Supporting Information for

Synthetic Host–Guest Assembly in Cells and Tissues: Fast, Stable and Selective Bioorthogonal Imaging via Molecular Recognition

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General

All the chemicals were purchased from either of the following company: Sigma Aldrich, Alfa Aesar, 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). Zeba™ spin desalting columns were purchased from Thermo Fisher Scientific (product no. 89883). Biotage® SNAP cartridge (KP–C18–SH, 12 g) was purchased from Biotage (part no. FSL0–1118–0012). Antibodies and fluorophores 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.

$^1$H NMR spectra were recorded using Bruker AVANCE III 400 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 instrument. 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 of fluorophore conjugates 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 Cy5–maleimide conjugate was carried out using a Biotage@ SNAP cartridge (KP–C18–SH, 12 g) and water/acetonitrile was used as eluent. The solvents used as eluent in HPLC purification of DNA conjugates were: solvent A (0.1 M TEAA buffer of pH 7.0 containing 5% acetonitrile) and solvent B (0.1 M TEAA buffer of pH 7.0 containing 50% acetonitrile). A gradient elution of solvent B in solvent A from 0–100% was used for the purification. Absorbance of fluorophores were measured using an Eppendorf BioSpectrometer. The structured illumination, DNA–PAINT and live cell images were captured using an inverted Zeiss ELYRA PS1 microscope. Bright field immunofluorescence microscopic imaging were carried out using Olympus inverted fluorescence microscope. Flow cytometry experiment was carried out using BD FACSAnalysis™ III flow cytometer.

Supplementary table S1: List of primary antibodies used for immunostaining
| Target                      | Antibody commercial sources       | Species |
|-----------------------------|-----------------------------------|---------|
| Microtubule (β–tubulin)     | DSHB (E7–s)                       | Mouse   |
| Microtubule (α-tubulin)     | Thermo Fischer Scientific (MA1-80017) | Rat     |
| VCAM1                       | BD Biosciences (Cat. no. 550547)   | Mouse   |
| Mitochondria (Tom 20)       | Santa cruz (sc–11415)             | Rabbit  |

**Supplementary table S2:** List of secondary antibodies used for immunostaining

| Target | Host | Specification and commercial source |
|--------|------|------------------------------------|
| Mouse  | Donkey | Donkey Anti–Mouse IgG (H+L)  Jackson ImmunoResearch Laboratories (Cat. No. 715-005-151) |
| Mouse  | Goat   | Goat anti–Mouse IgG (H+L)  Jackson ImmunoResearch Laboratories (Cat. No. 115-005-146) |
| Rabbit | Donkey | Donkey Anti–Rabbit IgG (H+L) Jackson ImmunoResearch Laboratories (Cat. No. 711-005-152) |
| Rat    | Donkey | Donkey Anti-Rat IgG (H+L)   Jackson ImmunoResearch Laboratories (Cat. No. 712-005-153) |

**Supplementary table S3:** List of Fluorophores

| Fluorophores | Commercial source |
|--------------|-------------------|
| Cy5 NHS ester| Lumiprobe (Cat. No. 23320) |
| Rhodamine NHS ester | ANASPEC (Cat. No. AS–81124) |
| Atto-647 NHS ester | ATTO-TEC (Cat. No. AD 647-31) |
| Bodipy NHS ester | Thermo Fischer Scientific (Cat. No. D2184) |

**Supplementary table S4:** Experimental set-up for imaging of cells and tissues
| Figures | Mode of imaging | Targeting molecules | fluorophores |
|---------|----------------|---------------------|--------------|
| Figure 1 | SIM | β–tubulin (anti–mouse) | CB[7] conjugated goat anti–mouse | ADA–Cy5 |
| Figure 2 | SIM | β–tubulin (anti–mouse) | CB[7] conjugated goat anti–mouse | ADA–Cy5 |
| Figure 3 | DNA–PAINT | β–tubulin (anti–mouse) | DNA conjugated donkey anti–mouse | DNA–Atto 655 |
| Figure 4 | SIM | Phalloidin–CB[7] | ADA–Cy5 |
| Figure 5b | Multiplexed SIM in cells | β–tubulin (anti–mouse) and Tom20 (anti–rabbit) | CB[7] conjugated donkey anti–mouse and TCO conjugated donkey anti-rabbit | ADA–Rhodamine and Tz–Cy5 |
| Figure 5c | Multiplexed SIM in Tissue | Phalloidin–CB[7] and β–tubulin (anti–mouse) | TCO conjugated donkey anti-mouse | ADA–Rhodamine and Tz–Cy5 |
| Figure 6b | SIM in Live cell | VCAM (anti–Rat) | CB[7] conjugated donkey anti–rat | ADA–Cy5 |
| Figure 6c & d | SIM in Live cell | Phalloidin–CB[7] | ADA–Rhodamine |
| Figure S26 | SIM | β–tubulin (anti–mouse) | CB[7] conjugated donkey anti–mouse | ADA–Cy5 |
| Figure S27 | SIM | β–tubulin (anti–mouse) | CB[7] conjugated donkey anti–mouse | ADA–Cy5 |
| Figure S28 | SIM | β–tubulin (anti–mouse) | CB[7] conjugated donkey anti–mouse | ADA–Cy5 |
| Figure S29a | SIM | Tom 20 (anti–rabbit) | CB[7] conjugated donkey anti–rabbit | ADA–Cy5 |
| Figure S29b | SIM | Phalloidin–CB[7] | ADA–Cy5 |
| Figure S30 | SIM | β–tubulin (anti–mouse) | CB[7] conjugated donkey anti–mouse | ADA–Cy5 |
| Figure S31 | SIM | β–tubulin (anti–mouse) | TCO conjugated donkey anti–mouse | Tetrazine–Cy5 |
| Figure | Description | Antibody / Reagent Details |
|--------|-------------|---------------------------|
| Figure S32 | Epi-fluorescence microscopy | β–tubulin (anti–mouse) CB[7] conjugated donkey anti–mouse ADA–Cy5 |
| Figure S33 | Epi-fluorescence microscopy | β–tubulin (anti–mouse) CB[7] conjugated donkey anti–mouse ADA–Cy5 |
| Figure S34 | DNA–PAINT | β–tubulin (anti–mouse) a) CB[7] conjugated donkey anti–mouse; b) DNA conjugated ADA DNA Atto 655 |
| Figure 35 | Multiplexed SIM | a) – b) Tom 20 (anti–rabbit) c) β–tubulin (anti–mouse) d) – a) – b) TCO conjugated donkey anti–rabbit c) CB[7] conjugated goat–anti–mouse d) Phalloidin–DBCO a) DAPI b) Tetrazine–Cy5 c) ADA–rhodamine d) Azide–Bodipy |
| Figure 36 | Sequential multiplexing | Tom 20 (anti–rabbit) and β–tubulin (anti–mouse) CB[7] conjugated donkey anti–rabbit and donkey anti–mouse; Phalloidin–CB[7] ADA–Cy5 |
| Figure S37 | SIM in live cell | – CB[7] conjugated donkey anti–rat ADA–Cy5 |
| Figure S38 | SIM in live cell | – – ADA–Cy5 |
Scheme S1. Synthetic scheme for preparation of CB[7] conjugated target/affinity ligands. (a) Preparation of trans-cyclooctene (TCO) conjugated CB[7]. (b) Preparation of tetrazine (Tz) conjugated target/affinity ligands and subsequent coupling with TCO conjugated CB[7].

Synthesis of CB[7]–OH: CB[7] has been prepared and purified by following literature reported protocols\(^1\)\(^2\) and characterized by \(^1\)H NMR and HRMS. Next, mono-hydroxylation of CB[7] has been done by following a literature reported procedure with some modification\(^3\). CB[7] (250 mg, 0.215 mmol) was taken in a 50 ml quartz tube. It was dissolved in 40 ml solution of miliQ water and 10 M HCl solution (1/1 vol. %) under argon atmosphere. Sample was sonicated for a brief period to ensure all the CB[7] is solubilized before the addition of a solution of hydrogen peroxide (49 µl from 30% solution, 0.215 mmol). Then the solution was subjected to UV irradiation at wavelength of 250-400 nm (450 W) under vigorous stirring condition for 7.5 h. After that, the acidic solution was evaporated using rotary evaporator upto 2 ml and 250 ml methanol was added to it. The resulted precipitate was allowed to settle down for overnight. Next, the supernatant was decanted and remaining volume was centrifuged at 5500 rpm to get a light-yellow colored precipitate. The precipitate was washed with 50 ml of methanol for 3 times and finally dried in vacuum oven for overnight. The product was characterised by HRMS. HRMS: (ESI-MS): calculated m/z 658.2235 [M + p-xylenediamine + 2H]^{2+}; found 658.2243 [M + p-xylenediamine + 2H]^{2+}. HRMS spectrum of CB[7]-OH has been shown in Fig. S1.
Synthesis of CB[7]-Allyl: Allyl modification of CB[7]-OH has been done by following a literature reported procedure with some modifications. NaH (64.8 mg, 60% dispersion in mineral oil, 1.62 mmol) was added to 6 ml anhydrous DMSO solution of CB[7]-OH (191 mg, 0.162 mmol) under dry argon atmosphere. The reaction was stirred for 1 h at room temperature. Allyl bromide (138.86 µl, 1.62 mmol) was then added to this reaction mixture maintaining the temperature of reaction mixture at 0°C during addition. The reaction was further stirred at room temperature for 18 h. After that the reaction mixture was poured into 200 ml diethyl ether, sonicated and allowed to stand for overnight. Then supernatant was decanted, and the remaining volume was centrifuged at 5500 rpm to get white precipitate. Then it was washed with 200 ml diethyl ether for 3 times and finally dried under high vacuum. The product was characterized by HRMS. HRMS: (ESI-MS): calculated m/z 678.2422 [M + p-xylenediamine + 2H]^{2+}; found 678.2430 [M + p-xylenediamine + 2H]^{2+}. HRMS spectrum of CB[7]-Allyl has been shown in Fig. S2.
Synthesis of CB[7]-NH₂: Amine modified CB[7] has been synthesized by following a literature reported procedure⁵ with some modifications. CB[7]-Allyl (124 mg, 0.1017 mmol) was taken in a quartz cuvette and dissolved in 3 ml miliQ water. Cystamine hydrochloride (115.53 mg, 1.017 mmol) was added to it and the reaction mixture was purged with N₂ gas followed by subjected to UV irradiation at wavelength of 254-400 nm (450 W) for 12.5 h. After completion of the reaction, 50 ml ethanol was added to get white precipitate. The precipitate was allowed to settle down for overnight. Then supernatant was decanted, and remaining volume was centrifuged at 5500 rpm to get solid precipitate. Next it was washed with 50 ml ethanol and dried under high vacuum. The product was characterized by HRMS. HRMS: (ESI-MS): calculated m/z 716.7583 [M + p-xylene diamine + 2H]²⁺; found 716.7572 [M + p-xylene diamine + 2H]²⁺. HRMS spectrum of CB[7]-NH₂ has been shown in Fig. S3.
Figure S3: HRMS spectrum and purity table of CB[7]-NH₂.

Synthesis of CB[7]-PEG-TCO: CB[7]-NH₂ (1.7 mg, 1.297 µmol) was taken in 3 ml glass vial and dissolved in 200 µl dry DMF. Et₃N (1.8 µl, 12.97 µmol) was added to it. TCO-PEG-NHS ester (1 mg, 1.945 µmol) was dissolved in 100 µl DMF in another vial and added to the reaction mixture. The reaction was stirred at room temperature for 28 h. After completion of reaction, 12 ml MeOH was added to the reaction mixture to get a white precipitate. It was allowed to settle down for 15 min. The precipitate was collected after centrifugation at 7200 rpm for 5 min. The above step was repeated twice to remove all the unreacted starting materials. The solid product was analysed by HRMS. HRMS: (ESI-MS): calculated m/z 916.3700 [M + p-xylenediamine + 2H]²⁺; found 916.3759 [M + p-xylenediamine + 2H]²⁺. HRMS spectrum of CB[7]-PEG-TCO has been shown in Fig. S4.
Synthesis of CB[7] conjugated antibody:

1. Secondary antibodies (Donkey anti-Rat, Donkey anti-Mouse, Donkey anti-rabbit and Goat anti-Mouse) were buffer exchanged by Zeba spin column pre-equilibrated with PBS containing 10% 1M NaHCO₃.
2. The concentration of the antibody was measured and used for the conjugation with tetrazine.
3. Buffer exchanged secondary antibody (150 µg, 1x10⁻³ µmol) was first taken in 1.5 ml microcentrifuge tube.
4. 1,2,4,5-tetrazine-NHS ester was prepared by following reported procedure⁶,⁷ and characterized by ¹H NMR. Then it was used for conjugation with secondary antibodies. 5 µl solution (Conc. of 4 mg ml⁻¹) of tetrazine-NHS ester in DMF was added to the antibody solution in two portions (2.5 µl each time).
5. The reaction was incubated at room temperature for 2 h.
6. After that tetrazine conjugated antibody was purified by Zeba spin column (pre-equilibrated with PBS).

Figure S4: HRMS spectrum and purity table of CB[7]–PEG–TCO.

| Compound             | m/z (Calculated) | m/z (found) | Intensity from mass spectrum | Yield(%) |
|----------------------|------------------|-------------|------------------------------|----------|
| CB[7]                | 650.2291         | 650.2297    | 49.30                        | 28.59    |
| CB[7]_(OH)ₙ          |                  |             |                              |          |
| CB[7]_OH             | 658.2235         | 658.2243    | 18.87                        | 10.61    |
| CB[7]_OH₂            | 666.2240         | 666.2249    | 8.65                         | 4.86     |
| CB[7]_Allyl          | 678.2422         | 678.2430    | 1.26                         | 0.73     |
| CB[7]_(NH₂)ₙ         |                  |             |                              |          |
| CB[7]_NH₂            | 716.7571         | 716.7582    | 9.43                         | 5.47     |
| CB[7]_NH₂₂           | 783.2582         | 783.2569    | 1.59                         | 0.92     |
| CB[7]_(PEG_TCO)ₙ     |                  |             | **83.35**                    | **48.33**|
| CB[7]_PEG_TCO        | 916.3700         | 916.3759    | 0.33                         | 0.19     |
| CB[7](OH)_PEG_TCO    | 924.3674         | 924.3682    | 51.87                        | 29.18    |
| CB[7](NH₂)_PEG_TCO   | 982.8981         | 982.8989    | 31.15                        | 17.52    |
7. CB[7]-PEG-TCO (21.2 µg, 6.5 µl from 3.33 mg ml\(^{-1}\) stock solution in H\(_2\)O, 12.5\(\times\)10\(^{-3}\) µmol) was added to antibody-tetrazine (75 µg, 5\(\times\)10\(^{-4}\) µmol) solution and the reaction mixture was stirred at room temperature for overnight to prepare CB[7] conjugated antibody through TCO-tetrazine ligation.\(^8\)

8. CB[7] conjugated antibody was characterized by MALDI mass spectrometry. MALDI mass spectrum has been shown in Fig. S5.

**Synthesis of TCO conjugated antibody:**

1. Secondary antibodies (donkey anti–rabbit, donkey anti–mouse, donkey anti-rat) was buffer exchanged by Zeba spin column pre–equilibrated with PBS containing 10% 1M NaHCO\(_3\).
2. The concentration of the antibody was measured and used for the conjugation with TCO.
3. Buffer exchanged secondary antibody (150 µg, 1\(\times\)10\(^{-3}\) µmol) was first taken in 1.5 ml micro centrifuge tube.
4. 5 µl solution (Conc. of 5.15 mg ml\(^{-1}\)) of TCO–PEG–NHS ester in DMF was added to the antibody solution in two portions (2.5 µl each time).
5. The reaction was incubated at room temperature for 2 h.
9. TCO conjugated antibody was then purified by Zeba spin column (pre–equilibrated with PBS) and characterized by MALDI mass spectrometry. MALDI mass spectrum has been shown in Fig. S6.

**MALDI–MS analysis for characterization of antibody–CB[7] conjugate**

Antibodies (1 µl) after desalting through Zeba spin column (~ 1 mg.ml\(^{-1}\) in miliQ water) was taken in micro centrifuge tube. 1 µl of Sinapinic acid (10 mg.ml\(^{-1}\) in 50:50 water (0.1% TFA)/acetonitrile) was mixed with the antibodies. The mixed solution was placed onto a MALDI plate and allowed to dry at room temperature for analysis. MALDI–MS analysis was performed using a Bruker autoflex system.
Figure S5: MALDI–MS spectra of free antibody and CB[7] conjugated goat anti–mouse secondary antibody. Mass shift after conjugation was used to calculate the degree of CB[7] conjugation. We estimated an average of six CB[7] molecules attached with a single antibody.

Figure S6: MALDI–MS spectra of free antibody and TCO conjugated donkey anti–mouse secondary antibody. Mass shift after conjugation was used to calculate the degree of TCO conjugation. We estimated an average of fifteen numbers of TCO was attached with a single antibody.
Synthesis of CB[7] conjugated Phallodin:

1. Amino phalloidin (120 µg, 60 µl from 2 mg ml\(^{-1}\) stock solution in dry DMF, 0.1523 µmol) was taken in 1.5 ml micro centrifuge tube.
2. 5 µl DMF solution containing 0.106 µl Et\(_3\)N was added to it.
3. 30.34 µl Tetrazine-NHS ester\(^9\) (from a stock solution of 10 mg ml\(^{-1}\) in DMF, 0.7615 µmol) was added to it and the reaction was stirred at room temperature for 3 h.
4. After the reaction, the reaction mixture was diluted using 120 µl of mili Q water and injected to HPLC for purification using water/acetonitrile containing 0.1% TFA as eluent. The purity of the isolated compound was again analysed by HPLC (see Fig. S8).
5. After purification it was characterized by HRMS. HRMS (ESI-MS): Calculated m/z 1071.4465 [M+H]\(^+\); found 1071.4470 [M+H]\(^+\). HRMS spectrum of Phallodin-tetrazine has been shown in Fig. S7.
6. Phallodin-tetrazine conjugate (30 µg, 0.038 µmol) in 50 µl water was taken in 1.5 ml micro centrifuge tube and CB[7]-PEG-TCO (130 µg, 39.1 µl from 3.33 mg ml\(^{-1}\) stock solution in H\(_2\)O, 0.076 µmol) was added to it and stirred at room temperature for overnight to prepare CB[7] conjugated phalloidin.
7. CB[7] conjugated phalloidin was then used for imaging of F-actin.
Figure S7: HRMS spectrum of Phalloidin–Tz.

Figure S8: HPLC chromatogram of tetrazine conjugated phalloidin. Acetonitrile gradient was changed from 5 to 35% in 17 min.

Synthesis of DBCO conjugated Phalloidin:

Scheme S3. Synthetic scheme for preparation of DBCO conjugated phalloidin.

1. Amino phalloidin (20 µg, 10 µl from 2 mg ml⁻¹ stock solution in dry DMF, 0.0254 µmol) was taken in 0.5 ml micro centrifuge tube.
2. 5 µl DMF solution containing 0.018 µl Et₃N was added to it.

3. 3.6 µl DBCO NHS ester (from a stock solution of 25 mg ml⁻¹ in DMF, 0.1270 µmol) was added to it and the reaction was stirred at room temperature for 3 h.

4. After the reaction, the reaction mixture was diluted using 80 µl of mili Q water and injected to HPLC for purification using water/acetonitrile containing 0.1% TFA as eluent. The purity of the isolated compound was again analysed by HPLC (see Fig. S9).

5. After purification it was characterized by LCMS. LCMS (ESI-MS): Calculated m/z 1366.60 [M+H]⁺, 683.10 [M+2H]²⁺; found 1366.97 [M+H]⁺; 684.14 [M+2H]²⁺. LCMS spectrum of Phalloidin-DBCO has been shown in Fig. S10.

6. DBCO conjugated phalloidin was then used for imaging of F-actin in multiplexed imaging.

Figure S9: HPLC chromatogram of phalloidin–DBCO conjugate. Acetonitrile gradient was changed from 5 to 35% in 17 min and upto 100 % in 25 min during hplc purification.

Figure S10: LCMS spectrum of phalloidin–DBCO conjugate.
Synthesis of compound 1
Mono tosylated tetraethylene glycol was synthesized following reported protocol with some modifications. Tetra ethylene glycol (10 g, 51.22 mmol) was dissolved in 130 ml acetonitrile in 250 ml RB Flask and Triethyl amine (7.13 ml, 51.22 mmol) was 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 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 purified using flash silica (230–400 mess) column (eluent: Hexane/ethyl acetate gradient elution from 0 to 80%). TEG–mono–OTs was obtained as colourless oil (6.019 g, 35%). Rf = 0.375 (EtOAc/Hexane: 7:3). Ditosylated derivative was formed as a side product (3.40 g, 20%). Rf = 0.75 (EtOAc/Hexane: 7:3); 1H NMR (400 MHz, CDCl3): δ 7.79(2H, d, ArH), 7.33(2H, d, ArH), 4.16 (2H, t, −CH2OSO2−), 3.69–3.59 (14H, m, −OCH2CH2O−), 2.44 (3H, s, −CH3).

1H NMR spectrum of TEG–mono–OTs is shown in Fig. S11.

Synthesis of compound 2
A solution of NaOH (0.864 g, 21.6 mmol) in water (8 ml) was added to a solution of triphenylmethanethiol (3.98 g, 14.402 mmol) in a mixture of EtOH/toluene (1:1 v/v, 50 ml). The
mono tosylated product (compound 1, 5.019 g, 14.402 mmol) was dissolved in a second solution of EtOH/toluene (1:1 v/v, 50 ml), which was then added to the Triphenylmethanethiol mixture in one portion. The reaction mixture was stirred for 18 hours at RT. After completion of reaction (monitored by TLC) the reaction mixture was poured into a saturated solution of NaHCO$_3$ (20 ml) and extracted with Et$_2$O (3 × 40 ml). The combined organic layers were washed with brine (3 × 40 ml), dried over Na$_2$SO$_4$ and solvent was removed under reduced pressure to give a pale−yellow oil. The crude product was purified by silica column (60–120 mess size) chromatography (eluent: hexane/EtOAc, 2:1→1:3) to give Trt−TEG−OH as a pale−yellow oil (5.442 g, 85%). R$_f$ = 0.42(Hexane/ EtoAc: 1:3); $^1$H NMR (400 MHz, CDCl$_3$): δ 7.41 (6H, d, ArH), 7.27 (6H, d, ArH), 7.20 (3H, m, ArH), 3.70 (2H, br, HOCH$_2$−), 3.63 (4H, t, −OCH$_2$CH$_2$O−), 3.58 (4H, t, −OCH$_2$CH$_2$O−), 3.45 (2H, t, −OCH$_2$CH$_2$OH), 3.30 (2H, t, −SCH$_2$−). $^1$H NMR spectrum of Trt−TEG−OH is shown in Fig. S12.

Synthesis of compound 3

A solution of Trt−TEG−OH (5.43 g, 12.0 mmol) and triethylamine (2.5 ml, 18 mmol) in dry DCM (60 ml) was cooled to 4 °C and stirred under argon. Methanesulfonyl chloride (1.68 ml, 24.0 mmol) was added drop wise, while maintaining the temperature below 5 °C. The reaction mixture was stirred for 30 minutes at 4 °C and then allowed to warm to RT and stirred for further 12 hours. After completion of reaction (monitored by TLC), the resulting residue was diluted to 100 ml with DCM and washed with 0.1M HCl (2 × 25 ml), saturated NaHCO$_3$ solution (2 × 25 ml), and brine (25 ml). The organic layer was dried over Na$_2$SO$_4$ and concentrated under reduced pressure. The crude product was purified by Flash silica column (eluent: hexane/EtOAc 1:1) to yield Trt−TEG−OMs as a pale−yellow oil (5.55 g, 88%). R$_f$ = 0.45(Hexane/EtoAc: 1:1); $^1$H NMR (400 MHz, CDCl$_3$): δ 7.41 (6H, d, ArH), 7.27 (6H, d, ArH), 7.21 (3H, m, ArH), 4.35 (2H, t, −CH$_2$OSO$_2$−), 3.73 (2H, br, −CH$_2$CH$_2$OSO$_2$−), 3.63 (4H, t, −OCH$_2$CH$_2$O−), 3.55 (2H, t, −OCH$_2$CH$_2$O−), 3.45 (2H, t, −OCH$_2$CH$_2$OH), 3.30 (2H, t, −SCH$_2$CH$_2$O−), 3.02 (3H, s, −OCH$_3$), 2.43 (2H, t, −SCH$_2$−). $^1$H NMR spectrum of Trt−TEG−OMs is shown in Fig. S13.

Synthesis of compound 4

Adamantane amine hydrochloride (176.2 mg, 0.9386 mmol) and potassium carbonate (260 mg, 1.8772 mmol) and Trt−TEG−OMs (500 mg, 0.9386 mmol) were dissolved in 2 ml of DMF placed in a 10 ml RB flask. The reaction mixture was heated to 85°C and stirred for 12h. After completion of reaction (monitored by TLC), the reaction mixture was cooled down at room temperature. 10 ml Methanol was added to the reaction mixture and the solid ppt was filtered off and filtrate was concentrated using rotary evaporator. 20 ml water was added to the crude product and product was extracted using ether (3x2 ml) from aqueous layer. Organic layer was dried over Na$_2$SO$_4$ and purified
by Flash silica column chromatography (Eluent: gradient eluent of 0–5% methanol in DCM v/v).
Yield 155 mg (0.2647 mmol, 65%). R<sub>f</sub> = 0.34 (eluent: DCM/MeOH: 19:1). ¹H NMR (400MHz, CDCl₃): δ 7.39 (6H, m, ArH), 7.27 (6H, m, ArH), 7.20 (3H, m, ArH), 3.90 (2H, t, −OCH₂CH₂O−), 3.65(4H, m, −OCH₂CH₂O−), 3.59 (2H, m, ), 3.46 (2H, m) 3.30 (2H, t, −SCH₂CH₂O−) 3.06(2H,m, −CH₂NH−), 2.42(2H, t, −SCH₂−), 2.14 (3H, br, −CH−), 1.94 (6H, br, −CH2−), 1.65 (6H, br, −CH2−).
¹H NMR spectrum of Trt–TEG–ADA is shown in Fig. S14.

**Synthesis of compound 5**

Trt–TEG–ADA (150mg, 0.2560 mmol) was dissolved in ml Dry DCM in 10 ml RB flask. The solution was purged with nitrogen and an excess of trifluoro acetic acid (TFA, 394μL, 5.12 mmol) was added. During the addition period of TFA the color of the solution was turned to yellow. Subsequently, triisopropylsilane (TIPS, 262μL, 1.28 mmol) was added to the reaction mixture and the color of mixture slowly recovered to colorless. The reaction mixture was allowed to stir at room temperature for 6 h under nitrogen atmosphere. The volatile components (solvent, TFA and TIPS) were then distilled off under reduced pressure. The pale–yellow residue was purified by washing with hexane (10 ml x 3) followed by vortexed. After final washing, the hexane was decanted out and liquid component was dried under high vacuum to a colorless viscous liquid. Yield: 45 mg (0.2896 mmol, 65%). R<sub>f</sub> = 0.15 (eluent: DCM/MeOH 19:1). ¹H NMR (400MHz, CDCl₃): 3.80 (2H, br, −OCH₂CH₂O−), 3.66(8H, br, −OCH₂CH₂O−), 3.16 (2H, t, −SCH₂CH₂O−), 2.90 (2H, t, −CH₂NH−), 2.42 (2H, t, −SCH₂−), 2.21 (3H, br, −CH−), 1.94 (6H, br, −CH2−), 1.71 (6H, br, −CH2−). HRMS: ESI–MS: m/z calculated 344.2254 [M+H]<sup>+</sup>, found 344.2241. ¹H NMR and HRMS spectrum of HS–TEG–ADA is shown in Fig. S15 and S16 respectively.
Figure S11: $^1$H NMR of compound 1 (400 MHz, CDCl$_3$) in Bruker AVANCE III 400 MHz NMR instrument.

Figure S12: $^1$H NMR of compound 2 (400 MHz, CDCl$_3$) in Bruker AVANCE III 400 MHz NMR instrument.
Figure S13: $^1$H NMR of compound 3 (400 MHz, CDCl$_3$) in Bruker AVANCE III 400 MHz NMR instrument

Figure S14: $^1$H NMR of compound 4 (400 MHz, CDCl$_3$) in Bruker AVANCE III 400 MHz NMR instrument
Figure S15: $^1$H NMR of compound 5 (400 MHz, CDCl$_3$) in Bruker AVANCE III 400 MHz NMR instrument

Figure S16: HRMS spectrum of compound 5.
Scheme S5. Synthetic scheme for preparation of ADA conjugated fluorophores.

**Synthesis of Cy5–Maleimide**

N-(2-aminoethyl) maleimide hydrochloride (278 µg, 27.8 µl from 10 mg ml\(^{-1}\) stock solution in DMSO, 1.575 µmol) was dissolved in 50 µl dry DMF in a 1.5 ml glass vial. Triethyl amine (4.57 µl from 100 µl ml\(^{-1}\) stock solution in DMF, 3.2815 µmol) was added to it. Then Cy5 NHS ester (1 mg, 1.3126 µmol) dissolved in 100 µL DMF was added to it and the reaction mixture was stirred at room temperature for 12 h. After that the reaction mixture was diluted to 1 ml using Mili Q water and purified by reverse phase column chromatography using Biotage\textsuperscript{@} SNAP C18 cartridge (eluent: a gradient elution using acetonitrile/water from 0 to 100%). The blue colored eluent from the column was collected, concentrated and lyophilized to yield Cy5 maleimide (0.66 mg, 68%, a blue coloured solid). HRMS: (ESI–MS): calculated m/z 765.2622 [M+2H–Na]+, 787.2442 [M+H]+, found 765.2552 [M+2H–Na]+, 787.2324 [M+H]+. HRMS spectrum of Cy5–maleimide is shown in Fig. S17.

**Synthesis of ADA–Cy5**

A stock solution of SH–TEG–ADA (22.22 µg, 50 µL from 0.9 mg ml\(^{-1}\) stock solution in PBS (pH 7.4), 0.1314 µmol) 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–Cy5 (90% from HPLC chromatogram). HRMS: ESI–MS: calculated m/z 1108.4804 [M+H]+, 554.7338 [M+2H]\(^{2+}\), found 1108.4652 [M+H]+, 554.7383 [M+2H]\(^{2+}\). HRMS spectrum of ADA–Cy5 is shown in Fig. S18.

**Synthesis of Rhodamine–Maleimide**

N-(2-aminoethyl) maleimide hydrochloride (804 µg, 80.4 µl from 10 mg ml\(^{-1}\) stock solution in DMF, 3.79 µmol) was dissolved in 50 µl dry DMF in a 1.5 ml glass vial. Triethyl amine (1.32 µl from 100 µl ml\(^{-1}\) stock solution in DMF, 9.48 µmol) was added to it. Then Rhodamine NHS ester (2 mg, 3.79
µmol) dissolved in 100 µL DMF was added to it and the reaction mixture was stirred at room temperature for 12 h. After that the reaction mixture was diluted to 1 ml using Mili Q water and purified by reverse phase column chromatography using Biotage® SNAP C18 cartridge (eluent: a gradient elution using acetonitrile/water from 0 to 100%). The blue colored eluent from the column was collected, concentrated and lyophilized to yield Rhodamine maleimide (1.4 mg, 68%, a blue coloured solid). HRMS: (ESI–MS): calculated m/z 553.2087 [M+H]⁺, found 553.4387 [M+H]⁺. HRMS spectrum of Rhodamine–maleimide is shown in Fig. S19.

**Synthesis of ADA–Rhodamine**

A stock solution of SH–TEG–ADA (93 µg, 50 µL from 1.86 mg ml⁻¹ stock solution in PBS (pH 7.4), 0.27 µmol) was added to the solution of Rhodamine maleimide (50 µg, 50 µL stock in PBS) and stirred for overnight at room temperature. After that the reaction mixture was directly injected in HPLC for purification to yield ADA–Rhodamine (90% from HPLC chromatogram). HRMS: ESI–MS: calculated m/z 896.4263 [M+H]⁺, 448.7168 [M+2H]²⁺, found 896.4230 [M+H]⁺, 448.7162 [M+2H]²⁺. HRMS spectrum of ADA–Rhodamine is shown in Fig. S20.

![Figure S17](image1.png)

**Figure S17:** HRMS spectrum of Cy5-maleimide.

![Figure S18](image2.png)

**Figure S18:** HRMS spectrum of ADA–Cy5.
Figure S19: HRMS spectrum of Rhodamine maleimide.

Figure S20: HRMS spectrum of ADA–Rhodamine.

Scheme S6. Synthetic scheme for preparation of tetrazine conjugated fluorophores.
**Synthesis of Rhodamine–Tetrazine conjugate**

Rhodamine NHS ester (500 µg, 0.947 µmol) was taken in 50 µl DMSO, amino tetrazine (196 µg, 40 µl from 4.9mg.ml⁻¹ in DMSO 0.947 µmol) and 6.2 µL triethylamine (10% in DMSO) were added to it. The reaction mixture was allowed to stir at dark for 3 h at room temperature. After that the product was purified by reverse phase HPLC to yield Rhodamine tetrazine conjugate (90% from HPLC chromatogram). HRMS: ESI–MS: Calculated m/z 600.2359 [M+H]⁺, found 600.2277 [M+H]⁺. HRMS spectrum of Rhodamine–Tz is shown in Fig. S21.

**Synthesis of Cy5–Tetrazine conjugate**

Cy5–NHS ester (250 µg, 0.328 µmol) was taken in 50 µl DMSO, amino tetrazine (300 µg, 30 µl from 10 mg.ml⁻¹ in DMF 0.656 µmol) and 1 µL triethylamine (10% in DMF) were added to it. The reaction mixture was allowed to stir at dark for 3 h at room temperature. After that the product was purified by reverse phase HPLC to yield Cy5–tetrazine conjugate (90% from HPLC chromatogram). HRMS: ESI–MS: Calculated m/z 812.2900 [M+H]⁺, found 812.2859 [M+H]⁺. HRMS spectrum of Cy5–Tetrazine is shown in Fig. S22.
Scheme S7: Synthetic scheme for preparation of bodipy–azide conjugate

**Synthesis of Bodipy–azide conjugate**

Bodipy fluorophore was conjugated to NHS ester of azido acetic acid in two steps. At first, bodipy NHS ester (20 µg, 2 µl from 10 mg.ml⁻¹ stock in DMF, 0.0514 µmol) was reacted with ethylene diamine (0.0103 µl, 0.1542 µmol) in presence of triethyl amine base (0.0215 µl, 0.1542 µmol) to form amine derivative of bodipy fluorophore. The conjugated product was purified by reversed phase HPLC using water/acetonitrile containing 0.1% TFA as eluent. The isolated compound was analysed by LCMS, LCMS: (ESI–MS) calculated 315.18 [M-F]⁺, found 315.33 [M-F]⁺. (see Fig. S23 and S24a). Then amine derivative of bodipy fluorophore (15 µg, 0.044 µmol) was conjugated to NHS ester of azido acetic acid (44.39 µg, 0.2239 µmol, 1.78 µl from 25 mg.ml⁻¹ stock in DMF) in presence of triethyl amine base (0.032 µl, 0.2239 µmol) for 3 hours in room temperature. Reaction mixture was purified using reversed phase HPLC using water/acetonitrile containing 0.1% TFA as eluent. The isolated compound was analysed by LCMS, LCMS: (ESI–MS) calculated 398.20 [M-F]⁺, found 398.26 [M-F]⁺. (see Fig. S23 and S24b).
Figure S23: HPLC purification of bodipy–amine and bodipy–azide conjugate. Polarity of acetonitrile was changed from 5% to 100% in 25 min.
Figure S24: (a) LCMS spectrum of Bodipy–amine conjugate; (b) LCMS spectrum of Bodipy–azide conjugate

Scheme S8. Synthetic scheme for preparation of ADA conjugated DNA.

DNA sequence for conjugation with ADA (docking): 5’ amine/ TTGATCTACAT/ DNA sequence for conjugation with Atto 655 (imager): / TATGTAGATC/3’ amine
DNA–PEG–maleimide
DNA–NH₂ (10 nmol, 10 µl from 1 mM stock in water) was taken in a micro centrifuge tube. 1.3 µl 10× PBS was added to it. Maleimide–PEG₂– succinimidyl ester (42.54 µg, 100 nmol, 1.7 µl from 25 mg.ml⁻¹ stock in DMF) was added to it and stirred at room temperature for 3 h. The conjugated product was purified by reversed phase HPLC after passing through Zeba spin column. The purified product was lyophilized and finally dissolved in PBS for ADA conjugation.

DNA–ADA conjugate
DNA–PEG–maleimide (5 nmol, 10 µl from 500 nM stock in PBS) was taken in a micro centrifuge tube. ADA–PEG–thiol (34.35 µg, 100 nmol, 17.18 µl from 2 mg.ml⁻¹ stock in PBS) was added to it and stirred at room temperature for 12 h. The conjugated product was purified by reversed phase HPLC. The purified product was lyophilized and characterized by MALDI mass spectrometry (Fig. S25).

Synthesis of DNA–Atto 655 conjugate
DNA–NH₂ (15 nmol, 15 µl from 1 mM stock in water) was taken in a micro centrifuge tube and diluted to 55 µl using water. 8 µl 1M NaHCO₃ was added to it. Atto 655 NHS ester (33.2 µg, 37.5 nmol, 16.6 µl from 2 mg.ml⁻¹ stock in DMF) was added to it and stirred at room temperature for 12 h. The conjugated product was purified by reversed phase HPLC after passing through Zeba spin column. The purified product was lyophilized and finally stored in water for DNA–PAINT imaging.

MALDI mass spectrometry analysis of DNA–ADA conjugate
ADA conjugated DNA (1 µl, 100 µM in miliQ water) was taken in a micro centrifuge tube. 4-hydroxy picolinic acid (10 mg.ml⁻¹ dissolved in 50:50 water/acetonitrile, 1 µl) was mixed with it. The mixed solution was plated onto the MALDI plate and allowed to dry at RT from analysis.
Cell Culture Protocol

1. HeLa / MEF cells were used for the experimental study.
2. The cells were cultured in a humidified atmosphere (5% CO\textsubscript{2}) at 37°C and grown in Dulbecco’s Modified Eagle’s Medium (DMEM, high glucose) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 2 mM Glutamax (Invitrogen, USA) and 1% antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin) (Gibco, USA).
3. At ~80% confluence, the cells were washed with DPBS (pH 7.3) (Gibco, USA), trypsinized and suspended in culture medium.
4. Cells were then counted and then in a typical experiment, ~20,000 cells/well/200 µL media were plated in 8-well chamber slide system (Eppendorf) / 35mm glass bottom cell imaging dish.
5. The cells were then maintained again in a humidified atmosphere (37°C, 5% CO\textsubscript{2}) for 24 h to reach ~60% confluence.

Thereafter, cells were used for imaging experiment.

CB[7]–ADA based microtubule labeling protocol in MEF/HeLa Cells:

1. Removal of culture media from 8-well chamber slide system.
2. Washing cells with 200 µL PBS (2×).
3. Fixation for 7 minutes with 200 µL chilled methanol at −20°C followed by washing for three times with PBS.
4. Blocking for 2 h with 200 µL 3% bovine serum albumin in PBS at room temperature.
5. Incubation for 24 h at 4°C with primary antibody (10 µg ml⁻¹, 200 µL) diluted in PBS containing 3% bovine serum albumin.
6. Washing with 200 µL PBS (3×) with 5 min incubation each time.
7. Incubation for 2 h with CB[7] conjugated secondary antibody (10 µg.ml⁻¹, 200 µL) in 3% bovine serum albumin in PBS.
8. Washing with 200 µL PBS (3×) with 5 min incubation each time.
9. Incubation with 200 µL of ADA–Cy5 conjugate (250 nM) in PBS (pH 7.4) for 30 min.
10. Removal of excess conjugate by washing with 200 µL PBS (3×) followed by SIM imaging.¹¹

**Multiplexed imaging protocol for HeLa cells:**

1. Removal of culture media.
2. Fixation for 15 min with 200 µL 4% paraformaldehyde in PBS.
3. Washing with 200 µL PBS (3×).
4. Permeabilization with 200 µL 0.25% v/v Triton X–100 in PBS for 10 min.
5. Washing with 200 µL PBS (3×).
6. Blocking for 2 h with 200 µL 3% bovine serum albumin and 0.1% v/v Triton X–100 in PBS at room temperature.
7. Staining for overnight at 4°C with primary antibodies against tubulin and mitochondria (10 µg.ml⁻¹, 200 µL) diluted in 3% bovine serum albumin and 0.1% v/v Triton X–100 in PBS.
8. Washing with 200 µL PBS (3×) with 5 min incubation each time.
9. Incubation for 2 h with secondary antibodies (10 µg.ml⁻¹, 200 µL) in 3% bovine serum albumin and 0.1% v/v Triton X–100 in PBS. CB[7] conjugated secondary antibody against tubulin and TCO conjugated secondary antibody against mitochondria were used in this step.
10. Washing with 200 µL PBS (3×) with 5 min incubation each time.
11. Incubation with 200 µL of imagers (ADA-Rhodamine and Tz-Cy5, 250 nM each) in PBS (pH 7.4).
12. Removal of excess conjugate by washing with 200 µL PBS (3×) followed by imaging.

**Structured illumination imaging**

Immunostained cells kept in an 8–well chamber slide was placed under the microscope and fluorescence microscopic images were captured by structured illumination method using an inverted Zeiss ELYRA PS1 microscope. Two lasers have been used for excitation: 561 nm (200 mW) and 642 nm (150 mW) for respective excitation of fluorophores. 10% laser power (255 W.cm⁻²) was used for
structured illumination imaging. Imaging was performed using a Zeiss oil–immersion objective (alpha Plan–apochromat DIC 63x/1.40 Oil DIC M27, numerical aperture (NA) 1.40 oil). Fluorescence light was spectrally filtered with emission filters (MBS– 561+EF BP 570–650/LP 750 for laser line 561 and MBS–642+EF LP 655 for laser line 642) and imaged using a PCO edge sCMOS camera. Structured illumination super–resolution 2D and 3D images were reconstructed using structured illumination analysis package for Zen software (Zeiss). Additional softwares have been used for color adjustment (ImageJ) and data analysis (Origin 9.0).

Figure S26. Stability of CB[7]-ADA based labeling against multiple buffer washing cycles. (a–e) Structured illumination images were captured after washing the labeled samples with PBS with 1 min incubation each time. (f) The plot of fluorescent intensity from microtubules vs number of washing step indicates host-guest interaction based labeling is stable during multiple washing cycles. Fluorescence intensity was measured from five different regions of microtubules and average intensity is shown in the plot. Scale bars: 2 µm (a–e).
**Figure S27.** Fast and efficient labeling of microtubule using CB[7]–ADA interactions in HeLa cells. Structured illumination imaging of microtubule was carried out after 5 s incubation with 1 µM ADA–Cy5 in CB[7] labeled microtubule in HeLa cells. Scale bar: 10 µm.

**Figure S28.** CB[7]–ADA interactions mediated labeling using low nM concentrations of ADA probe for no wash imaging. Structured illumination imaging was performed after incubating CB[7] tagged
microtubule in HeLa cells with 5 nM ADA–Cy5 for 15 min. Imaging was performed without removing the excess probe. Scale bar: 5 µm.

Figure S29. CB[7]–ADA interactions mediated labeling of (a) mitochondria and (b) actin using low nM concentrations of ADA probe for no wash imaging. Structured illumination imaging was performed after incubating CB[7] tagged mitochondria and actin in HeLa cells with 10 nM ADA–Cy5 imager for 15 min. Mitochondria was targeted via antibody based approach whereas actin was targeted using Phalloidin. Imaging was performed without removing the excess probe. Scale bar: 5 µm; inset scale bar 2.5 µm.
Figure S30. Stability study of CB[7]−ADA interaction based labeling platform. Labeling efficiency before (a) and after (b) incubation of the reactive reagents in physiologically relevant condition. 25 nM ADA−Cy5 and CB[7] labeled microtubules in HeLa cells were kept separately at 37°C in phenol red free culture media containing 10% FBS for 16 h. Fluorescence images were acquired after 15 min incubation of ADA−Cy5 with CB[7] labeled microtubules. Minimal change in labeling efficiency was observed from this experiment. Scale bars: 5 µm (a and b).

Figure S31. Stability study of TCO−Tz interaction based labeling platform. Labeling efficiency before (a) and after (b) incubation of the reactive reagents in physiologically relevant condition. 25 nM Tz−Cy5 and TCO labeled microtubules in HeLa cells were kept separately at 37°C in phenol red free culture media containing 10% FBS for 16 h. Fluorescence images were acquired after 15 min
incubation of Tz–Cy5 with TCO labeled microtubules. A significant reduction in labeling efficiency was observed from this experiment. Scale bars: 5 µm (a and b).

**CB[7]-ADA mediated labeling of microtubule in variable pH conditions:**

Microtubules in HeLa cells were targeted using CB[7] conjugated antibody against beta-tubulin. CB[7] labeled cells were incubated with ADA conjugated cy5 fluorophores (Conc. = 100 nM) in different pH solutions ranging from 4.5 to 9.2. The compositions of buffers used in this study are mentioned in the following table.

**Supplementary table S5:** List of buffers used to achieve a specific pH

| pH  | Buffers                      | Concentrations |
|-----|------------------------------|----------------|
| 4.5 | Citric acid/sodium citrate   | 10 mM          |
| 5.0 | Acetic acid/sodium acetate   | 10 mM          |
| 6.0 | 1x PBS                       | 10 mM          |
| 7.0 | 1x PBS                       | 10 mM          |
| 8.0 | HEPES                        | 25 mM          |
| 8.5 | 1x TAE                       | 40 mM          |
| 9.2 | NaHCO₃/Na₂CO₃                | 10 mM          |

**Figure S32.** Host–guest interaction based labeling of microtubule under various pH conditions (pH 4.5–9.2). Fluorescence microscopy images clearly depict the specific labeling of microtubule in HeLa cells, demonstrating the stable and robust nature of the CB[7]-ADA interaction based labeling method under various pH conditions. Scale bar: 5 µm; inset scale bar 1 µm.
CB[7]-ADA mediated labeling of microtubule in various cell culture medium:
Microtubules in HeLa cells were targeted using CB[7] conjugated antibody against beta-tubulin. CB[7] labeled cells were coupled with ADA conjugated cy5 fluorophores by incubating 100 nM solution of ADA conjugated cy5 fluorophores. The coupling experiments were performed in six commonly used cell culture media, including DMEM+F12, IMDM, F12, MEMα, Opti MEM, and RPMI.

Figure S33. CB[7]–ADA host–guest interaction based labeling of microtubule in various cell culture media. We observed that labeling experiment performed in all these media provides efficient microtubule staining via CB[7]-ADA interaction, indicating robust nature of the labeling platform. Scale bar 5 µm; inset scale bar 1 µm..

Protocol for DNA labeling via CB[7]–ADA interaction

1. Removal of culture media from 8-well chamber slide system.
2. Washing cells with 200 µL PBS (2×).
3. Fixation for 7 minutes with 200 µL chilled methanol at −20°C followed by washing for three times with PBS.
4. Blocking for 2 h with 200 µL 3% bovine serum albumin in PBS at room temperature.

5. Incubation for 24 h at 4°C with primary antibody (10 µg ml$^{-1}$, 200 µL) diluted in PBS containing 3% bovine serum albumin.

6. Washing with 200 µL PBS (3×) with 5 min incubation each time.

7. Incubation for 2 h with CB[7] conjugated secondary antibody (10 µg.ml$^{-1}$, 200 µL) in 3% bovine serum albumin in PBS.

8. Washing with 200 µL PBS (3×) with 5 min incubation each time.

9. Incubation with 200 µL of ADA–DNA conjugate (500 nM) in PBS (pH 7.4) for 30 min.

10. Removal of excess conjugate by washing with 200 µL PBS (3×) followed by DNA–PAINT imaging.

**DNA–PAINT imaging protocol:**

We used gold nanoparticle (~150 nm) as drift marker for the DNA–PAINT imaging studies. Gold nanoparticle was deposited on the imaging well by centrifuging the nanoparticle solution at 450 rcf for 3 min. Atto 655 conjugated DNA (1 nM) in PBS containing 500 mM NaCl was incubated with the cells for DNA–PAINT imaging. Single molecule blinking was recorded using a 642 nm excitation laser (150 mW). 50% laser power has been used for image acquisition using a Zeiss oil-immersion TIRF objective (alpha Plan-apochromat DIC 100×/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). EMCCD camera gain was kept at 50 during image acquisition. Exposure time of 100 ms was used for image acquisition.
Figure S34. Host–guest interaction assisted DNA–PAINT imaging of microtubule. (a) DNA–docking strand was immobilized onto microtubule using CB[7]–ADA interaction. DNA–PAINT image was reconstructed from 50,000 frames. Comparison with diffraction limited image shows distinguishable microtubules in densely packed structure. A closer look of single microtubule (zoomed region from i) shows hollow nature of microtubule. (b) Diameter of a single microtubule (42.2 nm) was calculated from Gaussian fitted intensity profile diagram. Scale bars: 1 µm (a) and 100 nm (i).

TISSUE SECTIONS

Dissection of thoracic muscle tissue and ovary 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.

**Ovary dissection**
1. For dissecting ovary tissues, female adult flies were taken and were submerged in PBS.
2. Fly was grabbed at its lower thorax with one forcep and were gently tugged at the lower thoracic abdomen with another forcep until the internal organs in the abdomen are exposed.
3. Once pair of ovaries were seen, they were detached from other organs and these ovaries were transferred in chilled PBS.

**Immunocytochemistry for thoracic muscle tissue and ovary 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 µM) was added to each chamber and incubated overnight at 4°C. Afterwards, samples were given six washes with 0.5% PBT for 5 min incubation each time.
5. ADA–Rhodamine (100 nM) was added to each chamber and incubated for 2 h at room temperature.
6. The samples were given six washes with 0.5% PBT for 5 minutes incubation each time. After all the washes, tissue samples were cleaned and mounted on slides using 70% glycerol in PBS with the ventral side facing upward.
7. For multiplexing, after blocking step, primary antibody (10 µg.ml⁻¹) against tubulin was incubated along with Phalloidin-CB (2 µM) for overnight at 4°C.
8. After overnight incubation, samples were given six washes as mentioned above followed by addition of the respective TCO-conjugated secondary antibody (against tubulin primary antibody) and were incubated for 2 h at room temperature.
9. After incubation, the samples were washed and the imagers (ADA-Rhodamine and Tz-Cy5, 100 nM each in PBS) were added and incubated for 30 min before mounting on glass slides for imaging.
Protocol for three color multiplexed imaging in cell

1. BSA blocked SVEC cells were stained for overnight at 4°C with primary antibodies against tubulin and mitochondria (10 μg.ml⁻¹, 200 μL) diluted in 3% bovine serum albumin and 0.1% v/v Triton X–100 in PBS.

2. Washing with 200 μL PBS (3×) with 5 min incubation each time.

3. Incubation for 2 h with CB[7] conjugated secondary antibodies against microtubule and TCO conjugated secondary antibodies against mitochondria (10 μg.ml⁻¹, 200 μL) in 3% bovine serum albumin and 0.1% v/v Triton X–100 in PBS.

4. Washing with 200 μL PBS (3×) with 5 min incubation each time.

5. Incubation with 200 μL of imagers (ADA-rhodamine and tetrazine–Cy5, 100 nM) in PBS (pH 7.4) for 30 min.

6. Removal of excess fluorophores with three times quick washing using PBS.

7. Incubation of phalloidin–DBCO (1 μM) with the cells for 1 h at room temperature.

8. Quick washing with 200 μL PBS (3×).

9. Incubation with 200 μL of imagers (Azide–Bodipy, 1µM) in PBS (pH 7.4) for 1 h.

10. Removal of excess fluorophores with three times quick washing using PBS.

11. Structured illumination images were recorded keeping the cells under the microscope using 642 nm (255 W.cm⁻²), 561 nm (425 W.cm⁻²) and 488 nm (455 W.cm⁻²) laser for excitation using respective band pass filter sets.

12. Nucleus was stained with DAPI and imaged using 405 nm laser.
Figure S35: Multiplexed imaging using bio–orthogonal non-covalent (CB[7]–ADA) and covalent (TCO–tetrazine and Azide–DBCO) recognition pairs. (a) DAPI staining of nucleus, (b) labeling of mitochondria using antibody–TCO and tetrazine–cy5 pair, (c) labeling of microtubule using antibody–CB[7] and ADA–rhodamine pair and (d) labeling of actin using phalloidin–DBCO and azide–bodipy pair. (e) merged image of DAPI, mitochondria, microtubule and actin. Scale bar: 5 µm (a–e).

Protocol for Sequential multiplexed imaging

1. BSA blocked MEF cells were stained for overnight at 4°C with primary antibodies against tubulin and mitochondria (10 µg.ml⁻¹, 200 µL) diluted in 3% bovine serum albumin and 0.1% v/v Triton X–100 in PBS.
2. Incubation with 200 µL PBS (3×) with 5 min incubation each time.
3. Incubation for 1 h with CB[7] conjugated secondary antibodies against mitochondria (10 µg.ml⁻¹, 200 µL) in 3% bovine serum albumin and 0.1% v/v Triton X–100 in PBS.
4. Washing with 200 µL PBS (3×) with 5 min incubation each time.
5. Incubation with 200 µL of imagers (ADA-Cy5, 100 nM) in PBS (pH 7.4) for 15 min.
5. Removal of excess fluorophores with three times quick washing using PBS.
6. Structured illumination images were recorded keeping the cells under the microscope using 642 nm laser for excitation (laser power 255 W.cm$^{-2}$).
7. Acquired region was photobleached using high power laser (765 W.cm$^{-2}$) for 30 s.
8. Incubation for 1 h with CB[7] conjugated secondary antibodies against microtubule (10 µg.ml$^{-1}$, 200 µL) in 3% bovine serum albumin and 0.1% v/v Triton X–100 in PBS.
9. Washing with 200 µL PBS (3×) with 5 min incubation each time.
10. Incubation with 200 µL of imagers (ADA-Cy5, 100 nM) in PBS (pH 7.4) for 15 min.
11. Removal of excess fluorophores with three times quick washing using PBS.
12. Structured illumination images of microtubule were recorded using 642 nm laser for excitation (laser power 255 W.cm$^{-2}$).
13. Acquired region was photobleached using high power laser (765 W.cm$^{-2}$) for 30 s.
14. Incubation for 1 h with CB[7] conjugated phalloidin (1 µM, 200 µL) in PBS.
15. Quick washing with 200 µL PBS (3×).
16. Incubation with 200 µL of imagers (ADA-Rhodamine and Tz-Cy5, 250 nM each) in PBS (pH 7.4).
17. Removal of excess conjugate by washing with 200 µL PBS (3×) followed by imaging.
Figure S36: Sequential labeling and imaging of mitochondria (b), microtubule (d) and actin (f) using CB[7]–ADA interaction. bright field (a) and post-photobleached image (c and e) of respective region of cell. Scale bar: 5 µm (a-f).

Antibody labeling based live cell imaging protocol:

1. Immunostaining of live cells (SVEC) were done after keeping the cells at ∼4°C.
2. ∼25000 cells in culture media were first taken in microcentrifuge tube and centrifuged at 4°C, 2000 rcf for 2 min.
3. Culture media was removed carefully and cells were washed 2 times with 100 µl PBS (pH 7.4) followed by centrifuge at 2000 rcf for 2 min at 4°C.
4. Cells were incubated with 100 µl primary antibody against VCAM (rat, 10µg ml⁻¹) in PBS for 30 min at 4°C.
5. Excess antibody was removed and washed the cells 2 times with 100 µl PBS (pH 7.4) followed by centrifuge at 2000 rcf for 2 min at 4°C.
6. Then cells were incubated with 100 µl CB[7] conjugated donkey anti–rat 2° Ab (10 µg  ml⁻¹) in PBS for 20 min at 4°C.
7. After subsequent removal of excess 2° Ab and centrifuge at 2000 rcf for 2 min at 4°C (2 times), 100 µl 250 nM ADA–Cy5 was added to the cells and incubated for 15 min in PBS at 4°C.
8. Excess fluorophores were removed by 2 times washing followed by centrifuged at 2000 rcf for 2 min at 4°C.
9. Cy5 stained cells were transferred to the 8–well chamber slide after dilution to 200 µl using PBS (pH 7.4) and structured illumination images were captured immediately using 642 nm laser.

**Protocol for bright field immunofluorescence microscopy and FACS analysis**

1. Immunostaining of live cells were done by following previously mentioned protocol.
2. For control I, primary antibody incubation step was omitted.
3. For control II, both primary and secondary antibody incubation steps were omitted.
4. Three differently labelled cells were transferred to 8–well chamber slide system and bright field immunofluorescence microscopic images were captured immediately using a motorized inverted microscope (IX81, Olympus, Tokyo, Japan) equipped with a CCD camera (FVII with CellP software, Olympus, Tokyo, Japan).
5. For FACS analysis, three sets of labelled cells were dispersed in 500 µl PBS, transferred to BD FACS tube and injected to FACS analysis instrument. The fluorescence response was monitored by exiting the fluorophores at 633 nm laser.

![Figure S37](image-url) **Figure S37.** Control experiment of live cell staining. (a) RFP labeled SVEC cell. (b) Fluorescence microscopic images of respective cells where primary antibody has been omitted in immunostaining. Scale bar 5 µm (a and b).
**Figure S38.** Control experiment of live cell staining. (a) RFP labeled SVEC cell. (b) Fluorescence microscopic images of respective cells where only ADA–Cy5 has been used for labeling. Scale bar 5 µm (a and b).

**Figure S39.** Fluorescence Activated Cell Shorting (FACS) for quantitatively analyse the live cell labeling data. Strong fluorescence response in presence of CB[7] targeting conjugate and ADA imager agent as compared to the controls clearly demonstrate the specificity of the ADA imaging agent for CB[7]-modified antibody in the presence of live cells.
Figure S40. Gold nanoparticle (AuNP) assisted delivery strategy for CB[7] conjugated Phalloidin derivatives. (A) HMD guest functionalized AuNP ($d \sim 2$ nm) was used to deliver CB[7] conjugated Phalloidin derivative inside live HeLa cell. (B) HMD guest binds with CB[7] with an affinity of $\sim 10^{-7}$ M$^{-1}$ ($K_d$) whereas ADA posses an affinity of $\sim 10^{-13}$ M$^{-1}$ ($K_d$) towards CB[7]. Therefore, the initial complex between HMD-CB[7] can be disassembled by presentation of the orthogonal guest molecule 1-adamantylamine (ADA), which has relatively high affinity for CB[7]. This guest exchange process leads to the formation of more stable CB[7]-ADA complex, leading to efficient target labeling.

Host–Guest mediated live cell actin labeling protocol:

1. Culture media was removed from the chamber well and cells were washed twice in DPBS (pH 7.3).
2. Cells were incubated in complete cell culture medium containing verapamil hydrochloride (10 µM) and chloroquine diphosphate (100 µM) with CB[7] conjugated Phalloidin (2 µM) and HMD–AuNP (200 nM). Cells were incubated at 37°C in a humid atmosphere (5% CO$_2$) for 3 h.
3. After 3 h incubation, the cells were washed twice in DPBS (pH 7.3).
4. 250 nM Rhodamine-ADA in complete cell culture medium was added as imager after removing the DPBS. Cells were incubated with the imager for 30–45 min at 37°C in a humid atmosphere (5% CO$_2$).
5. Excess imager was removed by washing with DBPS and cells were imaged by keeping them in phenol red free culture medium. For long term live cell imaging, cells were kept at 37°C in a humid atmosphere (5% CO\textsubscript{2}) by using incubator that is attached with the fluorescence microscope.

**Caption for Supporting Movie 1:** Live cell actin dynamics \textit{via} CB[7]–ADA mediated labeling.

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