Synthesis and Labeling of α-(2,9)-Trisialic acid with Cyanine Dyes for Imaging of Glycan-binding Receptors on Living Cells

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1. General methods for syntheses

All chemicals were purchased as reagent grade and used without further purification. Dichloromethane was distilled over calcium hydride (CaH_2). The sialylation reactions were carried out under anhydrous condition (argon atmosphere) with anhydrous solvent. Reactions were monitored by analytical thin-layer chromatography on silica gel F_{254} glass plates. Spots were detected under UV (254 nm) or by staining with the solution of acidic ceric ammonium molybdate or EtOH/H_2SO_4 (5%). Flash column chromatography was performed on silica gel (200-300 mesh). Gel filtration chromatography was performed using a column (50 cm × 1.7 cm) packed with BioGel P-2 fine resins (Bio-Rad, Hercules, CA). ^{1}H NMR spectra were recorded with a Bruker Avance III 400 MHz NMR spectrometer, and the chemical shifts (in ppm) were referenced to solvent peaks. ^{13}C NMR spectra were recorded with the 400 MHz NMR spectrometer (100 MHz) with chemical shifts referenced to solvent peaks. High resolution electrospray ionization mass spectra (HRMS-ESI) were recorded with a Waters LCT Premier XE mass spectrometer.

Abbreviations of the Chemicals and Solvents

THF Tetrahydrofuran

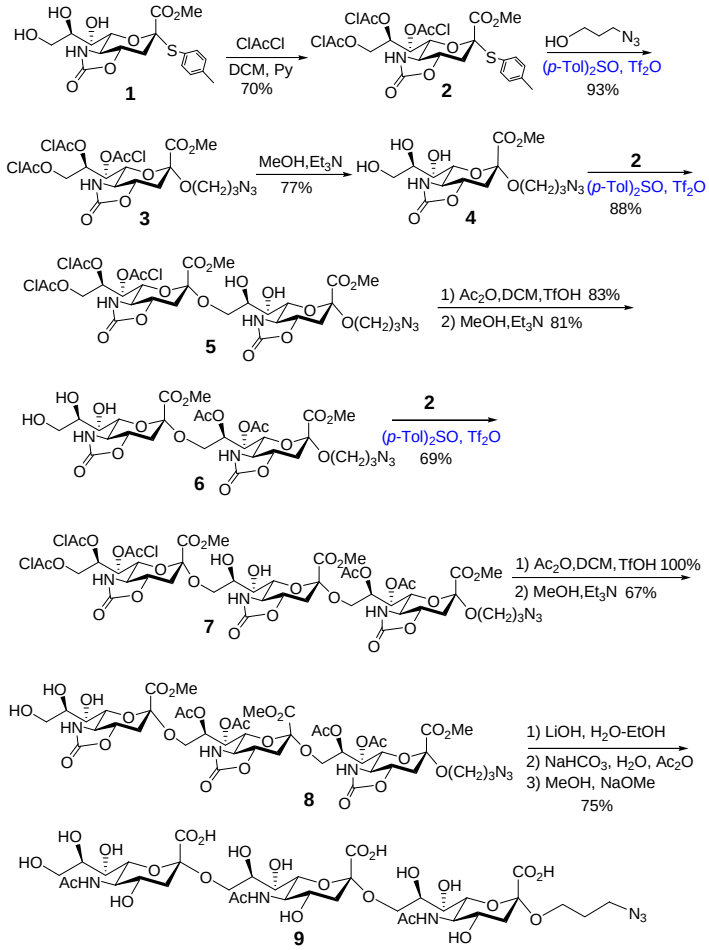
DMF *N*, *N*-dimethylformamide

DCC *N*, *N*-Dicyclohexylcarbodiimide

NHS *N*-Hydroxysuccinimide

TBTA tris-(benzyltriazolylmethyl)amine

2. Synthesis of triSia-N_3 (9)



Scheme S1 Synthesis of α-(2,9)-trisialic acid **9**.

*2.1 Preparation of donor **2***

Pyridine (3.2 mL, 39.6 mmol) was added to the solution of dissolved compound **1** [1] (516.8 mg, 1.25 mmol) in anhydrous dichloromethane (20 mL, 0.06 M) under argon. After cooled at 0℃ in 15 min, the fresh distilled chloroacetyl chloride (0.7 mL, 9.3 mmol) was added to the solution by syringe. After stirred for 1 h, the solvent was removed under reduced pressure. The residue was diluted by dichloromethane and washed by saturated brine, dried over anhydrous $MgSO_4$ then was subjected to column chromatography on silica gel (petroleum: ethyl acetate = 2: 1) to generate yellow oil, followed by recrystallization by petroleum/diethyl ether/ethyl acetate to give the desired product as white solid (560 mg, 70%). ^{1}H NMR (400MHz, $CDCl_3$): δ = 2.14 (t, *J* = 12.5Hz, 1H), 2.40 (s, 3H), 3.00 (t, *J* = 10.1Hz, 1H), 3.11 (dd, *J* = 3.5, 12.3Hz, 1H), 3.61 (s, 3H), 3.90 (m, 1H), 4.03-4.05 (m, 1H), 4.06 (s, 2H), 4.07 (dd, *J* = 15.2 Hz, 1H), 4.15 (dd, *J* = 15.2 Hz, 1H), 4.18 (d, *J* = 1.1Hz, 2H), 4.44 (dd, *J* = 3.6, 12.9Hz, 1H), 4.66 (dd, *J* = 1.9,

12.9Hz, 1H), 5.16 (dd, *J* = 1.2, 9.4Hz, 1H), 5.24 (s, 1H), 5.45 (m, 1H), 7.17 (d, *J* = 8.0Hz, 2H), 7.32 (d, *J* = 8.0Hz, 2H). ^{13}C NMR (100MHz, $CDCl_3$): δ = 21.3, 37.5, 40.3, 40.4, 40.7, 53.1, 57.6, 62.6, 69.2, 70.5, 74.9, 88.3, 124.5, 129.8, 136.3, 140.8, 158.7, 165.9, 167.0, 167.9, 168.0. HRMS(ESI): m/z calcd for $C_{24}H_{27}NO_{11}SCl_3$ ($[M+H]^+$): 642.0370; found: 642.0355.

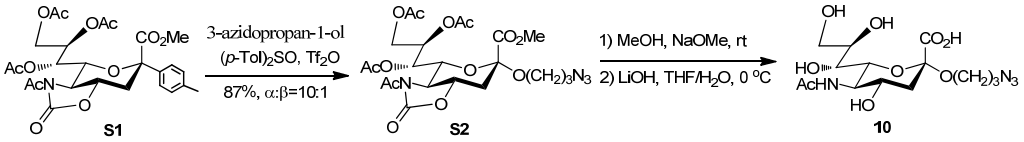
*2.2 General coupling protocol for the sialylations to prepare α-(2,9)-mono/di/trisialoside **3**, **5** and **7***

A solution of donor **2** (194.8 mg, 0.3 mmol), (p-Tol)$_2$SO (139.6 mg, 2.0 equiv) and activated 4 Å powdered sieves in anhydrous dichloromethane (10 mL) was stirred for 15 min at -70℃ under argon, followed by addition of Tf_2O (60.0 μL, 1.2 equiv). After 0.5 h, a solution of the acceptor 3-azidopropan-1-ol, **4** or **6** (1.1 equiv) in dichloromethane (5 mL) was added. The reaction mixture was stirred for 2 h at -70℃ with another 2 h at -50℃. After quenched by H_2O, the solution was diluted with dichloromethane, filtered through Celite, washed with saturated brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The glycosides were purified by column chromatography on silica gel eluting with a dichloromethane/methanol system to give the coupling products **3**, **5** or **7** in 93%, 88% or 69% yield, respectively. The NMR spectra of the sialylation products or their deprotected derivatives **3-8** are in good agreement with the literature data. [2]

*2.3 Global deprotection procedure to prepare triSia-N_3 (**9**)*

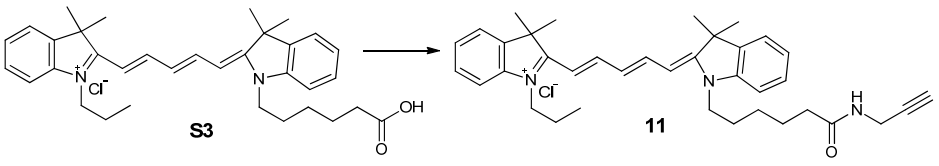
To a solution of compound **8** (62.1 mg, 0.054 mmol) in EtOH (6 mL) and H_2O (6 mL), LiOH·H_2O (69 mg, 1.6 mmol) was added under argon. After being stirred at 80 ℃ for 12 h, the reaction mixture was neutralized with a dilute aqueous HCl solution at 0℃ and then was concentrated under reduced pressure. The residue was dissolved in H_2O (2 mL), then $NaHCO_3$ (275.1 mg, 3.2 mmol) and Ac_2O (150 μL, 1.6 mmol) were added at room temperature under argon. After being stirred for 5 h, the solvent was removed and the residue was dissolved in MeOH (3.0 mL) followed by the addition of NaOMe (0.3 mL, 5.4 M in methanol) at room temperature under argon. After being stirred for 14 h, the mixture was neutralized by DOEWX resin, and the resulting solution was concentrated. The residue was purified by P2 biogel column chromatography (eluting with H_2O) and freeze-dried to give trisialoside **9** as white solid (38.7 mg, three steps 75%). ^{1}H NMR (400MHz, D_2O): δ = 1.61 (m, 3H), 1.71 (t, *J* = 6.4Hz, 2H), 1.90 (s, 3H), 1.91 (s, 6H), 2.61 (m, 3H), 3.29 (t, *J* = 6.7Hz, 2H), 3.39-3.45 (m, 2H), 3.50-3.60 (m, 11H), 3.68-3.85 (m, 11H). HRMS(ESI): m/z calcd for $C_{36}H_{58}N_6O_{25}Na$ ($[M+Na]^+$): 997.3349; found: 997.3353

3. Synthesis of monoSia-N_3 (10)



A solution of donor **S1**[1] (145.4 mg, 0.25 mmol), (*p*-Tol)$_2$SO (116.7 mg, 2.0 equiv) and activated 4 Å powdered sieves in anhydrous dichloromethane (7 mL) was stirred for 15 min at -70℃ under argon, followed by addition of Tf_2O (49.2 μL, 1.2 equiv). After 30 min, a solution of the acceptor (50.6 mg, 2.0 equiv) in dichloromethane (4 mL) was added. The reaction mixture was stirred for 2 h at -70℃ with another 2 h at -50℃. After quenched by Et_3N, the solution was diluted with dichloromethane, filtered through Celite, washed with saturated brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The glycosides were purified by column chromatography on silica gel (petroleum: ethyl acetate = 1:1) to give the product as colorless oil (121.1mg, 87%). α isomer (**S2**): ^{1}H NMR (400MHz, $CDCl_3$): δ = 1.85 (m, 2H) , 2.04 (s, 3H), 2.05 (t, *J* = 6.4Hz, 1H), 2.14 (s, 6H), 2.50 (s, 3H), 2.85 (dd, *J* = 3.5, 12.1Hz, 1H), 3.39-3.43 (m, 3H), 3.70 (dd, *J* = 9.4, 11.1Hz, 1H), 3.82 (s, 3H), 3.84 (d, *J* = 5.7Hz, 1H), 3.97-4.07 m, 2H), 4.39 (dd, *J* = 2.8, 12.3Hz, 1H), 4.64 (dd, *J* = 1.6, 9.4 Hz, 1H), 5.44 (dt, *J* = 2.8, 6.9Hz, 1H), 5.60 (dd, *J* = 1.6, 8.2Hz, 1H). ^{13}C NMR (100MHz, $CDCl_3$): δ = 20.7, 20.8, 21.1, 24.6, 29.1, 36.5, 48.1, 53.0, 59.1, 62.4, 63.1, 69.0, 71.7, 99.0, 153.6, 168.8, 170.0, 170.1, 170.6, 171.9. HRMS(ESI): m/z calcd for $C_{22}H_{30}N_4O_{13}Na$ ($[M+Na]^+$): 581.1697; found: 581.1707. Next, to a solution of **S2** (85.9 mg, 0.15 mmol) in methanol (3.0 mL), NaOMe (0.1 mL, 5.4 M in methanol) was added. After being stirred for 1 hour at room temperature, the reaction solution was neutralized by DOEWX resin and filtered through Celite. After the filtrate was concentrated, the residue was dissolved in THF/H_2O (3.2 mL, v: v = 2:1) and $LiOH{\cdot}H_2O$ (26.0 mg, 0.62 mmol) was added. Then, the mixture was stirred for 1 hour and neutralized by DOEWX resin, filtered through Celite and concentrated to give desired product **10** as white solid (51.0 mg, two steps 87%). ^{1}H NMR (400MHz, CD_3OD): δ = 1.63 (t, *J* = 12.4Hz, 1H), 1.71 (m, 2H), 1.92 (s, 3H), 2.62 (dd, *J* = 4.5, 13.0 Hz, 1H), 3.21 (bs, 2H), 3.30 (t, *J* = 6.6 Hz, 2H), 3.40-3.79 (m, 11H). HRMS(ESI): m/z calcd for $C_{14}H_{25}N_4O_9$ ($[M+H]^+$): 393.1622; found: 393.1614.

4. Synthesis of Cy5 alkyne (11)



To a solution of compound **S3**[3] (87.8 mg, 0.16 mmol) in DMF 1.0 mL, DCC (52.5 mg, 0.25 mmol) and NHS (30 mg, 0.26 mmol) were added. After being stirred at 50 ℃ for 0.5h, propargylamine (16.0 μL, 0.25 mmol) was dropped to the solution at room temperature. When the starting material disappeared, the solvent was removed under reduced pressure followed by silica gel column chromatography (dichloromethane: methanol = 30:1) to afford **11** (84.9 mg, 91%) as golden green solid. ^{1}H NMR (400MHz, $CDCl_3$): δ = 1.08 (t, *J* = 7.4Hz, 3H), 1.55-1.65 (m, 4H), 1.72 (s, 6H), 1.73 (s, 6H), 1.76-1.91 (m, 6H), 2.13 (t, *J* = 2.5Hz, 1H), 2.42 (t, *J* = 7.2Hz, 2H), 4.03 (m, 4H), 4.11 (t, *J* = 7.4Hz, 2H), 6.42 (d, *J* = 13.5Hz, 1H), 6.53 (d, *J* = 13.7Hz, 1H), 7.09 (m, 3H), 7.23 (m, 2H), 7.36 (m, 4H), 7.91(t, *J* = 13.0Hz, 2H). ^{13}C NMR (100MHz, $CDCl_3$): δ = 11.5, 20.8, 25.1, 26.4, 27.0, 28.1, 28.2, 28.7, 29.6, 36.0, 44.5, 45.8, 49.3, 49.4, 70.5, 80.4, 103.7, 104.0, 110.5, 110.8, 122.2, 125.1, 125.2, 126.4, 128.6, 128.7, 141.1, 141.2, 141.9, 142.2, 153.1, 153.3, 172.7, 172.9, 173.2. HRMS(ESI): m/z calcd for $C_{37}H_{46}N_3O^+$ ($[M-Cl]^+$): 548.3641; found: 548.3635.

5. Synthesis of monoSia-Cy5 (12)

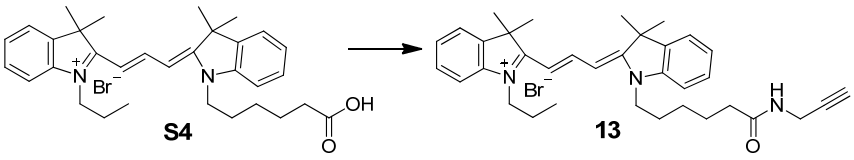
To the mixture of TBTA (6.4 mg, 0.2 equiv) and aqueous $CuSO_4 \cdot 5H_2O$ solution (1.2 mL, 0.01M, 0.2 equiv), the fresh prepared aqueous solution of sodium ascorbate (0.24 mL, 0.1M, 0.4 equiv) was added. Then the above Cu (I) catalytic system was added to the solution of Cy5 alkyne (**11**) (35.0 mg, 1.0 equiv) and monoSia-N_3 (**10**) (23.4 mg, 1.0 equiv) in *t*-BuOH/H_2O (2.0 mL, v: v = 1:1). As detected by TLC, the starting materials disappeared after 12h assisted by the ultrasonic irradiation at room temperature. The solvent was removed and the residue was purified by size exclution column chromatography on P-2 biogel by H_2O to give the monoSia-Cy5 (**12**) 50.1 mg in 86% yield. ^{1}H NMR (400MHz, CD_3OD): δ = 0.95 (t, *J* = 7.3Hz, 3H), 1.51-1.79 (m, 20H), 1.90 (bs, 4H), 2.12 (t, *J* = 7.1Hz, 2H), 2.58 (bs, 2H), 2.70 (d, *J* = 11.1Hz, 1H), 3.28(t, *J* = 7.0Hz, 4H), 3.40-4.02 (m, 23H), 6.19 (dd, *J* = 9.84, 13.4Hz, 2H), 6.53 (t, *J* = 11.8Hz, 1H), 7.15-7.22 (m, 4H), 7.31 (t, *J* = 7.64Hz, 2H), 7.40 (dd, *J* = 2,48, 7.64Hz, 2H), 8.15 (t, *J* = 12.6Hz, 2H). ^{13}C NMR (100MHz, CD_3OD): δ = 11.6, 21.9, 22.7, 26.3, 27.3, 28.1, 28.2, 29.4, 30.4, 36.4, 42.3, 44.9, 46.4, 49.6, 49.9, 50.6(2C), 54.2, 62.2, 64.7, 69.1, 70.4, 72.2, 72.9, 74.7, 74.8, 77.0, 77.1, 80.8, 89.3, 89.5, 104.4, 104.5, 112.1, 112.2, 123.4, 126.2, 126.7, 126.3, 129.8(2C), 142.7(2C), 143.6, 143.7, 155.4(2C), 174.7, 174.8, 174.9, 175.4, 175.5. HRMS(ESI): m/z calcd for $C_{51}H_{70}N_7O_{10}^+$ ($[M+H]^+$): 940.5188; found: 940.5184.

6. Synthesis of triSia-Cy5 (14)

To the mixture of TBTA (1.5 mg, 0.2 equiv) and aqueous $CuSO_4 \cdot 5H_2O$ solution (0.3 mL, 0.01M, 0.2 equiv) , the fresh prepared aqueous solution of sodium ascorbate (0.06 mL, 0.1M, 0.4 equiv) was added. Then the above Cu (I) catalytic

system was added to the solution of Cy5 alkyne (**11**) (8.0 mg, 1.0 equiv) and triSia-N_3 (**9**) (13.3 mg, 1.0 equiv) in *t*-BuOH/H_2O (2.0 mL, v: v = 1:1). As detected by TLC, the starting materials disappeared after 10h assisted by the ultrasonic wave. The solvent was removed and the residue was purified by size exclution column chromatography on P-2 biogel eluted with by H_2O to give triSia-Cy5 (**14**) 18.5 mg in 87% yield. ^{1}H NMR (400MHz, D_2O): δ = 0.83 (t, *J* = 6.7Hz, 3H), 1.12-1.32 (m, 14H), 1.45-1.62 (m, 9H), 1.90-1.96 (m, 10H), 2.17 (bs, 2H), 2.67 (m, 3H), 3.48-3.90 (m, 25H), 4.28 (bs, 4H), 5.89-5.98 (m, 2H), 6.30 (t, *J* = 13.6Hz, 1H), 7.29-7.05 (m, 8H), 7.66-7.74 (m, 3H). HRMS(ESI): m/z calcd for $C_{73}H_{104}N_9O_{26}^+$ ($[M+3H]^+$): 1522.7093; found: 1522.7097.

7. Synthesis of Cy3 alkyne (13)



To a solution of Compoud **S4**[4] (50.0 mg, 0.0884 mmol) in DMF 1.0 mL, DCC (27.4 mg, 0.1326 mmol) and NHS (15.3 mg, 0.1326 mmol) were added. Being stirred at 50 ℃ for 2h, propargylamine (8.5 μL, 0.1326 mmol) was dropped to the solution at room temperature. After the starting material disappeared by TLC, the solvent was removed under reduced pressure followed by silica gel column chromatography (dichloromethane: methanol = 30:1) to afford **13** (45.8 mg, 86%) as red solid. ^{1}H NMR (400MHz, $CDCl_3$): δ = 1.17 (t, *J* = 7.4Hz, 3H), 1.63-1.76(m, 14H), 1.79-2.00 (m, 6H), 2.10 (t, *J* = 2.5Hz, 1H), 2.44 (t, *J* = 7.2Hz, 2H), 4.02 (dd, *J* = 2.5, 5.6Hz, 2H), 4.17 (t, *J* = 7.8Hz, 2H), 4.29 (t, *J* = 7.4Hz, 2H), 7.12 (t, *J* = 8.7Hz, 2H), 7.21-7.29 (m, 2H), 7.30-7.45 (m, 6H), 8.45 (t, *J* = 13.4Hz, 1H). ^{13}C NMR (100MHz, $CDCl_3$): δ= 11.7, 21.2, 25.2, 26.4, 27.1, 28.2, 28.6, 36.2, 44.7, 46.3, 48.9, 49.0, 70.4, 77.3, 80.4, 103.9, 104.5, 110.9, 111.1, 122.1, 122.2, 125.3, 125.4, 128.8 (2C), 128.9, 129.5, 140.5, 140.7, 141.9, 142.2, 150.8, 173.4, 173.5, 174.0. HRMS(ESI): m/z calcd for $C_{35}H_{44}N_3O^+$ ($[M-Br]^+$): 522.3479; found: 522.3478.

8. Synthesis of triSia-Cy3 (15)

To the mixture of TBTA (5.7 mg, 0.0108 mmol, 0.4 equiv) and $CuSO_4 \cdot 5H_2O$ (0.4 equiv), the fresh prepared aqueous solution of sodium ascorbate (0.8 equiv) was added. Then the above Cu (I) catalytic system was added to the solution of alkynyl-modified Cy3 **13** (16.3 mg, 0.027mmol) and tri-sialioside **9** (26.3 mg, 0.027mmol) in *t*-BuOH/H_2O (3.0 mL, v/v = 1/1, took into account of the volume of aqueous solution added). As detected by TLC, the starting materials almost disappeared after 10h assisted by the ultrasonic wave at 15-25 ℃. The solvent was removed through freeze

drying and the residue was purified by size exclution column chromatography on P-2 biogel eluted with redistilled H_2O to give the corresponding conjugate triSia-Cy3 (**15**) (32.6 mg, 76%). ^{1}H NMR (400MHz, D_2O): δ = 0.87 (t, *J* = 7.5Hz, 3H), 1.10-1.28 (m, 5H), 1.53-1.67 (m, 10H), 1.68-1.79 (m, 4H), 1.85-1.98 (m, 10H), 2.16-2.23 (m, 2H), 2.57-2.68 (m, 5H), 3.32 (t, *J* = 7.0Hz, 2H), 3.40-3.50 (m, 5H), 3.51-3.67 (m, 14H), 3.68-3.83 (m, 10H), 3.83-4.05 (m, 7H), 4.22-4.30 (m, 2H), 6.22 (t, *J* = 13.9Hz, 2H), 7.17-7.29 (m, 3H), 7.32-7.40 (m, 2H), 7.47 (d, *J* = 6.8Hz, 2H), 8.35-8.45 (m, 2H). HRMS(ESI): m/z calcd for $C_{71}H_{102}N_9O_{26}^+$ ($[M+3H]^+$): 1496.6931; found: 1496.6962.

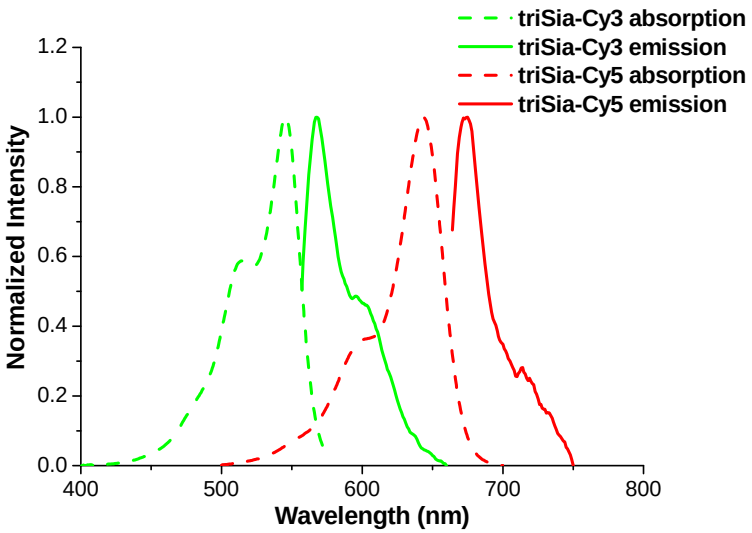


Figure S1 Absorption and emission spectra of triSia-Cy3 (green) and triSia-Cy5 (red) in HEPES buffer (pH 7.40).

Table S1 Spectroscopic data of the cyanine alkynes and cyanine tagged oligosialic acids.

entry[a]	probe	$\lambda_{abs,\ max}$ (nm)	ε ($M^{-1}cm^{-1}$)	λ_{em} (nm)	Φ^b
1	Cy5 alkyne, **11**	642	1.5×10^5	662	0.123
2	triSia-Cy5, **14**	642	2.1×10^5	666	0.113
3	Cy3 alkyne, **13**	544	5.4×10^4	562	0.100
4	triSia-Cy3, **15**	545	6.7×10^4	560	0.168

[a]The corresponding spectral datas were measured in HEPES buffer (10 mM , pH 7.40). [b]Relative quantum yield was determined using cresyl violet in methanol (Φ = 0.54) as reference,[3] the corresponding excitation wavelength of Cy3 series and Cy5 series were 500 nm and 560 nm, respectively.

9. Cell culture and fluorescence imaging

PC-12 cells (purchased from Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences) were cultured in modified RPMI 1640 medium (HyClone Corporation) supplemented with 10% fetal bovine serum. Fluorescence imaging of cells was performed using a confocal microscope (Olympus FV1200 with a 100 × oil objective).

Incubating PC-12 cells in humidified incubator (37℃, 5% CO_2) overnight to make them adhere to the glass bottom of culture vessels (Nest Corporation, Φ 20 mm). After washing once with phosphate buffer solution (PBS, pH 7.4), the cells were treated with triSia-Cy3 or/and triSia-Cy5 and incubated for 1 h, the final concentrations of target compounds were 5 μM. Then the cells were washed thrice with PBS, and added new culture medium to observe the fluorescence. Corresponding parameters of optical path were set as below: Cy3 channel (excitation: 559 nm/emission range: 570-635 nm); Cy5 channel (excitation: 635 nm/emission range: 650-720 nm); FRET channel (excitation: 559 nm/emission range: 650-720 nm).

For PEI transfection, target compounds were introduced into the cytosol by means of transfection reagent PEI (0.1 mM in H_2O). The beforehand mixed solution of target probes and PEI (wt/wt = 1:3) were added to living cells and incubated for 1 h. The final concentrations of target compounds were 5 μM for triSia-Cy3 or triSia-Cy5. Then, the cells were washed thrice with phosphate buffer solution (PBS, pH 7.4) and added new culture medium to observe the fluorescence.

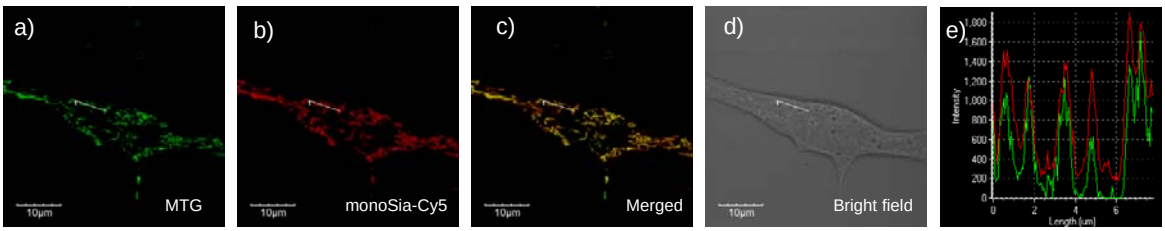


Figure S2 Colocalization experiments involving monoSia-Cy5 (**12**) and MitoTracker Green (MTG) in PC-12 cells. The cells were incubated with MTG (0.1 μM) for 30 min, and then the medium was replaced with fresh medium containing monoSia-Cy5 (5 μM) and incubated for 1 min. Images for monoSia-Cy5 (a) and MTG (b) recorded using excitation wavelengths of 488 and 635 nm, and band-path emission filters at 495-555 nm and 650-720 nm, respectively. (e) Intensity profile of region of interest (ROI 1) across the PC-12 cell costained with monoSia-Cy5 and MTG. Scale bars, 10 μm.

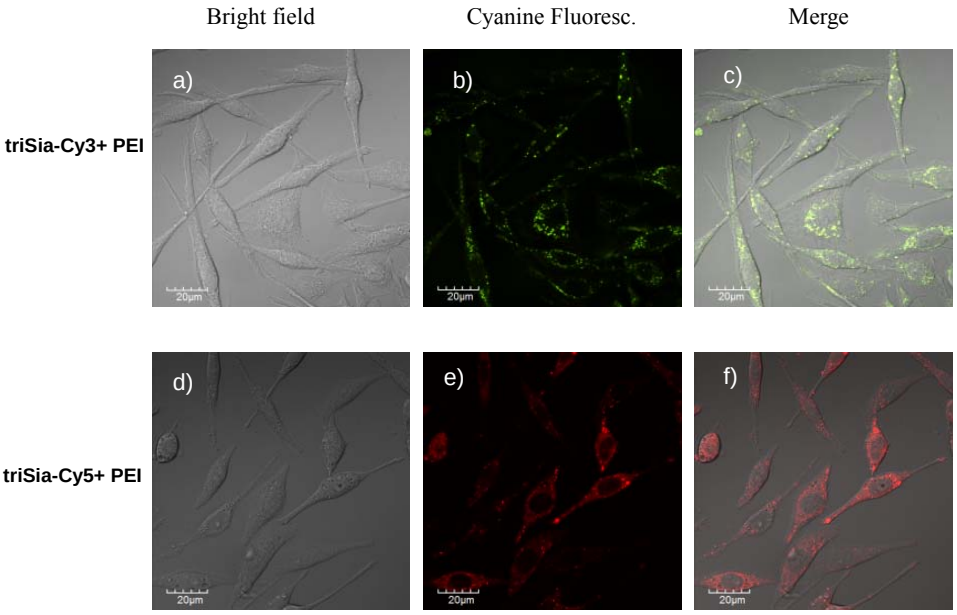


Figure S3 Confocal laser scanning microscopy images of PC-12 cells incubated with triSia-Cy3/triSia-Cy5 (5 μM) in the presence of PEI (the weight ratio of triSia-Cy3/triSia-Cy5 to PEI was 1:3) for 1h. Middle column: Cy3 fluorescence (excitation: 559 nm/emission: 570-635 nm) or Cy5 fluorescence (excitation: 635 nm/emission: 650-720 nm). Scale bars, 20 μm.

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