Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

ExoTracker：A Low-pH-activatable Fluorescent Probe for Labeling Exosomes and Monitoring Endocytosis and Trafficking

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Experimental Section

Chemicals and reagents

All chemicals and solvents were of analytic grade and from commercial sources without further purification. All reagents used for cell culture and animal treatment were of cell culture grade. All chemicals and regents were purchased from Sigma-Aldrich unless otherwise declared.

Cell lines and cell culture

Cells were cultured in corresponding medium supplemented with 10% fetal bovine serum (FBS, Biological Industries, Israel) and 1% penicillin-streptomycin (HyClone, USA). All cells were maintained and propagated at 37°C in 5% CO_2 atmosphere. Cells source and cultural medium were summarized in **Table 1**

Table 1 Cell information

Cell name	Type	Source	Culture Medium
HCV29	Normal bladder epithelial cells	Gifted from The Biomembrane Institute, Seattle, WA, USA	RPMI 1640
YTS-1	Bladder cancer cells	Gifted from The Biomembrane Institute, Seattle, WA, USA	RPMI 1640
KG1a	Human leukemia-derived cells	Gifted from Fred Hutchinson Cancer Research Center, Seattle, WA, USA	RPMI 1640
HEK293T	Human embryonic kidney cells	Purchased from ATCC #CRL-11268	DMEM

a) ATCC: American Type Culture Collection (Manassas, VA, USA).

Compound synthesis

Compound 2: A mixture of 1,1,2-trimethyl-1H-benzo[e]indole **1** (3.13 g, 15.0 mmol), 3-bromopropanoic acid (2.43 g, 16.0 mmol) were dissolved in toluene (5.0 mL) under nitrogen atmosphere, and refluxed for 3 h with a Dean-Stark condenser. After cooling to room temperature, the reaction mixture was poured into ether (Et_2O, 100 mL). The resulting solid was collected without further purification. Yield compound **2**: 4.11 g, (76%). HRMS (ESI, m/z) Calcd. for [$C_{18}H_{20}BrNO_2$−Br^-], 282.1489; Found, 282.1613. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.37 (d, J = 8.4 Hz, 1H), 8.28 (d, J = 9.2 Hz, 1H), 8.22 (d, J = 7.6 Hz, 1H), 8.17 (d, J = 8.8 Hz, 1H), 7.82-7.71 (m, 2H), 4.78 (t, J = 7.2 Hz 2H), 3.05 (t, J = 7.2 Hz, 2H), 2.97 (s, 3H), 1.76 (s, 6H).

Compound 3: To a solution of compound **2** (72.2 mg, 0.2 mmol) was added 4-(diphenylamino) benzaldehyde (54.6 mg, 0.2 mmol). The mixture was stirred at 80^{o}C under nitrogen atmosphere for 9 h. After cooling to room temperature, the reaction mixture was poured into ether (Et_2O, 100 mL). The resulting solid was collected and purified by silica gel column chromatography (CH_2Cl_2:MeOH =

40:1) to afford the desired product **3**. (107 mg, yield 87%). HRMS (ESI, m/z) Calcd. for [$C_{37}H_{33}BrN_2O_2$−Br^-], 537.2537; Found, 537.2604. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.41 (d, J = 4.8 Hz, 1H), 8.35 (s, 1H), 8.23 (d, J = 7.6 Hz, 1H), 8.18 (d, J = 8.8 Hz, 1H), 8.05 (m, 3H), 7.66(t, J = 6.8 Hz, 1H), 7.57(t, J = 7.2Hz, 1H), 7.38-7.22 (m, 5H), 7.18-7.03 (m, 6H), 6.86 (d, J = 7.6 Hz, 2H), 4.89 (t, J = 7.2 Hz, 2H), 3.18 (t, J = 7.6 Hz, 2H), 1.97 (s, 6H).

Exosome preparation

Bladder cancer cells YTS-1 were cultured in RPMI-1640 medium as described in *Cells and culture condition* to reach the confluency at about 80%. Cells were washed three times with sterilized PBS, and cultured in serum-free RPMI-1640 medium supplemented with 1% penicillin-streptomycin for 48 h. Exosomes were enriched and purified by three-steps centrifugation (500 g for 10 min, 2000 g for 20 min, 11,000 g for 30 min) to remove cell fragments and other vesicles. Finally, the supernatant proceeded with 105,400 g for 120 min twice to precipitate exosomes.[1, 2] Exosomes were resuspended by sterilized PBS and quantified by BCA protein assay kit (Beyotime biotechnology, China). Exosomes were sterilized through 0.22 μm filter (Merck Millipore, USA) and stored at 4℃ for further use.

Characterization of exosomes

The size of purified exosomes was analyzed using Malvern ZEN3600 equipped with a blue laser and a quartz chamber for sample injection (O-Ring top plate model). Transmission electron microscope (TEM) images were obtained on a H-7650 transmission electron microscope (HITACHI, Japan) with an accelerating voltage 80 kV.[3] Fluorescence detection was carried out on microplate reader (SYNERGY 4, BioTek, USA). Super-resolution imaging was performed on N-SIM (Nikon, Japan).

Western Blot

Exosomes were dissolved by RIPA lysis buffer (including protease inhibitor) and the protein concentration was quantified by BCA kit. Exosomal proteins were denatured by adding 5× SDS-loading buffer, separated by 10% SDS-PAGE and transferred onto PVDF membranes (Merck Millipore, USA). Then membranes were blocked with 3% bull serum albumin (BSA, Sigma-Aldrich, USA) in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature, and incubated overnight at 4°C in 5% nonfat dry milk/ TBS-T containing either anti-Alix (1:1000, mAb, #2171s, Cell Signaling Technology, USA), anti-TSG101 (1:1000, mAb, #ab83, Abcam, UK), anti-Calnexin (1:1000, rAb, #2433s, Cell Signaling Technology, USA), or anti-CD63 (1:1000, rAb, #ab134045, Abcam, UK), followed by addition of HRP-conjugated secondary Ab (1:5000; Beyotime).

Exosome labeling

ExoTracker was added to the 1 μg/μL exosome solution to a final concentration of 10 μM, and reacted for 30 min in dark at room temperature. The redundant probes were neutralized by adding glycine to a final concentration of 100 μM. ExoTracker labeled exosomes were washed with PBS and filtered by 10 kDa ultrafiltration tube (Merck Millipore, USA). CFDA-SE labeling was performed, as per manufacturer's protocol. For exosomes labeling efficiency test, labeled exosomes were captured by CD63-beads (Abcam, UK) and detected by Flow cytometer.

ExoTracker fluorescence at different pH

Exosomes were labeled with ExoTracker or CFDA-SE as described above. 20 μg exosomes diluted in 200 μL Britton-Robinson (BR) buffer with pH ranging from 1.81 to 11.92.[4, 5] The fluorescence at Ex/Em = 550 nm/670 nm for

Exotracker or Ex/Em = 494 nm/521 nm for CFDA-SE was immediately recorded on microplate reader (SYNERGY 4).

Cytotoxicity assay

Normal bladder cell HCV29 and bladder cancer cells YTS-1 (2×10^3 /well) were cultured in 96-well plate overnight. Different concentration of ExoTracker was added and gently mixed. Cells were cultured for 48 h, and their viability was assayed by Cell Counting Kit-8 (CCK-8, EnoGene, China).

Imaging endocytosis of living cells

HCV29 cells were seeded on confocal dish (Jet Biofil, China) up to 40% confluence. LysoTracker green DND-26 (1:10000, #8783, Cell Signaling Technology, USA) and Hoechst 33258 (5 μg/mL, #B1155, Sigma-Aldrich, USA) were added to culture media. Exosomes were labeled and purified as mentioned above. 50 μg labeled exosomes were added to the confocal dish carefully and the real-time endocytosis procession was photographed by Nikon A1 (Nikon Corporation, Japan).

Data analysis

Data analysis was processed by GraphPad prism software (GraphPad Software, USA, v8.0.1), Image data were analyzed and quantified by Image J (https://imagej.nih.gov/, v1.8.0)

Supplementary Figures

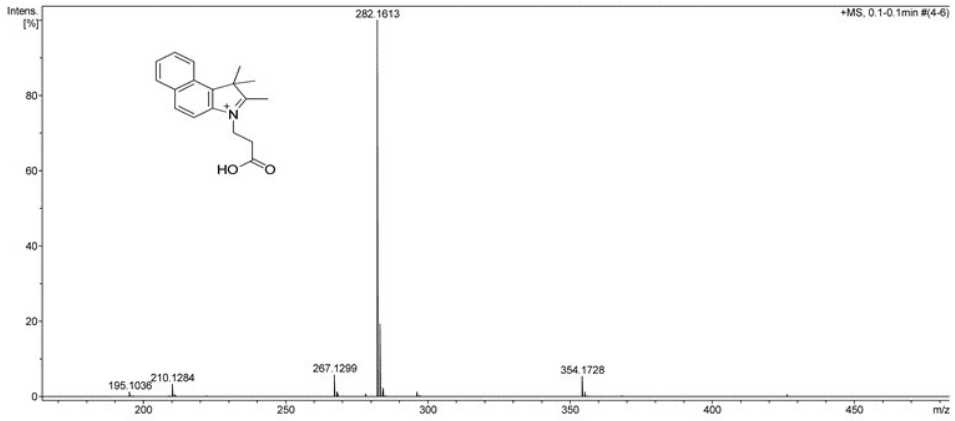

Fig. S1. LC-MS spectrum of compound **2** in **Figure 1**.

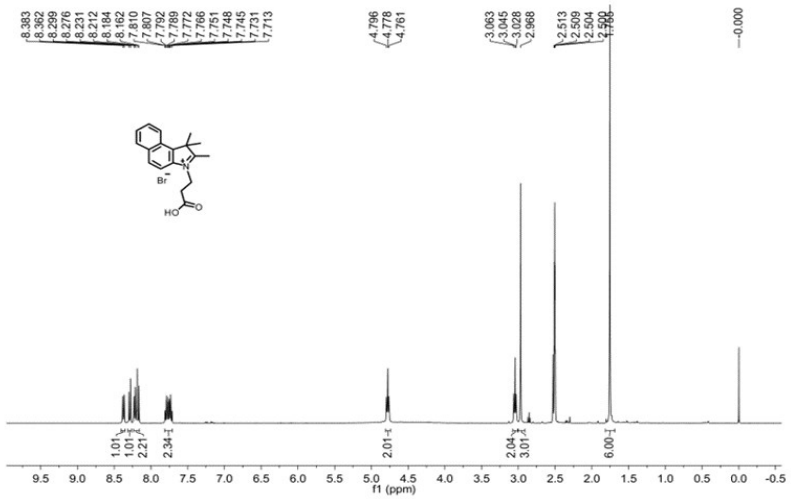

Fig. S2. NMR spectrum of compound **2.**

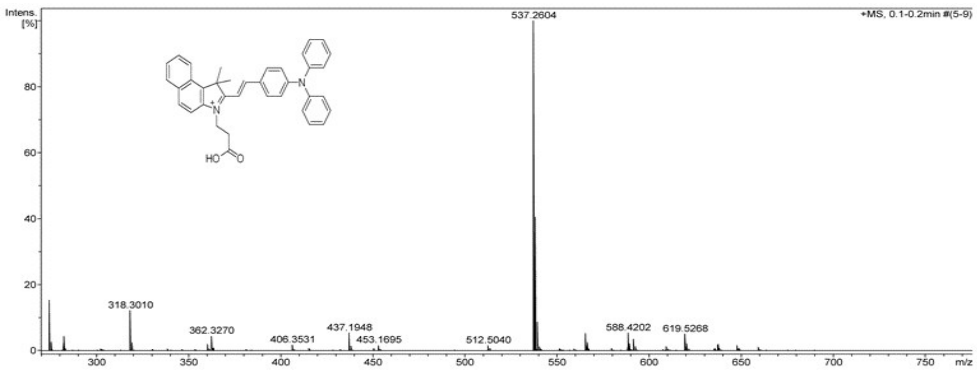

Fig. S3. LC-MS spectrum of compound **3** in **Figure 1**.

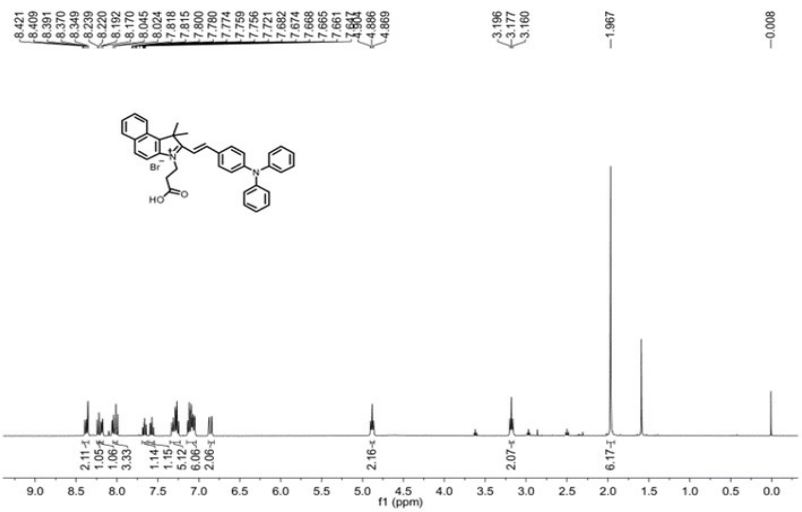

Fig. S4. NMR spectrum of compound **3.**

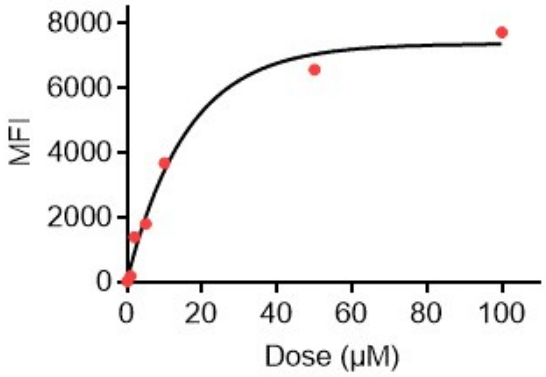

Fig. S5. Relationship between ExoTracker dose and mean fluorescence intensity (MFI) of ExoTracker-labeled exosomes. Exosomes were capruted by CD63-beads and analyzed by flow cytometry.

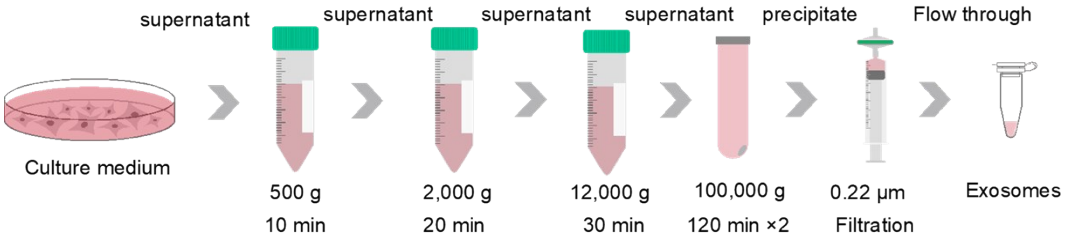

Fig. S6. Procedure for exosome enrichment and purification.

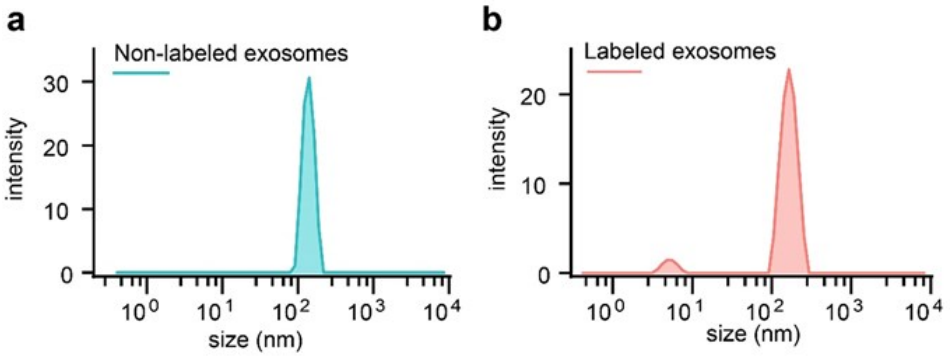

Fig. S7. Nanoparticle tracking analysis (NTA) of exosome size range before **a)** and after **b)** ExoTracker labeling.

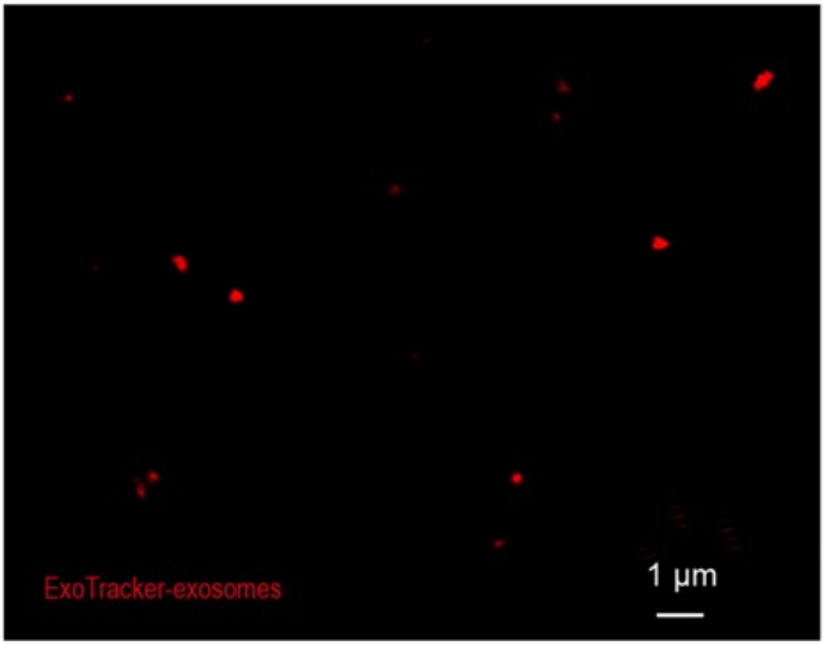

Fig. S8. Nikon structured illumination microscopy (SIM) image of ExoTracker-labeled exosomes. Scale bar: 1 µm.

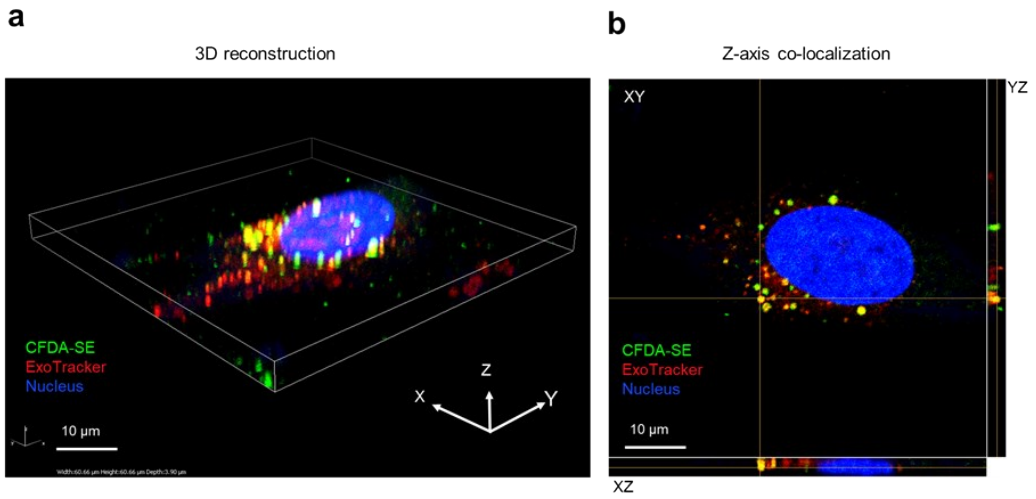

Fig. S9. Confocal 3D reconstruction of CFDA-SE/ ExoTracker dual-labeled exosomes in HCV29 cells in pH 7.4 PBS solution. **a)** 3D reconstruction (by ImageJ program) of confocal image. **b)** Z-axis segmentation of 3D reconstruction image. Scale bar: 10 μm.

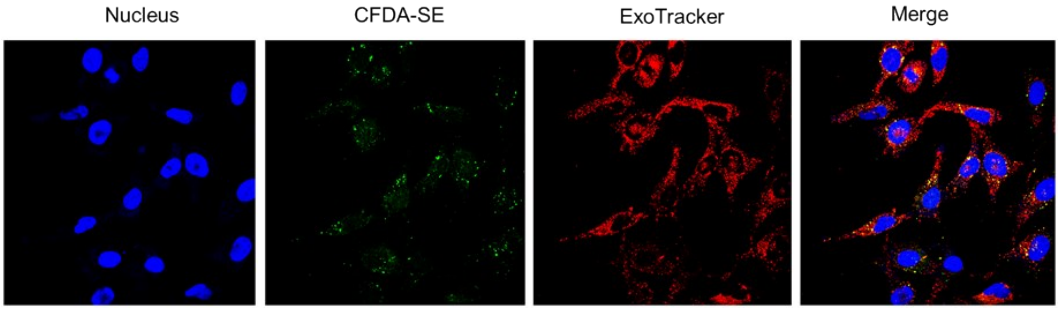

Fig. S10. Confocal image of CFDA-SE/ ExoTracker dual-labeled exosomes in HCV29 cells in pH = 5.6 PBS solution. Scale bar: 50 μm.

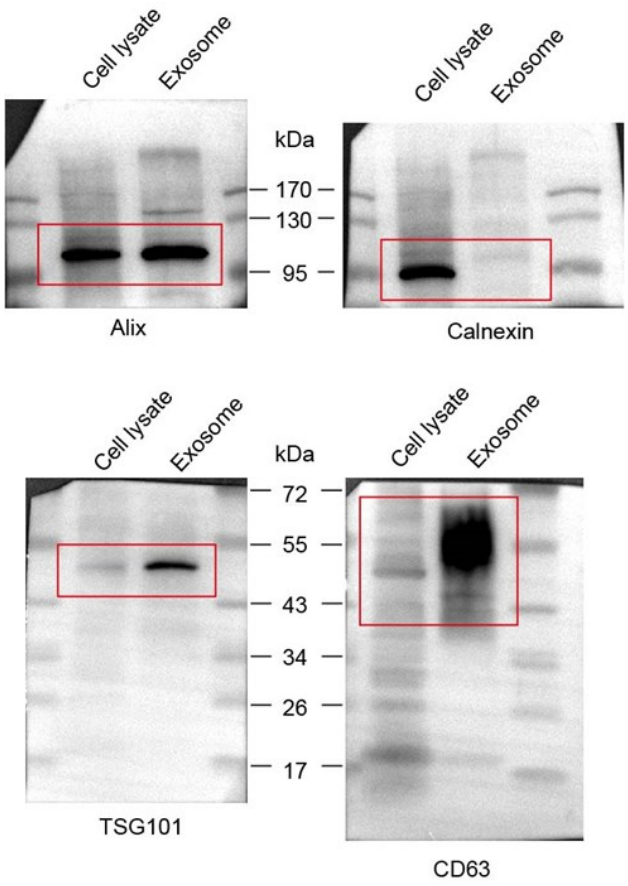

Fig. S11. Uncropped images of Western blotting results. Areas indicated by red boxes are shown in **Fig. 2a.**

References

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