A Flow Cytometry Assay to Quantify Intercellular Exchange of Membrane Components

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Supporting Information

Supplementary Figures

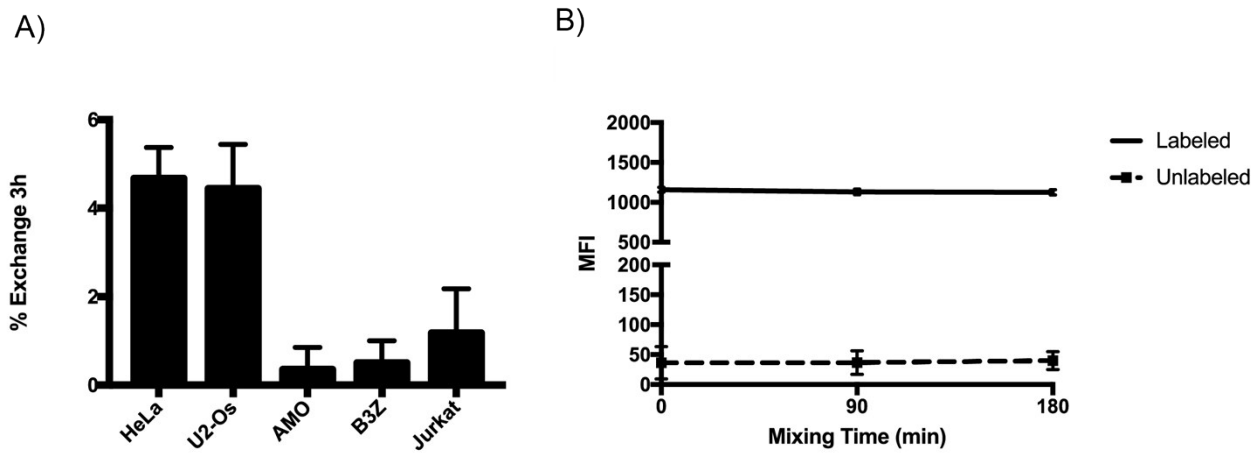


Figure S 1 Exchange rates of bodipy cholesterol (bdp-Ch 1) are cell-type and live cell-dependent. Cells were incubated overnight with bodipy-cholesterol 1 and then mixed with unlabelled cells for 3 h. Flow cytometry results were analyzed and showed differences in the lipid exchange rates for the different cell lines; A) expression of fluorescent signal exchange after 3 h for different cell lines indicating that the lipid exchange depends on the cell line. B) Fixation abolished lipid exchange of HeLa cells; HeLa cells fixed using 2% PFA for 15 mins prior to co-culture. Data represent mean ± SD. Error bars SD.

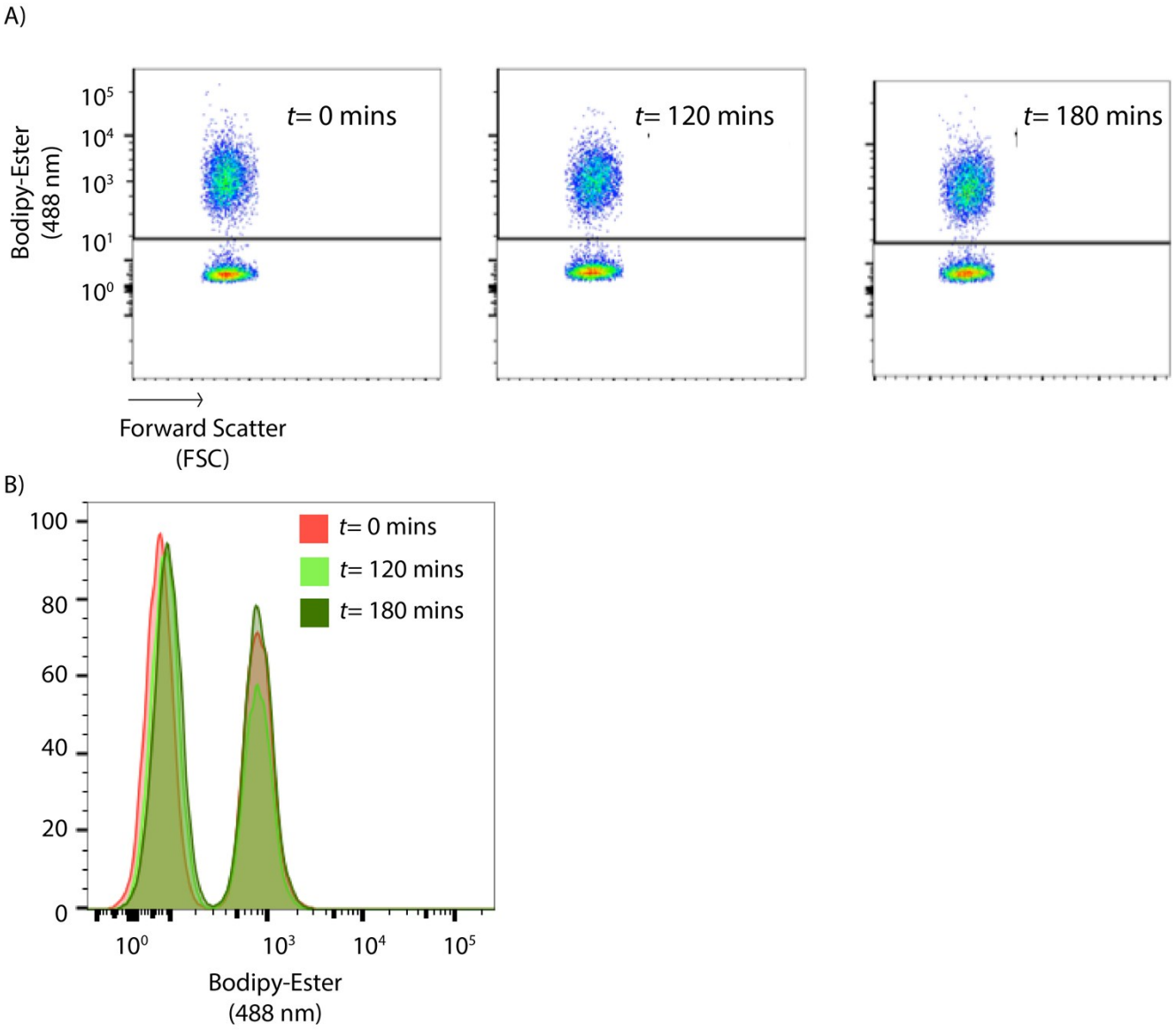


Figure S 2 Exchange of Bodipy-488. Flow cytometry assay indicates no bodipy is exchanged between labelled and unlabelled HeLa-cells. Cells treated with bodipy-ester (5µM for 24h) were mixed with unlabelled HeLa cells. A) scatter plots and B) overlay histograms of mixing experiments (t=0, 120 and 180 mins) showing any shift.

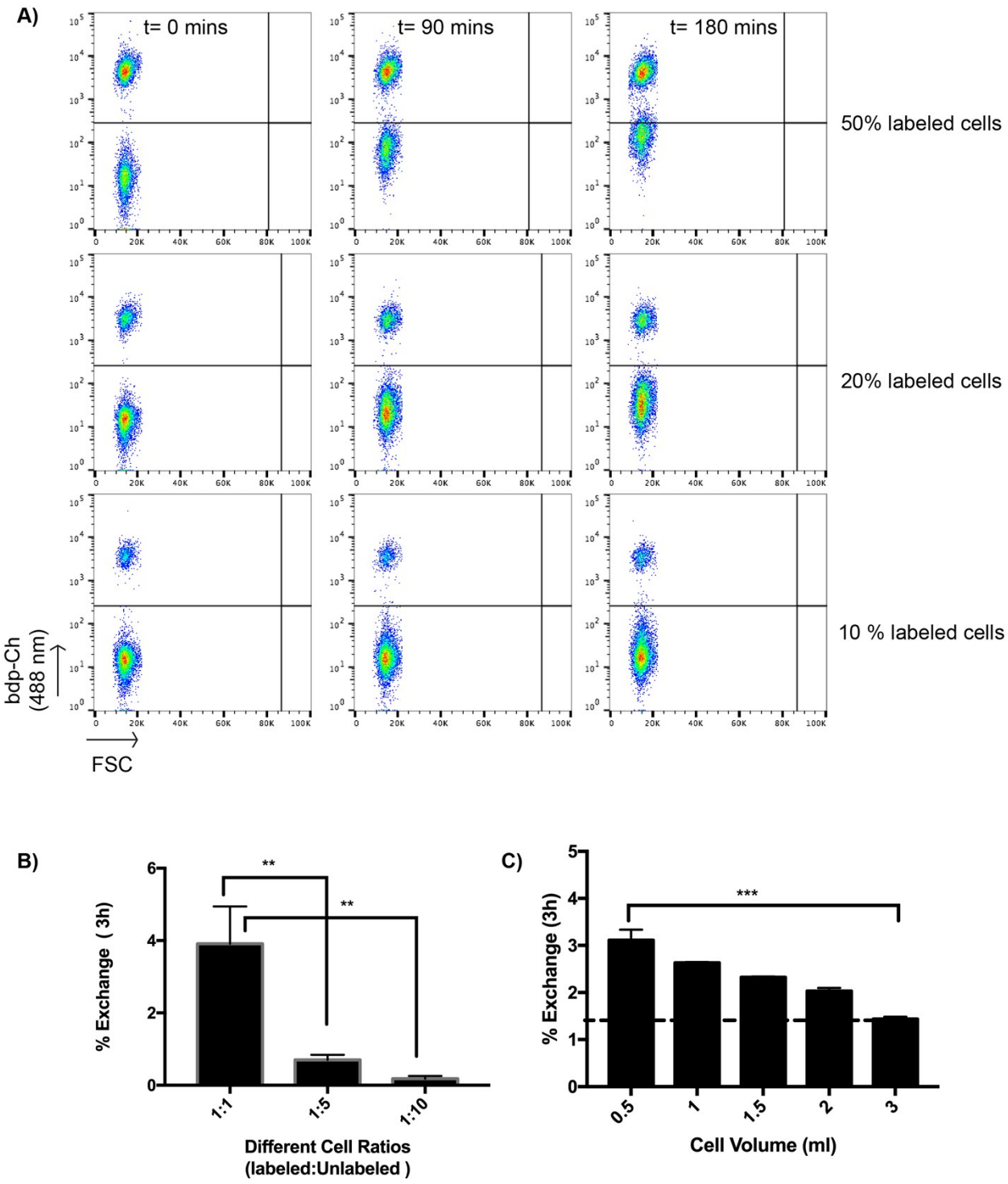


Figure S 3 Cell ratios affect cholesterol exchange: HeLa cells were mixed at either different ratios of labelled vs. unlabelled cells (1:1, 1:5, 1:10) or different culture volume. A, B) Flow cytometry analysis (t=60, 90, 180 mins) shows exchange rates to be dependent on fluorescent cell fraction. C) Flow cytometry analysis after 180 mins showed rated to be dependent on the culture media volume. Data are representing as mean ± SD. Error bars SD; *p<0.05; **p<0.01; ***p<0.001; unpaired t-tests

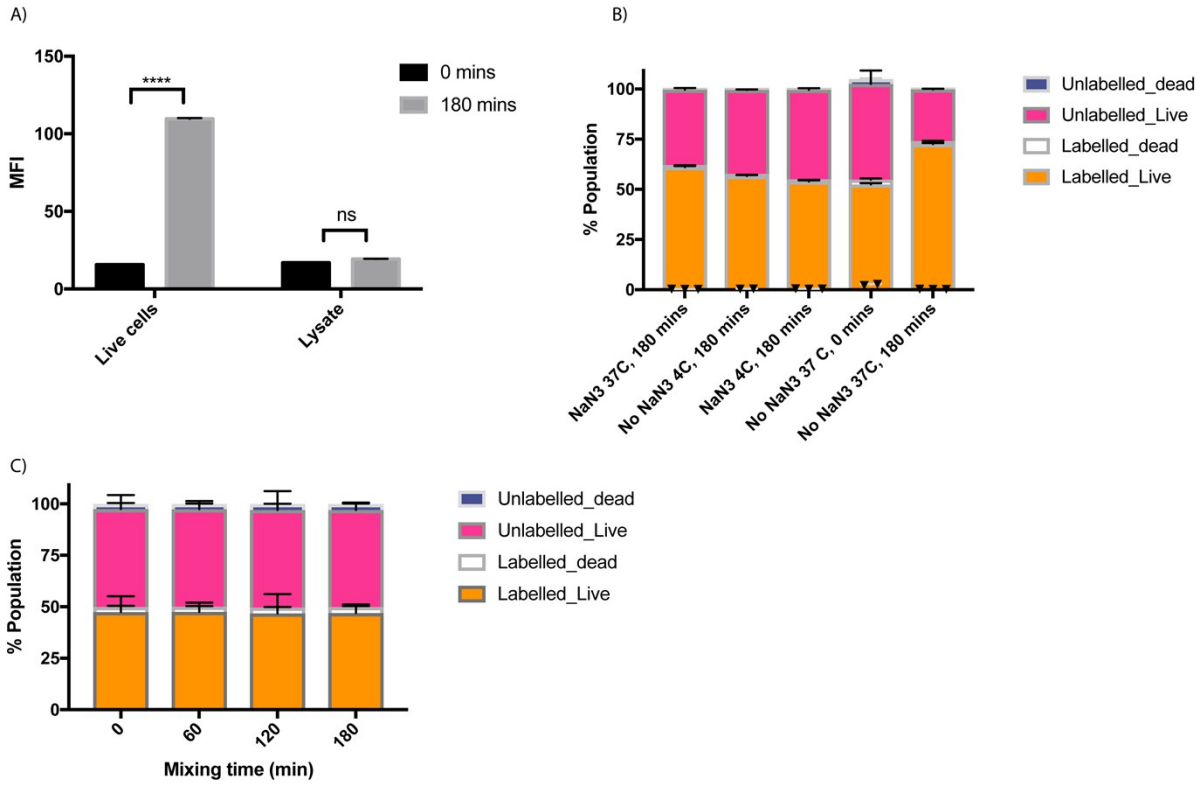


Figure S 4 Cell debris doesn't affect the lipid exchange in HeLa cells. A) Labelled HeLa-cells with bdp-Ch 1 were lysed by ultra-sonication and co-cultured with unlabelled cells at 37 °C for 3 h. MFI was calculated using flow cytometry and results showed any uptake of the fluorescent lipid difference B) Labelled HeLa-cells with bdp-Ch 1 were co-cultured for 3 h with unlabelled cells with or without 1 mM sodium azide at 37 °C or 4 °C. Propidium Iodide (PI) used as live/dead dye. Flow cytometry analysis indicates <2% cell debris and lipid exchange independent of cell debris and only at 37 °C .C) Labelled HeLa-cells with bdp-Ch 1 were co-cultured with unlabelled cells with 1 mM sodium azide at 4 °C for metabolic and energetic inhibition; flow cytometry analysis indicates <2% cell debris, the absence of toxicity during these conditions and the absence of lipid exchange due to cell debris.

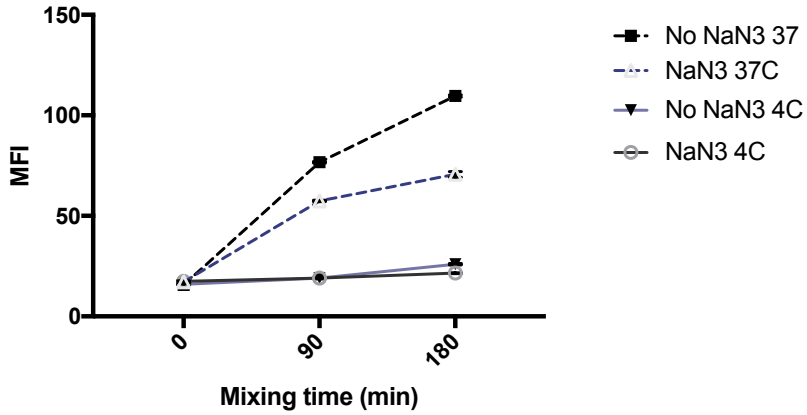


Figure S 5 Cellular energy is necessary for membrane lipid exchange. Labelled HeLa-cells with bdp-Ch 1 were we co-cultured with unlabelled cells with 1 mM sodium azide for ATP depletion at 37 °C or 4 °C. Flow cytometry analysis indicates the absence of lipid exchange at 4 °C or the decrease of the exchange at 37 °C with sodium azide.

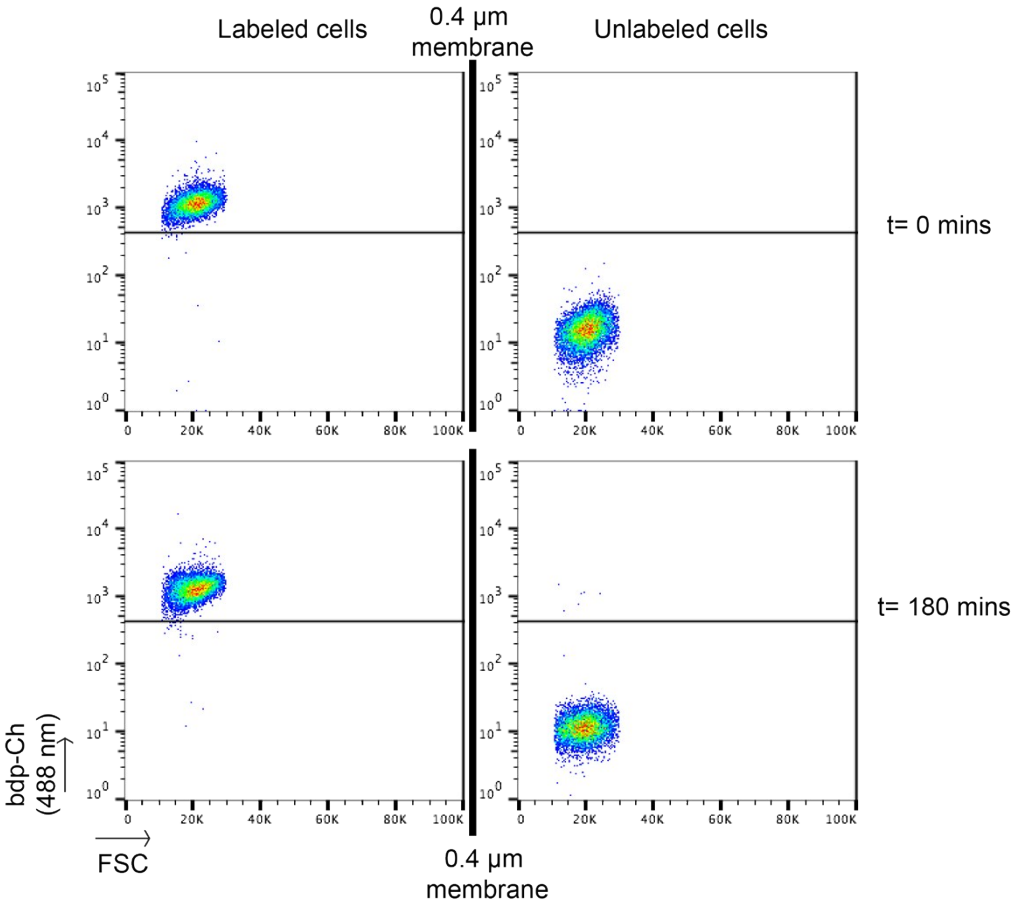


Figure S 6 HeLa cells show no lipid exchange when co-cultured in a trans-well plate. Labelled HeLa-cells with bdp-Ch 1 were separated by a 0.4µm membrane from unlabelled cells and incubated for 3 h. Flow cytometry analysis indicates the absence of lipid exchange.

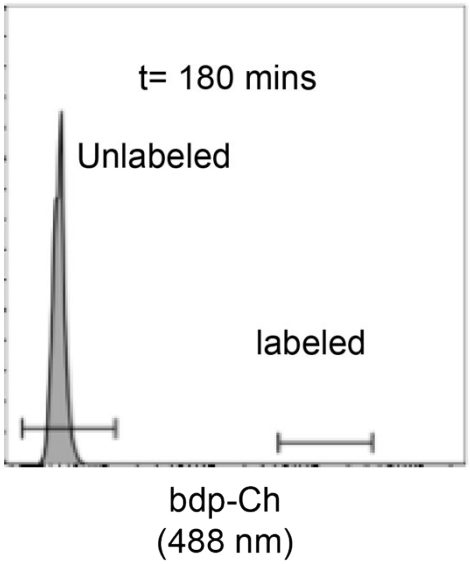


Figure S 7 Supernatant Exchange. HeLa-cells were incubated with bdp-cholesterol (bdb-Ch 1) and washed with PBS. After 1h, the supernatant was collected and added to an unlabelled population of HeLa-cells for 3 h. No labelling was observed.

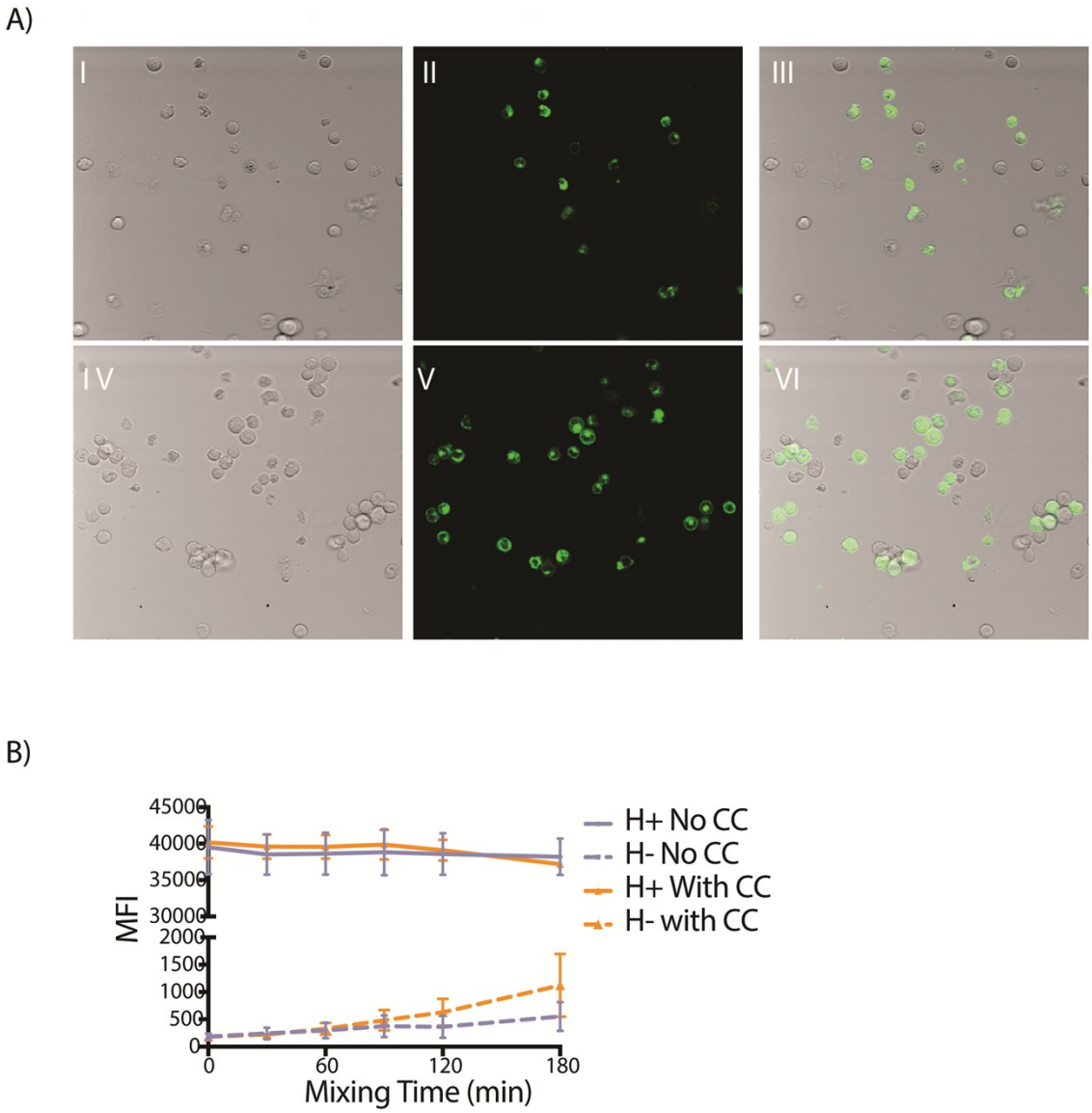


Figure S 8 Flow cytometry of cholesterol exchange between jurkat cells in presence or absence of coiled coils. Forcing cells in close proximity using lipidate coiled-coil (CC) peptides enhance sterol exchange. A) Confocal microscopy of Jurkat cells with or without coiled coil: I-III) Jurkat cells were labelled with 1 and co-cultured with unlabelled cells without the presence of lipidated coiled coil peptides; IV-VI) Jurkat cells were labelled with 1 and treated with 5 μM CPE co-cultured with unlabelled cells, pre-treated with 5 μM CPKCells treated with bdp-Cholesterol 1 (5 μM for 24 h) and CPE (5 μM) were mixed with unlabelled cells incubated with CPK (5 μM). Cholesterol exchange is only observed for CC-forming pairs. Data are representing as mean ±SD. Error bars SD. H+: Labelled cells; H-: Unlabelled cells

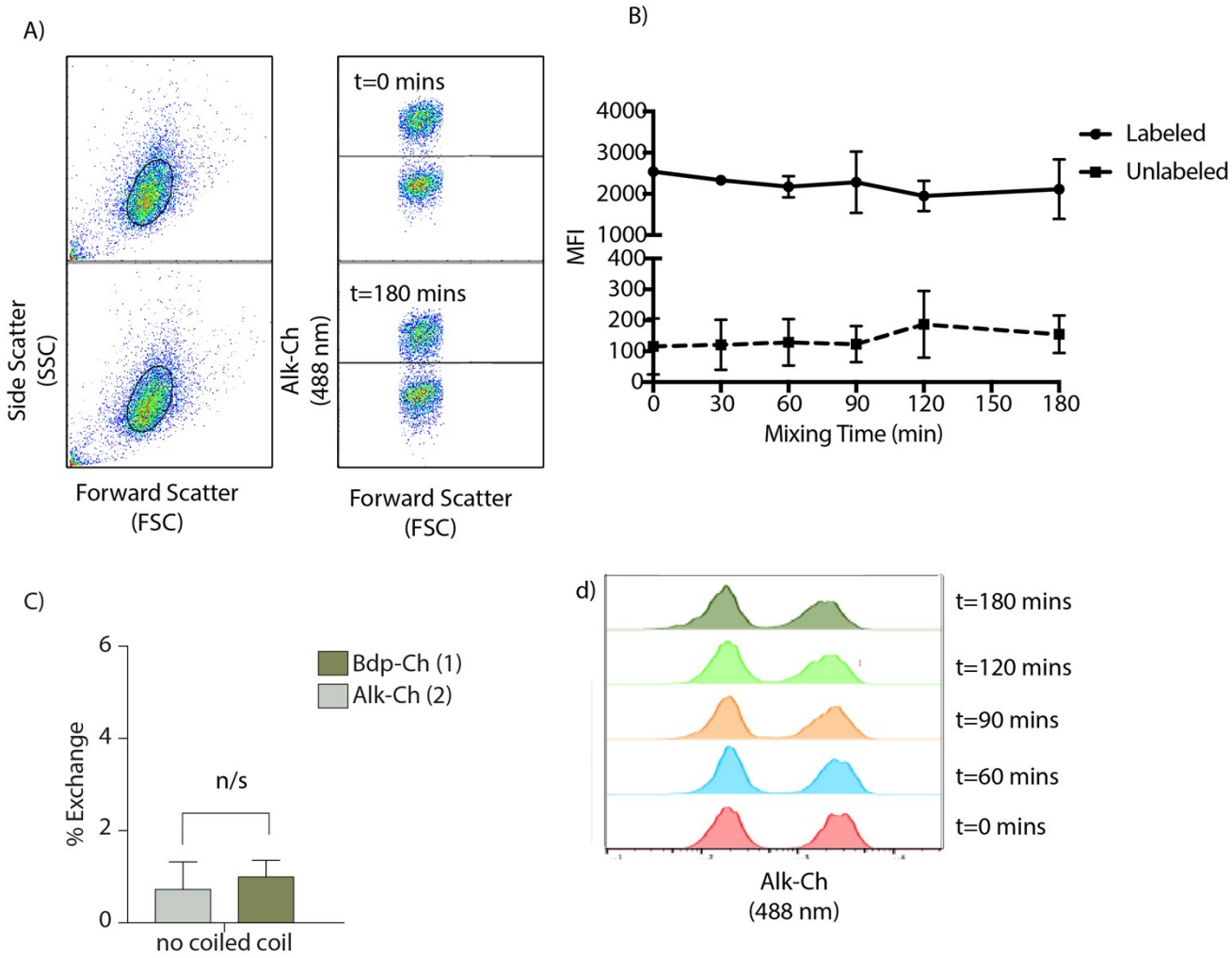


Figure S 9 Flow cytometry assay indicates no cholesterol exchange between live jurkat cells. Cells were treated with Cholesterol-Alkyne (Alk-Ch 2, Avanti, 10 µM for 24 h). Labelled and unlabelled live cells mixed and labelled using CuAAC and flow cytometry assay showed any cholesterol exchange. A) Dot plots and histograms of mixing cells at t=0 mins, and t=180 mins B) MFI in different times. C) Exchange rates of Alk-Ch (2) and bdp-Ch (1) in jurkat cells in the absence of CC peptides. D) Histograms in different times, showing no Alk-Ch (2) exchange. Data are representing as mean ± SD. Error bars SD.

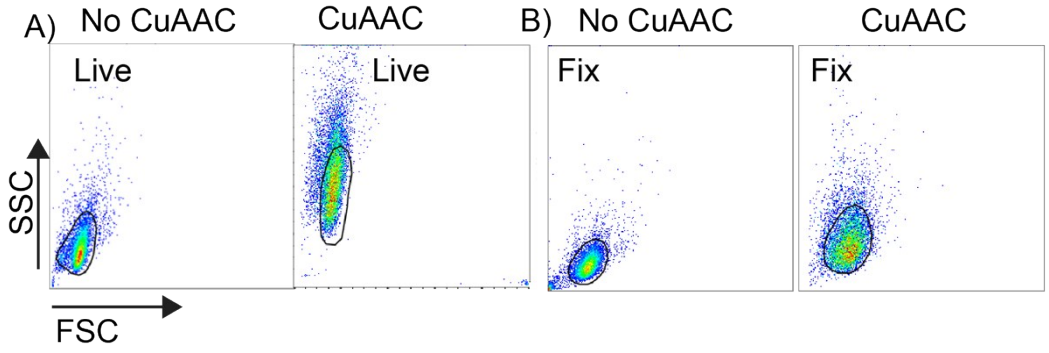


Figure S 10 Live-cell CuAAC affects cellular morphology. HeLa-cells treated with cholesterol-alkyne were subjected to CuAAC conditions with Alexa Fluor® 488 azide. A) FSC/SSC before addition of CuAAC-reagent mix of unfixed and fixed cells; B) FSC/SSC after addition of reagents to unfixed and fixed cells. Fixed cells retain their FSC/SSC profile, whereas live cells broaden their FSC/SSC value distribution.

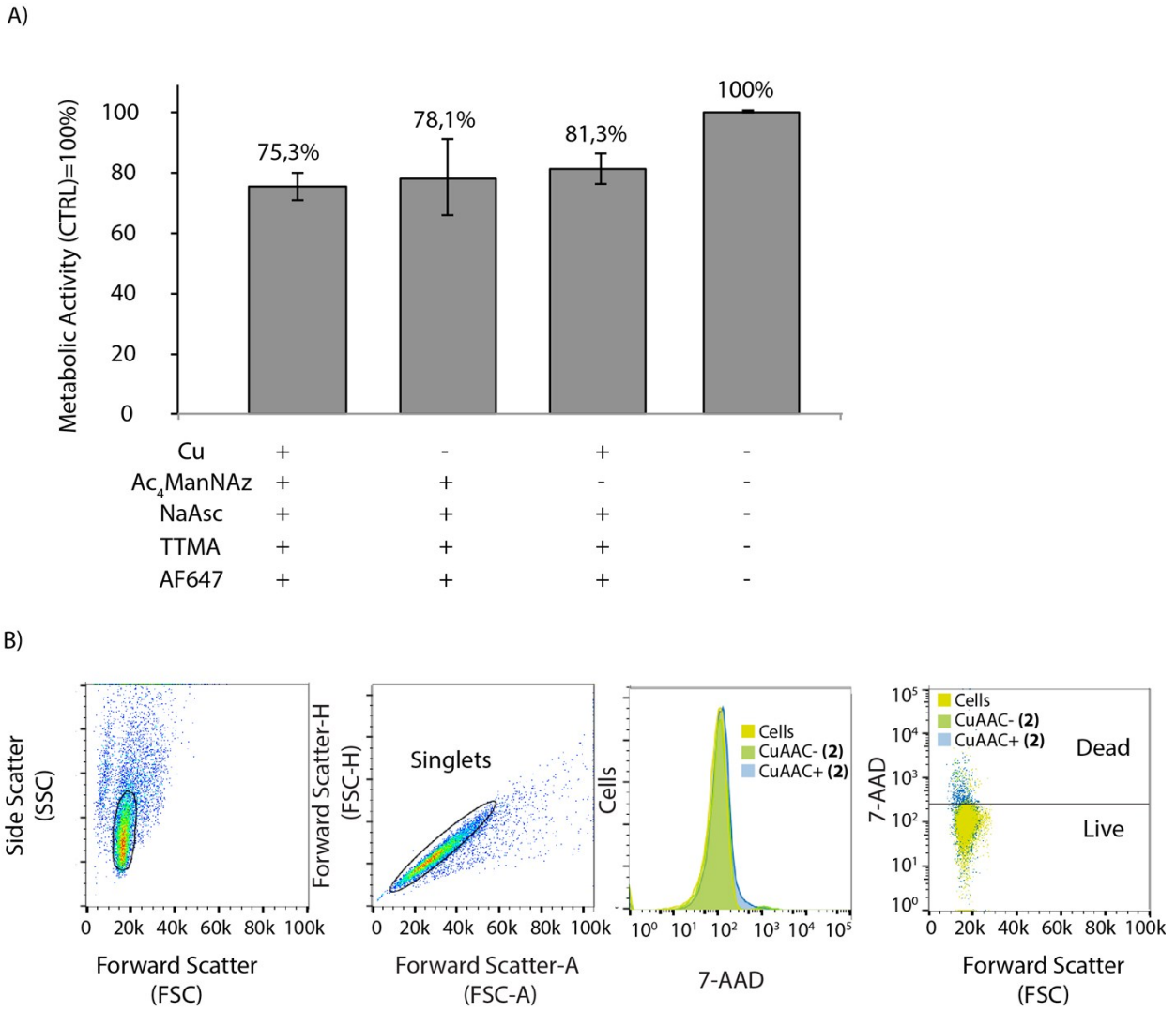


Figure S 11 CuAAC does not major impact in cell viability. Cells were treated with/without Ac4ManNAz for 72h or with Alk-Ch (2, Avanti) 10 uM. Then subjected to CuAAC (with or without catalyst) conditions for 5 mins (100 µM CuSO4, 250 µM TTMA [(Tris((1-((O-ethyl)carboxymethyl)-(1,2,3-triazol-4-yl))methyl)amine] ligand, 2.5 mM sodium Ascorbate and 3 µM Alexa Fluor® 647 Alkyne or Alexa Fluor® 488 azide). Cells were washed three times. The cell WST-1 viability assay[1] shows no difference in cell viability under these CuAAC conditions, despite the observed morophological changes in S10. B) Cells treated with Alk-Ch (2, Avanti) 10 uM and were subjected to CuAAC; 7-AAD viability dye was used and not major cell toxicity (<4 %) was defined.

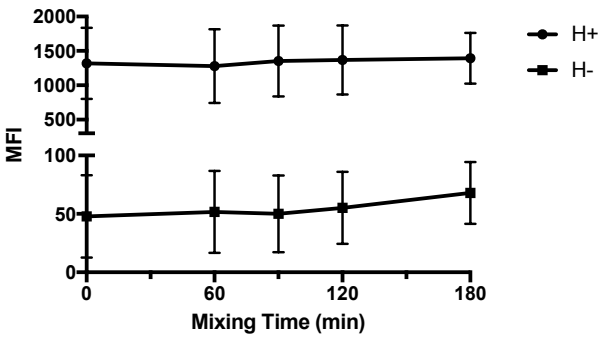


Figure S 12 Flow cytometry assay indicates slow glycan exchange between live HeLa cells. First, cells treated with Ac_4ManNAz 50 µM for 72 h. Labelled (H+) and unlabelled (H-) live cells were mixed, fixed with 2% PFA and labelled using optimized click conditions. MFI calculated after 3h indicates very slow glycan exchange. Data represent as mean ± SD. Error bars SD. H+: Labelled cells; H-: Unlabelled cells

Materials and Methods

Reagents

Cholesterol and all other chemical reagents were purchased at the highest grade available from Sigma Aldrich and used without further purification. All solvents were purchased from Biosolve Ltd. Phosphate buffered saline (PBS): 5 mM KH_2PO_4, 15 mM K_2HPO_4, 150 mM NaCl, pH 7.4.

Silica gel column chromatography was performed using silica gel grade 40-63 µm (Merck). TLC analysis was performed using aluminium-backed silica gel TLC plates (60_F 254, Merck), visualisation by UV absorption at 254 nm and/or staining with $KMnO_4$ solution. NMR spectra (^1H and ^{13}C) were measured on a Bruker AV-400MHz spectrometer at ambient temperature at the Leiden Institute of Chemistry NMR Facility. Chemical shifts are recorded in ppm. Tetramethylsilane (TMS) is used as an internal standard. Multiplicity was reported as follows: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, m = multiplet, br = broad.
Electrospray LC-MS analysis was performed on a PE SCIEX: API 3000 LC/MS/MS system using a Gemini 3u C18 110A analytical column (5µ particle size, flow: 1.0 ml/min), on which the absorbance was measured at 214 and 254 nm. Solvent system for LC-MS: A: 100% water, B: 100% acetonitrile, C: 1% TFA (aq).

MALDI-TOF mass spectra were acquired using an Applied Biosystems Voyager System 6069 MALDI-TOF mass spectrometer. α-Cyano-4-hydroxycinnamic acid (CHCA) was used as matrix in all cases. Sample concentrations were ~0.3 mg/ml.

HPLC-ELSD analysis was performed using a Shimadzu HPLC setup equipped with two LC-8A series pumps coupled to a Shimadzu ELSD-LT II detection system. Separation (Vydac 214 MS C4 column, 5u, 100 × 4.6 mm, flow rate: 15 mL/min), in all instances, was carried out over a linear gradient of 10-90% B over 20 minutes with an initial 5 min hold at 10% B. HPLC buffers: A – H_2O (0.1% TFA); B – Acetonitrile (0.1% TFA). The drift tube temperature for ELSD was set at 37°C and the nitrogen flow-rate at 3.5 bar.

Flow Cytometry[2,3]

Flow cytometry assays were performed using the Merck Guava® EasyCyte 12HT Benchtop Flow Cytometer and all flow cytometry data was analyzed using FlowJo v10.1 (FlowJo, LLC). Counting and Characterization was performed by measuring 10,000 events in triplicate and concatenation of this data. For manual gating, the outermost ring of the dot plot was selected. Quadrants were manually selected to illustrate fluorescence plots. No compensation was required.

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence.

In the flow cytometer, particles are carried to the laser intercept in a fluid stream. Any suspended cell from 0.2–150 micrometers in size is suitable for analysis. When particles pass through the laser intercept, they scatter laser light. The scattered and fluorescent light is collected by appropriately positioned lenses. A combination of beam splitters and filters steers the scattered and fluorescent light to the appropriate detectors. The detectors produce electronic signals proportional to the optical signals striking them.

Light scattering occurs when a particle deflects incident laser light. The extent to which this occurs depends on the physical properties of a particle, namely its size and internal complexity. Factors that affect light scattering are the cell's membrane, nucleus, and any granular material inside the cell. Cell shape and surface topography also contribute to the total light scatter. Correlated measurements of FSC and SSC can allow for differentiation of cell types in a heterogeneous cell population.

Forward-scattered light (FSC) is proportional to cell-surface area or size. FSC is a measurement of mostly diffracted light and is detected just off the axis of the incident laser beam in the forward direction by a photodiode. FSC provides a suitable method of detecting particles greater than a given size independent of their fluorescence.

Side-scattered light (SSC) is proportional to cell granularity or internal complexity. SSC is a measurement of mostly refracted and reflected light that occurs at any interface within the cell where there is a change in refractive index. SSC is collected at approximately 90 degrees to the laser beam by a collection lens and then redirected by a beam splitter to the appropriate detector.

Cholesterol Exchange Essay

For the study of cholesterol exchange, cells were incubated with Bdp-Cholesterol 1 (TopFluor, Avanti) for 18 h at 37 °C. Cells were washed 6 times, before co-culture with unlabelled cells. For the study of exchange of adherent cells, the cells were detached prior to exchange using EDTA/PBS for 15 mins and seeded in a 96-V-plate for the mixing and exchange.

Coiled-coil Enhancement of Exchange Reaction

For the coiled-coil formation labelled cells treated with 5 μM CPE and the unlabelled cells with 5 μM CPK for 10 mins at 37 °C. Cells washed twice with PBS and were resuspended in fresh media. Cell were mixed and co-cultured for different time-periods (20,000 cell/100μl treated+ 20000 cell/100μl untreated) in media with or without serum. Then fluorescence measured using Guava® easyCyte 12HT Benchtop Flow Cytometer and our results were analysed using FlowJo v10.1 (FlowJo, LLC).

Bioorthogonal Sialylated Glycan Exchange Essay

For the glycan studies, cells incubated with 50 μM Ac_4ManNAz for 72 h at 37 °C. Adherent cells were detached using 2 mM EDTA/PBS for 15 mins and mix in 96-V-plate with unlabelled cells.

For the coiled-coil mediated exchange enhancement, cells were treated with 5 μM CPE and 5 μM CPK for 10 mins at 37 °C prior to mixing. Cells washed twice with PBS and were resuspended in fresh media. Cell were mixed and co-cultured for different time-periods (20,000 cell/100μl treated+ 20,000 cell/100μl untreated) in media with or without serum. Prior to labelling cells were fixed with PFA (paraformaldehyde) 2% for 15 mins at room temperature. The paraformaldehyde was removed (2 x washing) and the cells were resuspended in PBS. Then CuAAC mix was added. Click solution comprised of 1 mM CuSO4, 100 μM TTMA [(Tris((1-((O-ethyl)carboxymethyl)-(1,2,3-triazol-4-yl))methyl)amine] ligand, and 2 mM sodium ascorbate and 2 μM alexa-fluor-647 alkyne (Invitrogen). After 20mins, the cells were washed three times with PBS, prior to incubation with 3% BSA (Bovine serum albumin) for 30 mins to remove unreacted fluorophore. The cells were then washed and flow-cytometry performed.

For the cholesterol-alkyne assay, the cells were incubated for 18 h at 37 °C with 5 μM cholesterol-alkyne 2 (Avanti) in full media[4]. Cells were then co-cultured, fixed and labelled using the above biorthogonal labelling protocol but with 2 μM Alexa-fluor-488 azide (Invitrogen).

Mammalian Cell Culture

Cells were cultured in 25 cm^2 flasks and split at 70-80% confluence (three times per week). The flasks were incubated at 37 °C at 7.0% CO_2. The medium was refreshed three times a week. Cells used in all biological experiments were cultured for a maximum of 8 weeks. Adherent cell cultures with a maximum confluence of 70-80% were trypsinized and centrifuged (1.5 min, 2000/4000rcf (live/fix cells), and the cells were re- suspended using fresh media.
10 μL of cell suspension and 10 μL of trypan blue were mixed and pipetted into a cell counting slide, and cells were counted using a BioRad TC10 automated cell counter. The cell suspension was diluted to the appropriate seeding density.
HeLa[5], U2Os[6] cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum, 2mM L-glutamine, 1% penicillin and 1% streptomycin. Cells were cultured in an atmosphere of 7% CO_2 at 37°C. Medium was refreshed every two days and cells passaged at 70% confluence by treatment with trypsin-EDTA (0.05% trypsin).

Jurkat[7]1 and AMO cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, penicillin 100 I.U./mL and streptomycin 50 µg/mL.

CTL hybridoma, B3Z[8] was cultured in IMDM medium supplemented with 10% FCS, 2 mM glutamax, 0.25 mM 2-Mercaptoethanol, penicillin 100 I.U./mL and streptomycin 100µg/mL in the presence of hygromycin B (500µg/ml)[9].

Live cell confocal microscopy

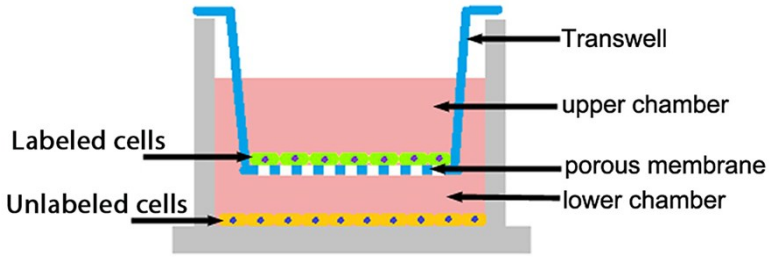
Cells were seeded on a 35 mm dish (3 x 10^5) in a complete media after the addition of 5 µM bdp-Ch 1 for 18 h. Next day, prior to the concfocal microscopy, lipidate coiled-coil peptides were added as follows: 5 µM final concentration of CPE was added to bdp-Ch treated cells and 5 µM of CPK was added to unlabelled cells, both were incubated at 37 °C for 10 mins. After three washing steps, fresh media was added and cells were transfered into a 8-well µ-slide (ibidi, cat. 80826) by mixing 1 x 10^4 bdp-Ch-CPE-modified cells with an equal amount of CPK-modified cells per well. Samples were imaged with a Leica TCS SP8 confocal microscope (63x oil lens, N.A.=1.4).

WST-1 Cytotoxicity Assay[1]

The cell proliferation reagent WST-1 (CAS 150849-52-8) was used to assess cell viability. This assay is based on the cleavage of a tetrazolium salt (WST-1) to soluble formazan dye by the mitochondrial dehydrogenase of living cells. At indicated time-points, 10ul of a freshly made mixture of WST-1 and PMS-OMe (90 uM WST-1 and 181 µM PMS-OMe) were added to each well, and the plates were incubated at 37°C for 4 h. Subsequently, the optical densities of the plates were detected at 450 nm (formazan formation) as measured using 96 well plate reader. The cytotoxicity was expressed as percentage over control.

Transwell assay[10]

Labelled cells were prevented from directly contacting unlabelled cells using a transwell 0.4 µm-pore membrane (Costar). Cells were seeded in a 6-well plate with full DMEM media with or without the addition of 5 µM bdp-cholesterol (TopFLuor, Avanti) and cells incubated 24h at 37 °C. Cells were detached with 2.5 mM PBS/EDTA, washed and then re-suspended in DMEM media and then counted. Labelled cells (in 0.3 mL of medium) were added in the upper compartment (done in 6-well plates) and unlabelled cells (in 0.5 mL of medium) placed in the lower chamber separated from targets. The inserts are then picked up using gloves and transferred onto the top of the unlabelled HeLa cell culture with the addition of 2 ml of fresh media into the inserts. The cells were incubated for 3 h at 37°C, and then collected and analyzed with flow cytometry.



Synthesis of Ac_4ManNAz

Ac_4ManNAz (Tetra-*O*-Acetyl-*N*-azidoacetylmannosamine) was synthesized in full accordance with the reported procedure[11].

^{1}H NMR (400MHz, $CDCl_3$), δ= 6.04 (d, 1H), 5.91 (d, 1H), 5.49 (d, 1H), 5.33 (d, 1H), 5.18 (dd, 1H), 5.00-4.97 (m, 1H), 4.81 (d, 1H), 4.77 (d, 1H), 4.55 (d, 1H), 4.54 (dd, 1H), 4.52 (d, 1H), 4.4 (m, 2H), 4.32 (m, 7H), 4.1 (m, 1H), 2.14 (s, 3H), 2.11 (s, 3H), 2.04 (s, 6H), 1.99 (s, 6H), 1.5 (s, 3H), 1.34 (s, 3H). LC-MS (ESI): m/z [M+H]+, calc. for $C_{16}H_{22}N_4O_{10}$: 431.37; found 431.37

Synthesis of peptides

CPE (cholesterol-PEG_{12}-peptideE) and CPK (cholesterol-PEG_{12}-peptideK) were synthesized and purified as previously reported[12]. Peptide sequences were $(EIAALEK)_3$ and $(KIAALKE)_3$ for E and K respectively.

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