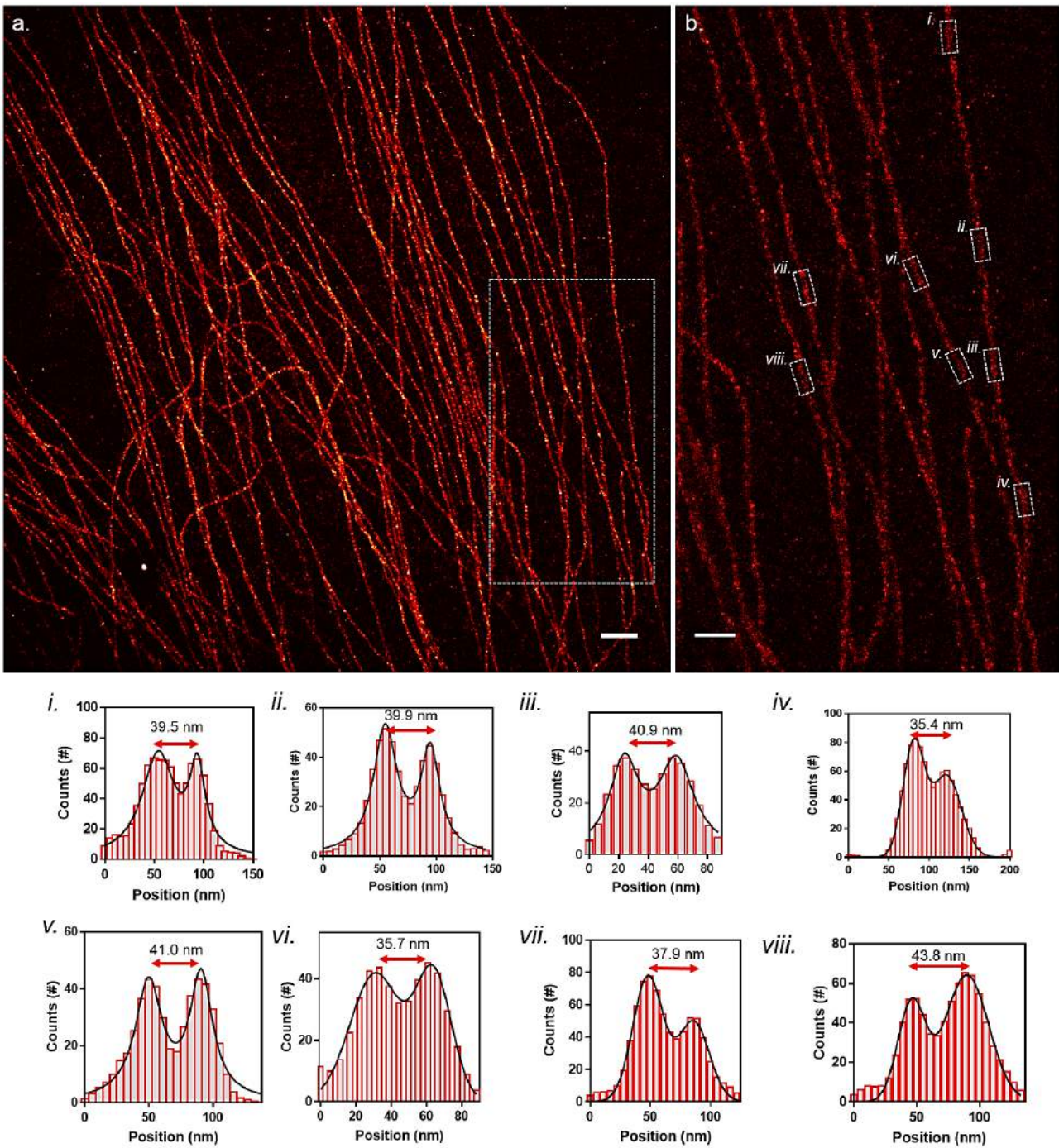


Figure S46 Super-resolved image analysis protocol using ImageJ for finding the diameter of the microtubule. Scale bars: (a) 500 nm, and (b and c) 100 nm.



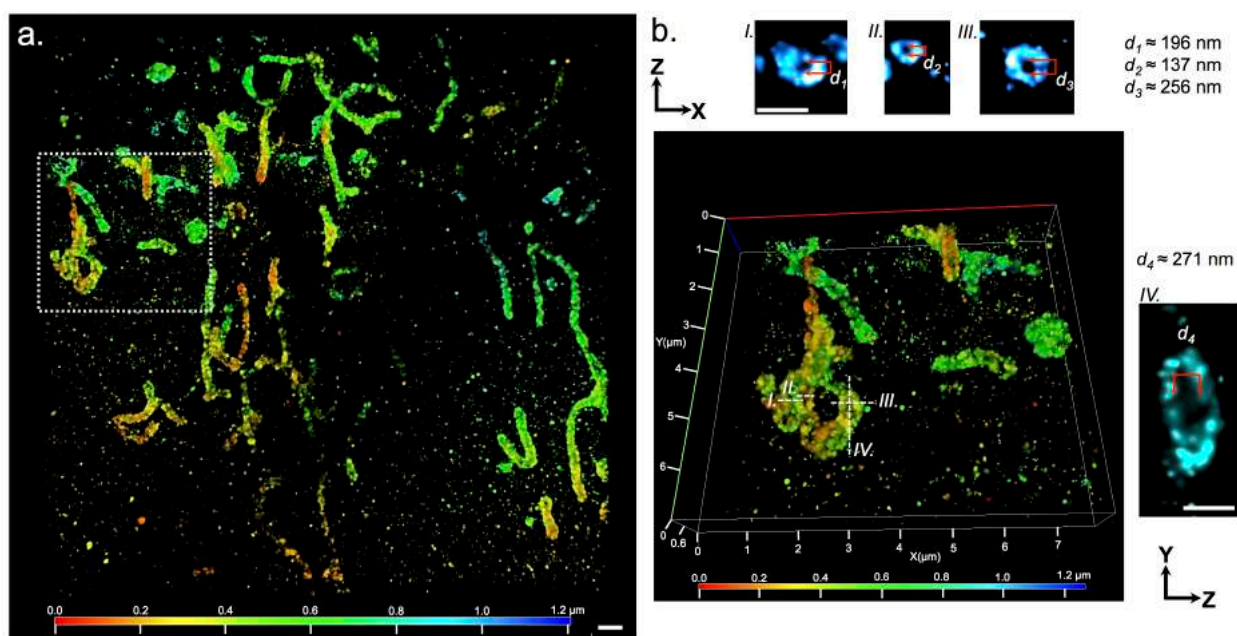


Figure S48| 3D super-resolution imaging using host-guest probe. (a) Tom20 in MEF cells were stained with CB[7], as described previously for 2D imaging. For 3D imaging, a phase ramp is introduced over one-half of the detection beam path with a glass wedge. host-guest based imaging was performed using 2.5 nM PBS solution of HMD-Cy5 imager. Reconstructed super resolved image shows the 3D view of the mitochondria in MEF cells, where z -direction position is color-coded. (b) Zoomed-in view of the boxed area in **a**. x - z cross-section along the dotted lines (*I*, *II* and *III*) and y - z cross-section along the dotted lines (*IV*) shows the localization of Tom20 on the outer mitochondrial membrane. Scale bars: (a) 1 μm and (b) 500 nm.

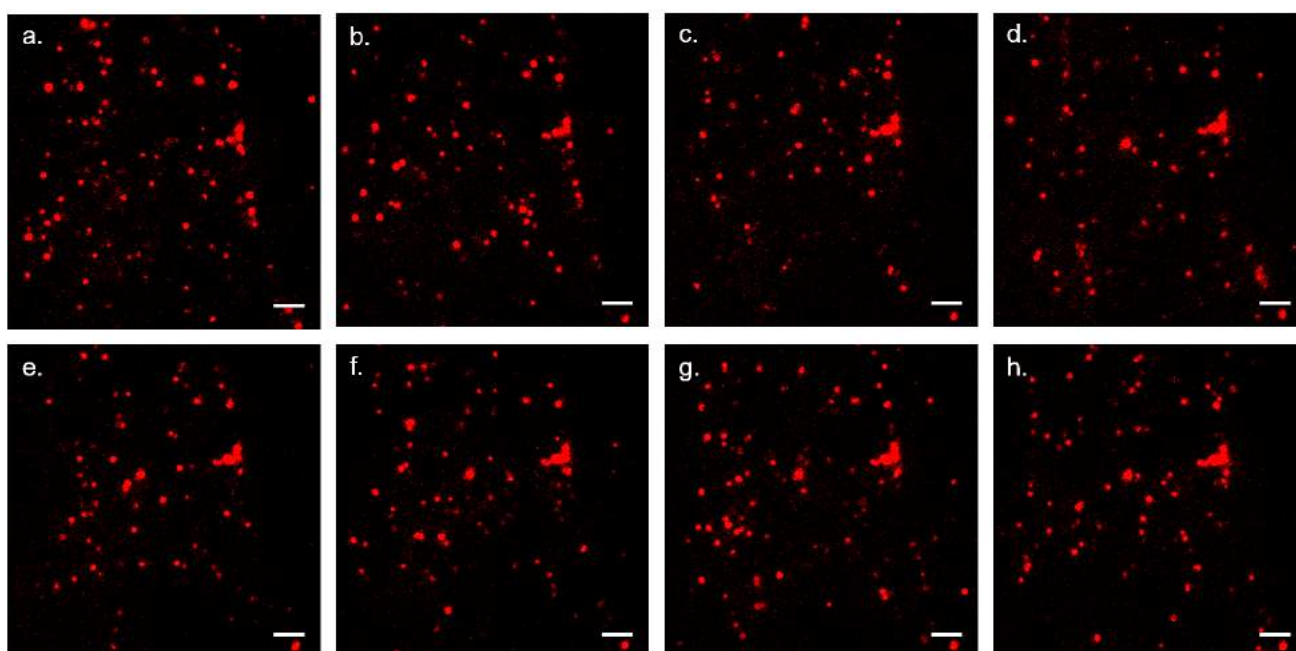


Figure S49| Single molecule blinking events from the host-guest based imaging of actin from figure 4b. Each frame corresponds to the single molecule blinking frames at 9500 interval, which clearly showed the reversible blinking pattern in entire set of image acquisition. Scale bar 2.5 μm (a-h).

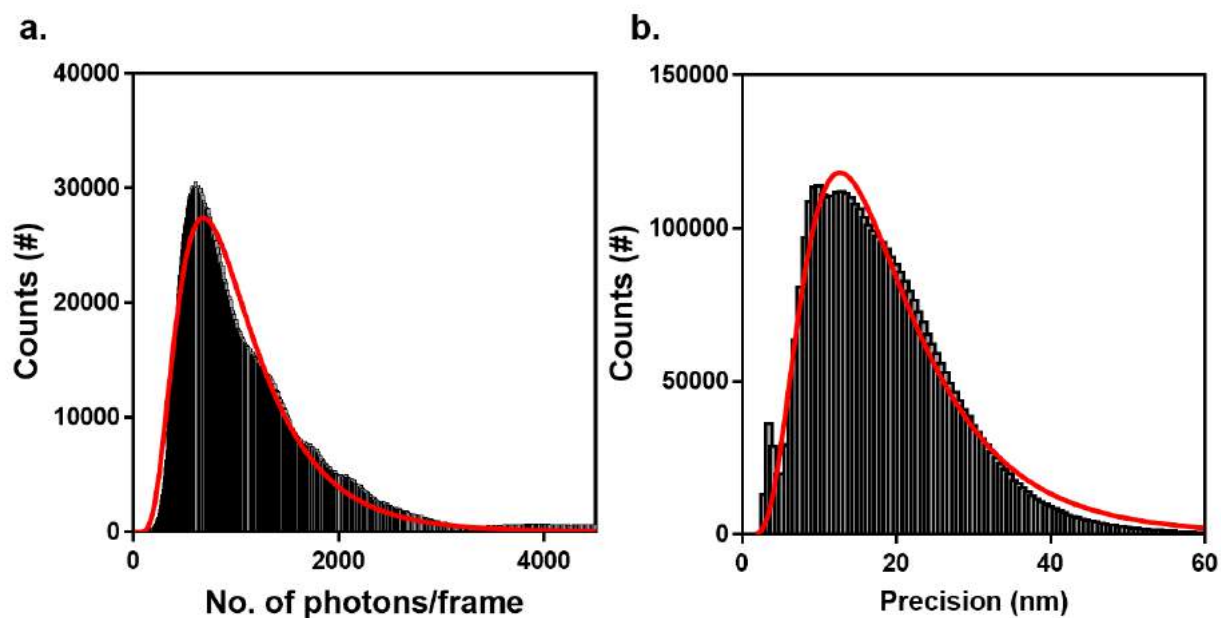


Figure S50 | (a) Number of photons per frame and (b) localization precision data for the super resolution imaging of actin from figure 4b.

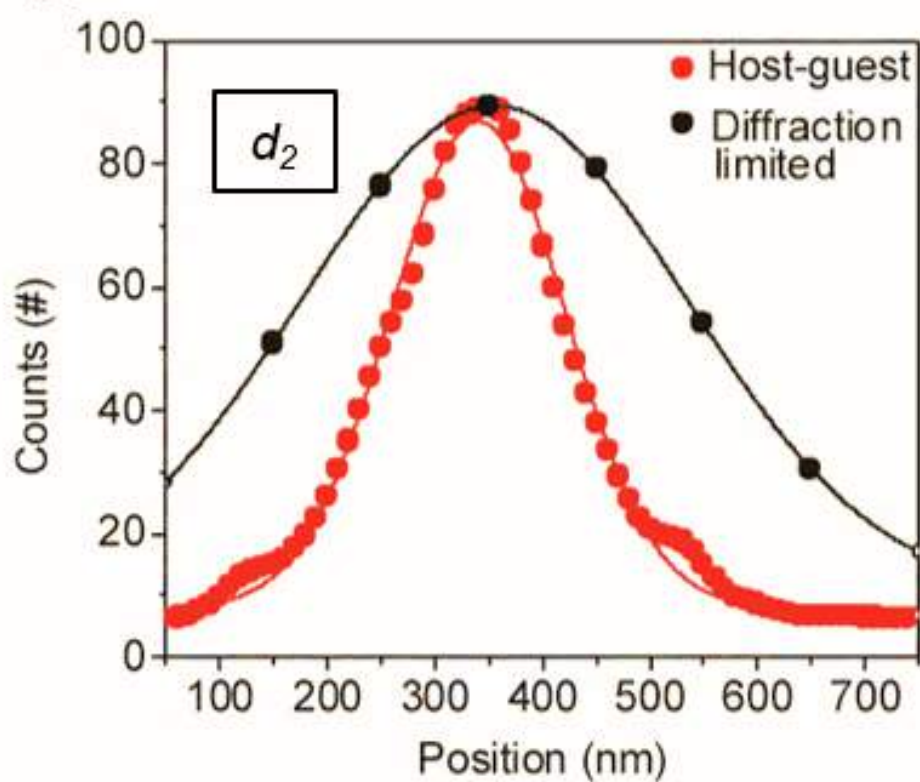


Figure S51 | Improvement of diameter calculation of actin filaments of thoracic muscle tissues from *drosophila melanogaster*.

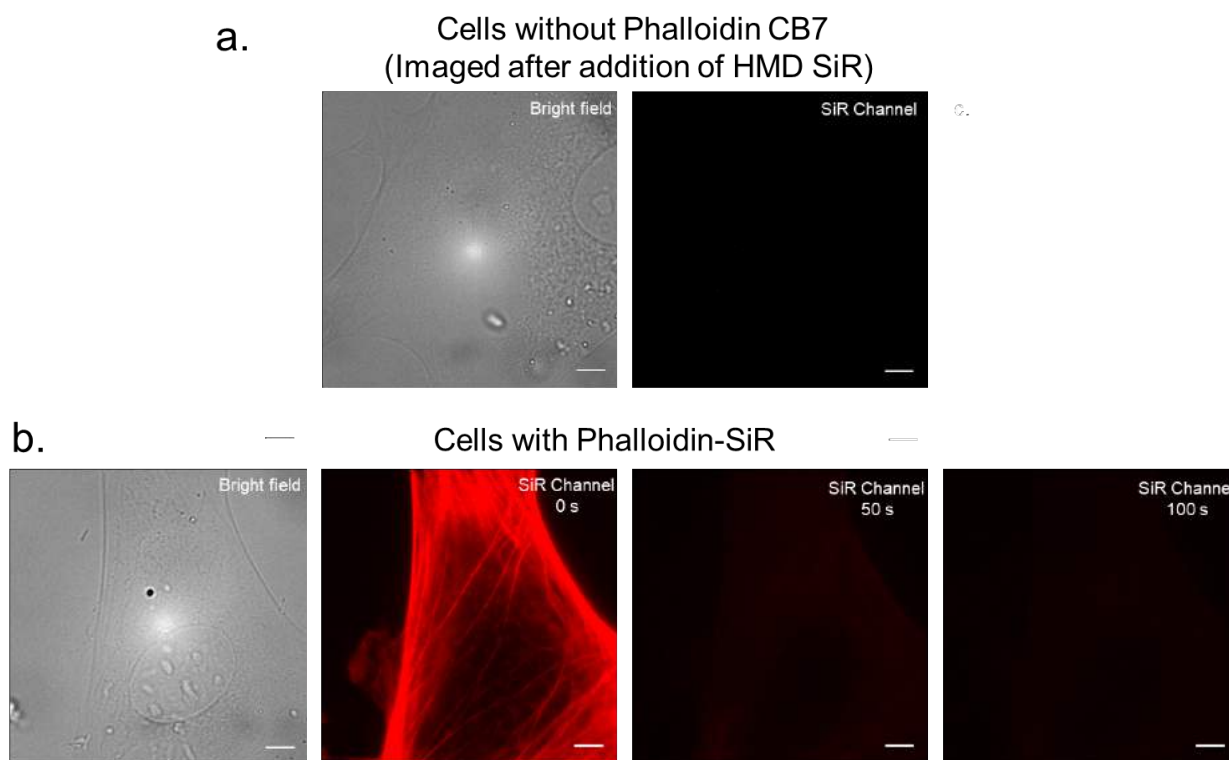


Figure S52 Minimal non-specific binding and Non-blinking behavior of SiR fluorophore in absence of CB[7]. (a) Imaging performed in presence of SiR-labeled HMD imager but without prior phalloidin-CB[7] incubation showed no fluorescence from the HeLa cells. (b) HeLa cells that are directly labelled with phalloidin-SiR showed diffraction limited actin image and subsequent bleaching of the signal. No blinking was observed in this case. Scale bars: 2.5 μm .

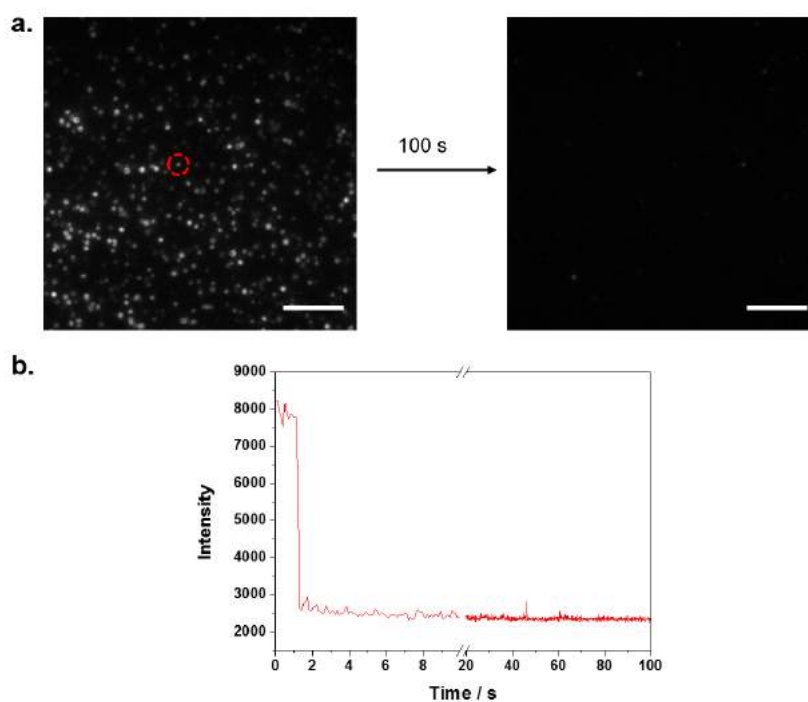


Figure S53 Non-blinking behaviour of HMD TEG SiR fluorophore after non-specifically immobilizing on glass surface. (a) HMD-TEG-SiR fluor was allowed to absorb on glass surface for 30 min preincubated with BSA (30 min). Single molecule images were recorded over a time period of 100 s after quick wash with PBS.

Two frames correspond to the images at 100 s interval (1000 frames apart). TIRF images were recorded with 100 ms integration time with laser power density of 245 W.Cm^{-2} . (b) Single molecule traces of the indicated region from (a), shows non-blinking behaviour of SiR fluor over a time period of 100 s. This indicates that dynamic host-guest interaction is the important criteria for inducing blinking behaviour of SiR imager. Scale bar: $5 \mu\text{m}$.

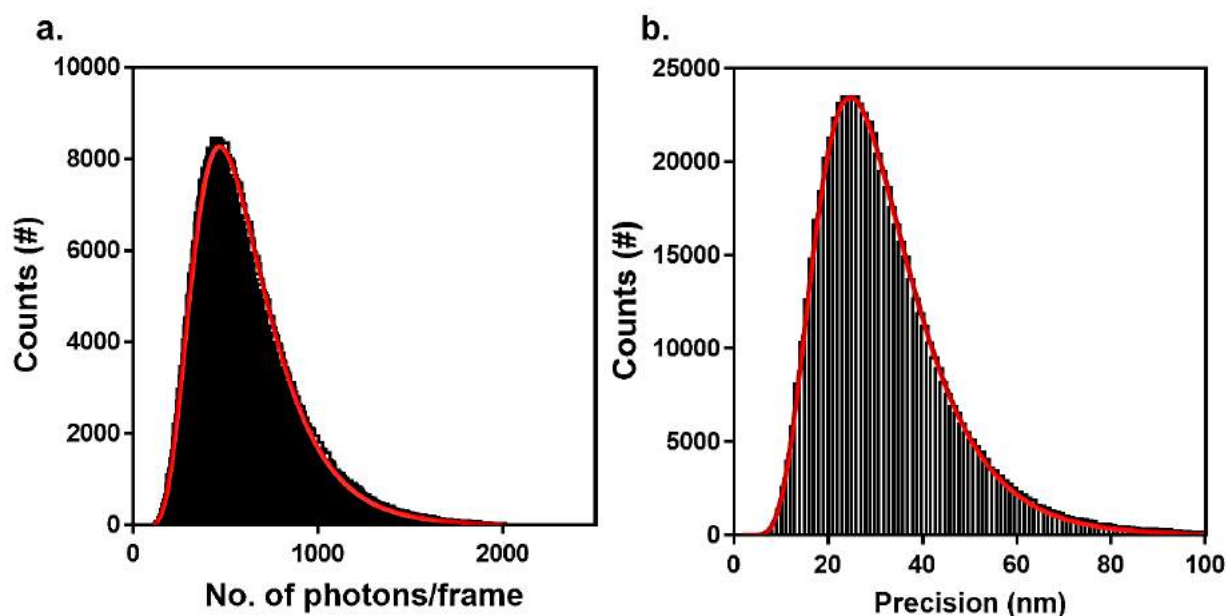


Figure S54 | (a) Number of photons per frame and (b) localization precision data for the super resolution imaging of actin in live cell from figure 5b.

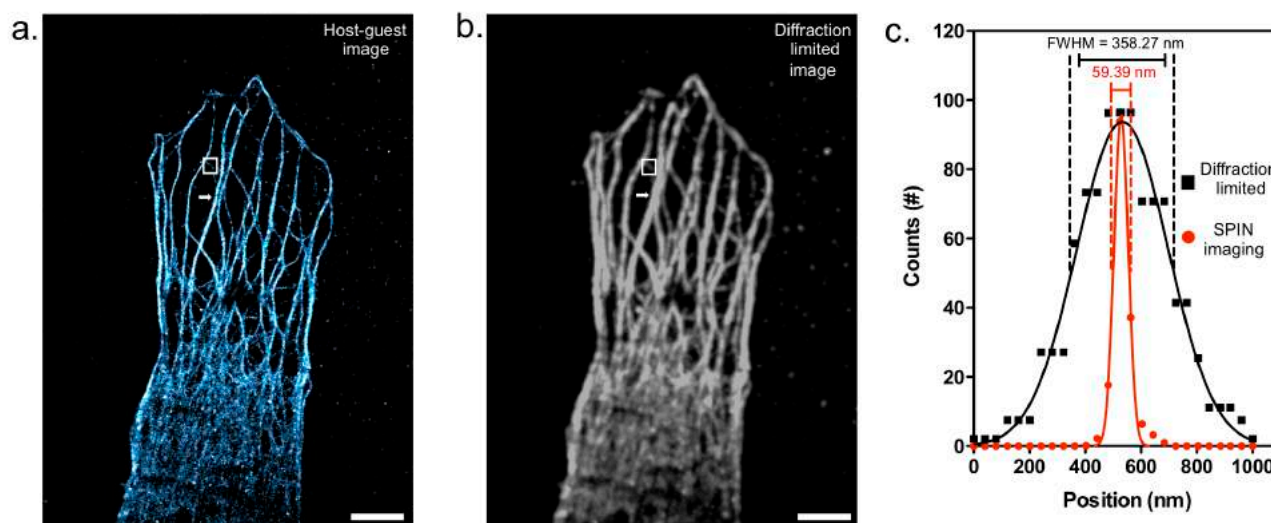


Figure S55 | Additional live cell super-resolution fluorescence microscopy images of phalloidin targeted actin cytoskeleton using host-guest probe. (a) Reconstructed super-resolved SPIN image of actin fibers from live cells. Non-covalent interaction mediated delivery allows the access to the CB[7] functionality for imaging via

SPIN technique. After target binding, incubation of the cells with SiR labeled HMD imager resulted in spontaneous blinking. To construct the SPIN image a 1 min video was recorded with an exposur time of 30 ms (excitation at 642 nm). (b) The corresponding diffraction limited image shows the comparison with SPIN image. A significant improvement of resolution and much sharper image from SPIN technique was achieved from live cell microscopy experiment. (c) Cross-section profile of a actin filament (region marked with a square in image b and c) shows a Gaussian fit with FWHM of 59.39 ± 1.86 nm for SPIN image as compare to a FWHM of 358.27 ± 8 nm for the diffraction limited image. Scale bars: (b) and (c) 5 μ m.

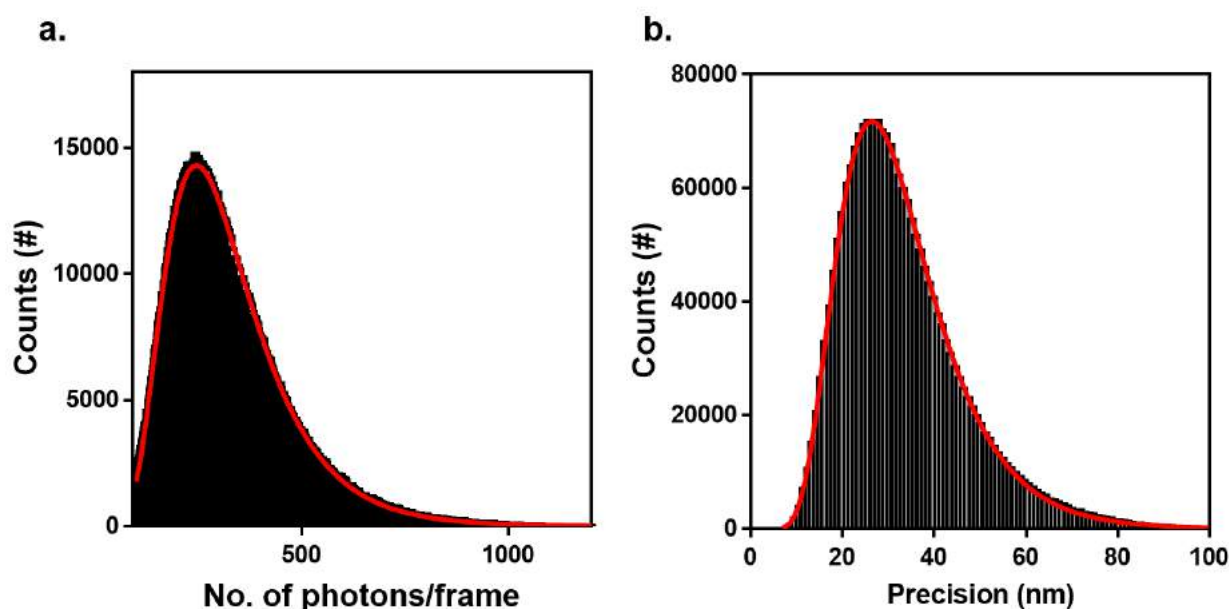


Figure S56 (a) Number of photons per frame and (b) localization precision data for the super resolution imaging of actin in live cell from figure S54.

Captions for Supporting Movies:

Supporting Movie 1: Blinking video for host-guest imaging of F-actin in fixed cell using CB[7]-phalloidin conjugate and Cy5 conjugated HMD imager.

Supporting Movie 2: Blinking video for live cell host-guest imaging of F-actin using CB[7]-phalloidin conjugate and silicon rhodamine conjugated HMD imager.

Supporting Movie 3: Bleaching free long-time imaging of actin in live cell via host-guest mediated interaction. This video is composed of 10 subsequent reconstructed super-resolution images recorded over 10 min. To construct each super-resolution image, a 60 s video was recorded with an exposur time of 30 ms (excitation at 642 nm).

Supporting References:

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