Supplementary Material

Dual-Monomer Solvatochromic Probe System (DSPS) for Effectively Differentiating Lipid Raft Cholesterol and Active Membrane Cholesterol in the Inner-Leaflet Plasma Membrane

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Preparation of SNAP-D4 plasmid constructs

D4 domain of perfringolysin O was designed to be fused to the C-terminal of SNAP-tag without linker. For prokaryotic expression of SNAP-D4, the gene was codon optimized for E. coli and commercially synthesized in Sangon Biotek (Shanghai, China). Then the gene was cloned into the pET-22b (+) vector with a C-terminal His6-tag at the NdeI/XhoI site. For eukaryotic expression of SNAP-D4, the D4 gene was codon optimized for mammalian cells and commercially synthesized in the same company. Then D4 was cloned into the pcDNA3.1 (+) vector at the EcoRI/XhoI site and subcloned into the pSNAPf vector at the C-terminal of SNAP-tag seamlessly. Then the eukaryotic SNAP-D4 gene was subcloned into a pCMV-HIV lentiviral vector (Obio Technology, Shanghai, China) saved in our laboratory, respectively. All D4 was Y415A/D434W/A463W (YDA) mutated in this study as described previously [1].

Expression and purification of prokaryotic SNAP-D4 proteins

The pET-22b-SNAP-D4 construct was transformed into E. coli Rosetta (DE 3) cells. After overnight culture, a single colony was chosen and cultured in 5 mL of Luria-Bertani (LB) medium (containing 100 μg/mL ampicillin) and incubated in a shaker at 37℃ until it got cloudy. The culture was amplified in 100 mL LB medium and then transferred to 2000 ml of LB medium with 100 μg/ml ampicillin, which was incubated in a shaker at 37℃ until OD 600 reached around 0.6. Then SNAP-D4 expression was induced at 30℃ with 0.1-0.5 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) for 8 h. The induced culture was centrifuged at 8000 rpm for 3 min. The cell pellets were resuspended with 50 ml of cold NTA lysis buffer (20 mM Tris, 500 mM NaCl, 10% glycerol, 0.1 mg/ml lysozyme, pH 8.0) After sonication, the lysate was centrifuged at 16,000 rpm for 50 min. The pellets were sonicated for a few more times to remove supernatant proteins and then resuspended in 3 mL of inclusion body lysis buffer (6 M guanidine hydrochloride, 20 mM Tris, 5 mM dithiothreitol, pH8.0). After centrifugation, the supernatant was diluted with 6 mL diluting buffer (3 M guanidine hydrochloride, 10 mM natrium aceticum, 10 mM EDTA·2NA, pH 4.2). Then it was added to 200 mL of refolding buffer (0.5 mM oxidized glutathione, 5 mM reduced glutathione, 2 mM EDTA·2NA, 100 mM Tris, pH8.0) drop by drop and stirred for 48 h. After completely refolding of the inclusion bodies, the protein solution was transferred to a dialysis bag and

concentrated to a final volume of 10-20 mL after incubation in PEG 20000 and were dialyzed in the NTA lysis buffer. Then the proteins were bound to the Ni NTA agarose resin (Invitrogen, USA). The resin was washed with NTA lysis buffer, and the protein was eluted with NTA lysis buffer containing 20-500 mM imidazole. The fractions were analyzed for SNAP-D4 by R-250 staining of SDS-PAGE gels.

Cell culture and transfection

The human cell line Huh-7 (human hepatoma cells) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in high-glucose DMEM (Hyclone, USA) supplemented with 5% fetal bovine serum (FBS, NTC, Argentina) at 37 °C with 5% CO2. Cells were transiently transfected with mammalian expression plasmids using the jetPIME kit (Polyplus Transfection) according to the manufacturer's instructions. For stable cell line establishment, the lentivirus construct of SNAP-D4 was co-transfected with psPAX2 packaging plasmid and pMD2.G expressing plasmid into HEK293T cells. Supernatants containing lenviruses were collected at 24 h post-transfection and centrifuged at 140000 g for 1 h 45 min at 4℃ by an Optima XPN-100 Ultracentrifuge (Beckman Coulter). The lentivirus pellets were resuspended in high-glucose DMEM. Huh-7 cells and L02 cells were infected with the lenviruses and selected by G418 (1 mg/ml) for at least a month.

EGFP-D4 expression and fluorescence protease protection (FPP) assay [2]

EGFP-D4 gene (D4 not mutated) was codon optimized for E. coli and commercially synthesized in Sangon Biotek (Shanghai, China). Then the gene was cloned into the pET-22b (+) vector with a C-terminal His6-tag at the NdeI/XhoI site. E. coli strain BL21 (Plyss) was used for the overexpression of the EGFP-D4 proteins. E. coli transformed with EGFP-D4 were cultured in LB medium at 37˚C with shaking until the OD600 reached 0.4-0.8. Cultures were induced with 0.5 mM IPTG for 20 h at 19˚C, then E. coli cells were harvested by centrifugation and resuspended in PBS, followed by sonication. Cell lysate supernatants containing EGFP-D4 were used to stain cholesterol in the outer leaflet of plasma membrane (OPM). SNAP-D4 stably expressing cells were stained with TMR-BG first, then the cells were incubated with supernatants containing EGFP-D4 recombinant proteins for 30 min at

room temperature. Cells in PBS were treated with Protease K (50 μg/ml) first and then digitonin (20 μM), both incubated for 5 min. Cells were observed live under confocal microscopy at room temperature. Protease K was used to digest proteins in the OPM, while digitonin was used to permeabilize the plasma membrane.

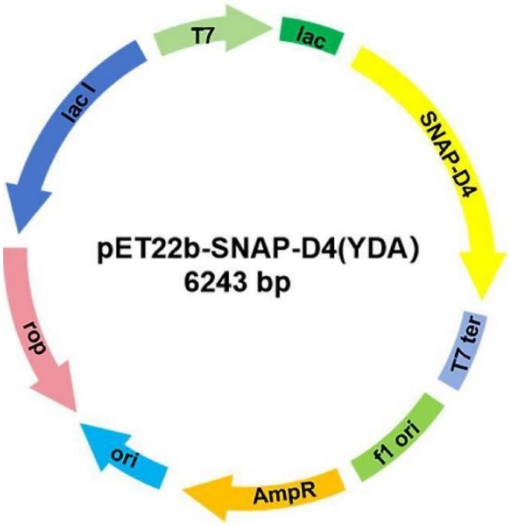

Fig. S1 Construction of the prokaryotic expression plasmid encoding SNAP-D4 in pET-22b vector.

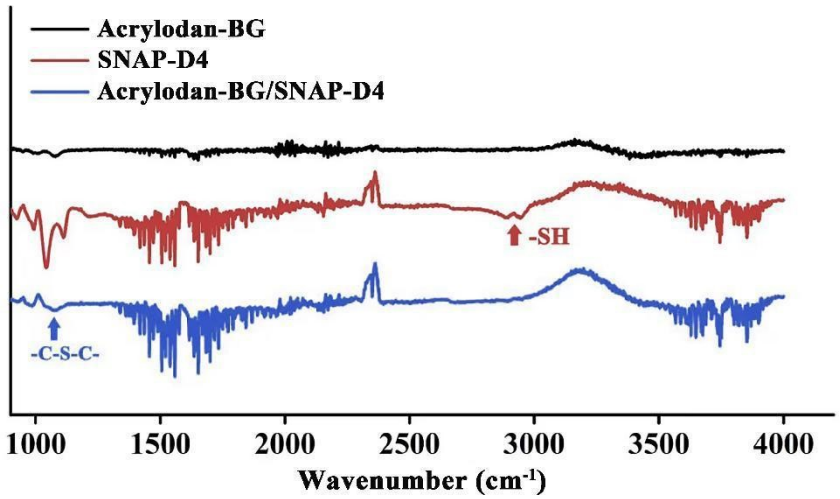

Fig. S2 The infra-red spectra of Acrylodan-BG conjugated with SNAP-D4 by -SH group.

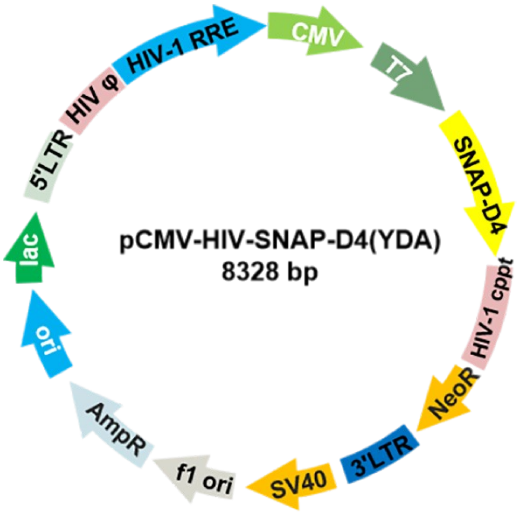

Fig. S3 Construction of the eukaryotic stable expression plasmid encoding SNAP-D4 in pCMV-HIV lentiviral vector.

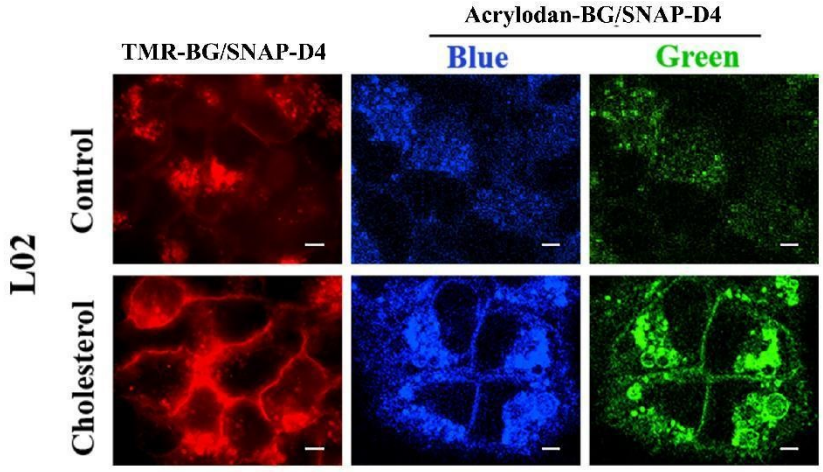

Fig. S4 Physiological low cholesterol in the inner-leaflet PM of L02 cells stably expressing SNAP-D4 and significantly increased after exogenous cholesterol treatment (5 mM). Scale bar = 5 μm.

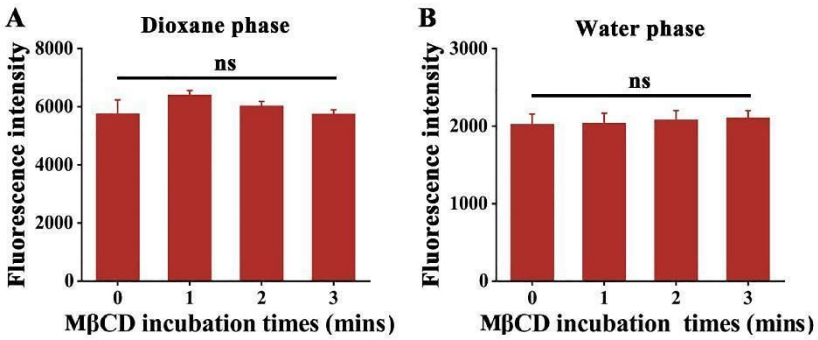

Fig. S5 MβCD not affecting Acrylodan-BG/SNAP-D4 probe itself. After Acrylodan-BG (0.5 μM) were reacted with SNAP-D4 purified protein (0.1 mg/ml) at 37℃ for 30 min, the conjugated Acrylodan-BG/SNAP-D4 was dialyzed and diluted into dioxane or water (100 x), then the fluorescence was detected by a microplate reader before or after MβCD (10 mM) treatment. A. Acrylodan-BG/SNAP-D4 in dioxane phase, λex: 405 nm, λem: 470 nm. B. Acrylodan-BG/SNAP-D4 in water phase, λex: 405 nm, λem: 550 nm. N≥3, ns, not significant, Mean ± SD.

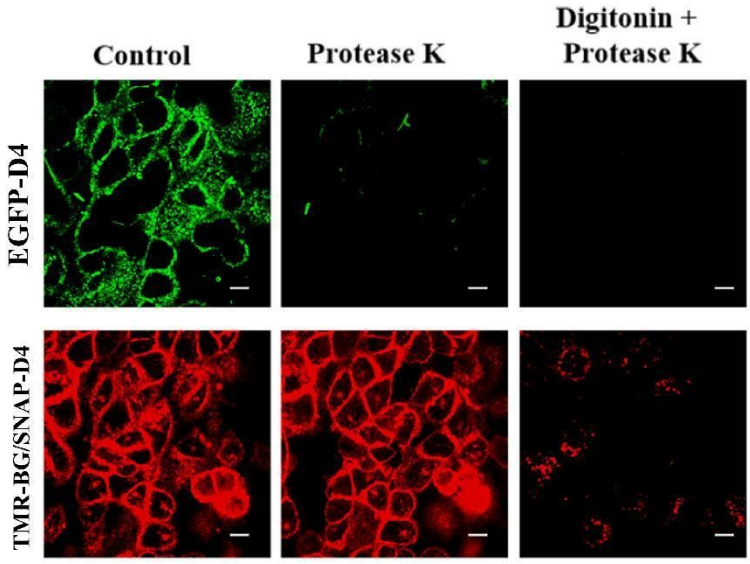

Fig. S6 SNAP-D4 expressed in cells could not cross plasma membranes. SNAP-D4 stained with TMR-BG as described. Red fluorescence indicates TMR-BG/SNAP-D4, green fluorescence indicates EGFP-D4 proteins. Scale bar = 10 μm.

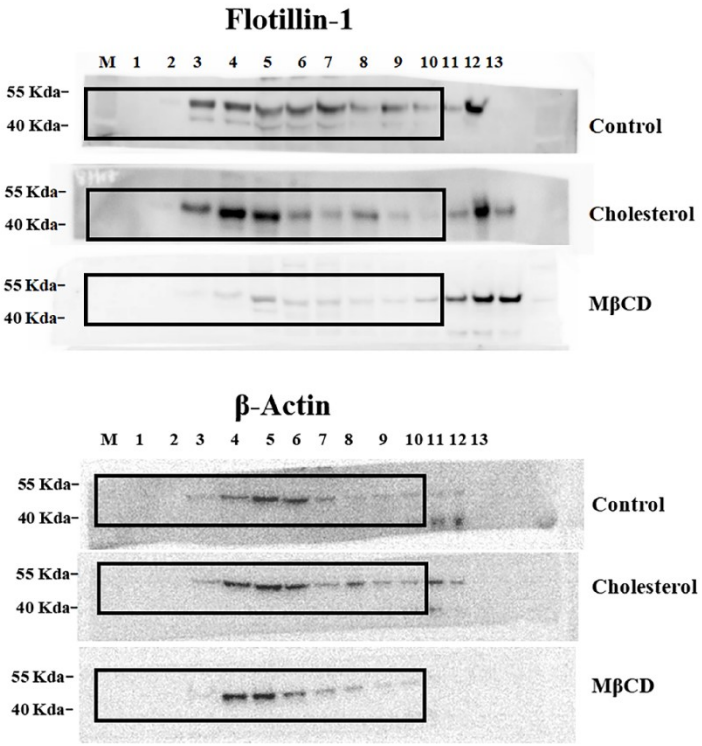

Fig. S7 The Western blot raw data for Fig. 6A, bands in black boxes indicate marker and top-to-bottom fractions (5%-40% sucrose) of lipid rafts extracted from stable SNAP-D4 expression Huh-7 cell lysates. β-actin was used to indicate total protein of each group were the same. Notably, flotillin-1 localized not only in lipid raft fractions, but also in non-raft fractions (high density fractions from lane 10-12, lane 13 was extra fraction if any remained). It was not due to unsuccessful isolation of the lipid raft, but a phenomenon observed in previous studies[3].

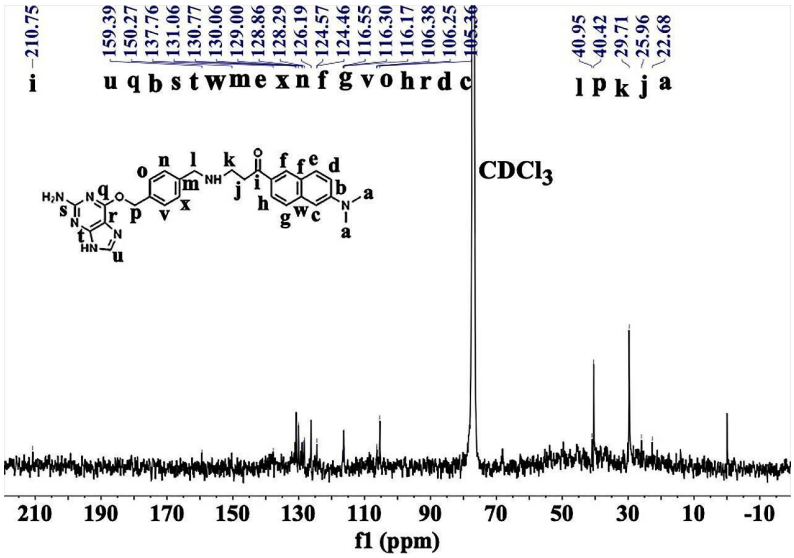

Fig. S8 The ^{13}C NMR spectrum of Acrylodan-BG.

Reference

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2. M. Maekawa and G. D. Fairn, J Cell Sci, 2015, 128, 1422-1433.
3. J. Song, L. Y. Ping, D. M. Duong, X. Y. Gao, C. Y. He, L. Wei and J. Z. Wu, Molecular medicine reports, 2016, 13, 2087-2093.