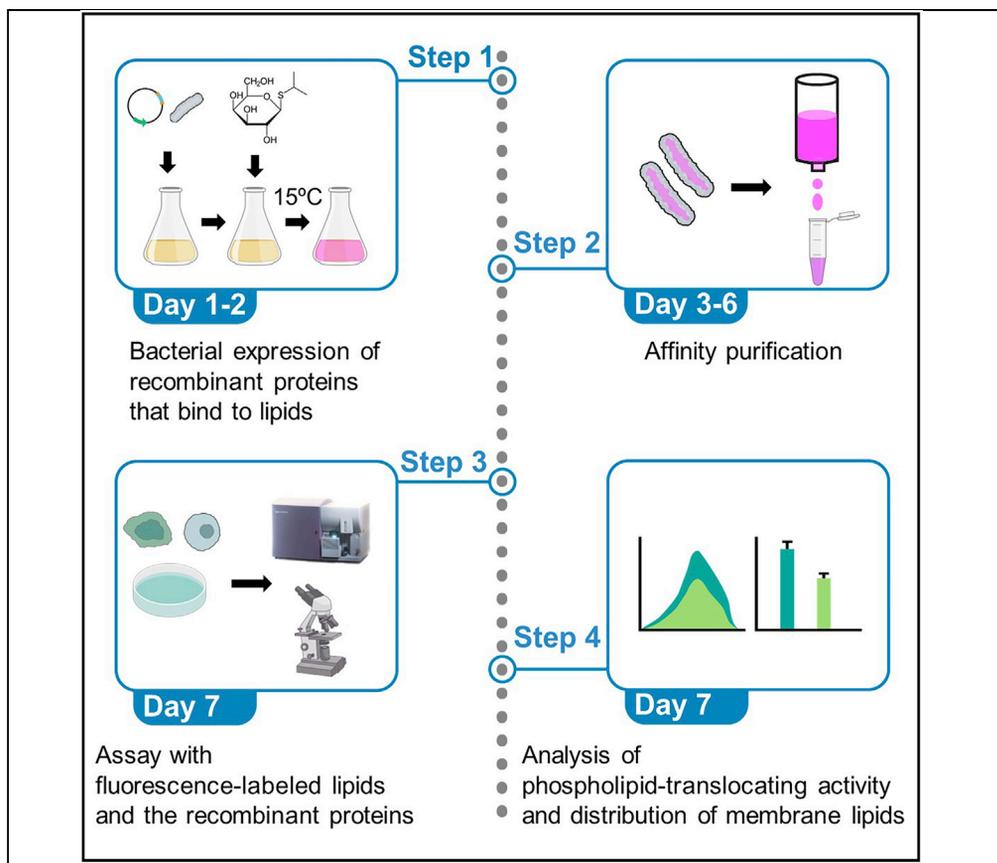


Protocol

Protocol to analyze lipid asymmetry in the plasma membrane



The plasma membrane containing cholesterol exhibits phospholipid asymmetry, with phosphatidylcholine and sphingomyelin enriched in its outer leaflet and phosphatidylserine (PtdSer) and phosphatidylethanolamine (PtdEtn) on the cytoplasmic side. We herein describe steps for bacterial expression of recombinant proteins that bind to membrane lipids, followed by affinity purification. Using fluorescence-labeled phospholipid analogs, we further detail the assay to detect flippase activity, which maintains the single-sided distribution of PtdSer and PtdEtn, in mammalian cells.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Flippases translocate phosphatidylserine to the inner leaflet of the plasma membrane

Assay for detecting the flippase activity and PtdSer exposure on the cell surface

Analysis of sphingomyelin and cholesterol in the outer leaflet of the plasma membrane

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Protocol

Protocol to analyze lipid asymmetry in the plasma membrane

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SUMMARY

The plasma membrane containing cholesterol exhibits phospholipid asymmetry, with phosphatidylcholine and sphingomyelin enriched in its outer leaflet and phosphatidylserine (PtdSer) and phosphatidylethanolamine (PtdEtn) on the cytoplasmic side. We herein describe steps for bacterial expression of recombinant proteins that bind to membrane lipids, followed by affinity purification. Using fluorescence-labeled phospholipid analogs, we further detail the assay to detect flippase activity, which maintains the single-sided distribution of PtdSer and PtdEtn, in mammalian cells.

For complete details on the use and execution of this protocol, please refer to Segawa et al. (2021).¹

BEFORE YOU BEGIN

Phospholipids are asymmetrically distributed between the lipid bilayer of the plasma membrane. Typically, sphingomyelin (SM) and phosphatidylcholine (PtdCho) are abundant in the outer leaflet, whereas amino phospholipids such as phosphatidylserine (PtdSer) and phosphatidylethanolamine (PtdEtn) are compartmentalized in the inner leaflet.² Cholesterol, a major neutral lipid in the plasma membrane, has diverse biological functions, and its dynamics and distribution are affected by the surrounding phospholipids such as SM and PtdSer.^{3–5} The asymmetry of these phospholipids is disrupted in various biological processes such as apoptosis, blood clotting, and lymphocyte activation, exposing PtdSer and PtdEtn to the cell surface.⁶ However, the mechanisms by which the amount and distribution of these diverse membrane lipids are regulated remain unclear. Three types of molecules are known to regulate the distribution of membrane lipids across the lipid bilayer^{7,8}: flippase specifically translocates PtdSer and PtdEtn from the outer to the inner leaflet of the plasma membrane in an ATP-dependent manner; floppase translocates PtdCho or cholesterol from the inner to the outer leaflet in an ATP-dependent manner; and scramblase randomizes membrane phospholipids by transferring them in both directions without expending energy. Herein, we describe a protocol for detecting lipids such as SM, PtdSer, and cholesterol embedded in the outer leaflet of the plasma membrane using lipid-binding proteins and a protocol to detect flippase activity using fluorescence-labeled phospholipid analogs.

Institutional permissions

Experiments using recombinant DNA and bacteria must be performed according to relevant institutional and national guidelines and regulations. The experiments described in these protocols were conducted with the approval of the Tokyo Medical and Dental University Safety Committee for Recombinant DNA Experiments.



Preparation of plasmid DNA

⌚ Timing: 3–4 days

Plasmid DNA for the bacterial expression of non-toxic lysenin (NT-Lys) that binds to SM-rich domains

1. Obtain the plasmid DNA, pQE30/His6-mRFP-NT-Lys (RIKEN BRC, Catalog No. RDB13960), from RIKEN BRC.⁹
2. Excise a DNA fragment encoding sequences for NT-Lys-mRFP with the restriction enzymes BamHI and HindIII and introduce it into a pCold DNA I vector (TAKARA, Catalog No. 3361) using the digested sites to generate the plasmid DNA, pCold I-His6-mRFP-NT-Lys, for efficient *E. coli* expression by cold shock.

Plasmid DNA for the bacterial expression of Domain 4 (D4) that binds to cholesterol-rich domains

3. Obtain the plasmid DNA, pET28/His6-mCherry-D4 (RIKEN BRC, Catalog No. RDB14300), from RIKEN BRC.^{10,11}
4. Excise a DNA fragment encoding sequences for mCherry-D4 with the restriction enzymes KpnI and XhoI and introduce it into the pCold DNA I vector (TAKARA, Catalog No. 3361) using the digested sites to generate the plasmid DNA, pCold I-His6-mCherry-D4, for efficient *E. coli* expression by cold shock.

Preparation of stock solutions for phospholipid assays

⌚ Timing: 2 h

Preparation of aliquots of stock solutions

Organic solvents, such as chloroform and methanol, are generally evaporated in a stream of nitrogen and re-dissolved in DMSO for cell-based assays.

Reagent	Storage concentration	Working concentration	Storage condition
NBD-PS	1 mM	0.1–1 μM	–30°C
NBD-PE	1 mM	0.1–1 μM	–30°C
NBD-PC	1 mM	0.1–1 μM	–30°C
NBD-SM	1 mM	0.1–1 μM	–30°C
BSA	5 mg/mL	2.5 mg/mL	–30°C
IPTG	100 mM	0.1–0.2 mM	–30°C
L-arabinose	100 mg/mL	0.8 mg/mL	–30°C
A23187	10 mM	1–3 μM	–30°C
Propidium iodide	1 mg/mL	5 μg/mL	4°C

5. One milligram of NBD-PS, NBD-PE, NBD-PC, and NBD-SM is dissolved in 1.22, 1.32, 1.25, and 1.35 mL of DMSO, respectively, and 50- to 100-μL aliquots are stored at –30°C with protection from light.
6. BSA is dissolved in Hanks' Balanced Salt Solution (HBSS) at 5 mg/mL, filtered through a PVDF membrane (0.22 μm, MERCK), and stored at –30°C. Once thawed, the solution is kept at 4°C and used within two months.
7. A total of 238 mg of IPTG is dissolved in 10 mL of distilled water, filtered through a PVDF membrane (0.22 μm, MERCK), and 0.5- to 1-mL aliquots are stored at –30°C.
8. One gram of L-arabinose is dissolved in 9 mL of distilled water, filtered through a PVDF membrane (0.22 μm, MERCK), and 0.5- to 1-mL aliquots are stored at –30°C.
9. Ten milligrams of A23187 is dissolved in 1.91 mL of DMSO, and 50- to 100-μL aliquots are stored at –30°C.

10. Twenty milligrams of propidium iodide (PI) is dissolved in 20 mL of distilled water, filtered through a PVDF membrane (0.22 μm , MERCK), and 0.5- to 1-mL aliquots are stored at 4°C with protection from light.

Note: All aliquots can be stored at -80°C .

Note: IPTG, L-arabinose, A23187, and PI can be stored at their respective temperatures for at least six months. We usually limit the number of freeze-thaw cycles of IPTG, L-arabinose, and A23187 to 10.

△ CRITICAL: The number of freeze-thaw cycles of NBD-lipids in DMSO is limited to 10, and they are used within two months after thawing. Without thawing, NBD-lipids in DMSO may be stored at -30°C for at least four months.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and viral strains		
<i>E. coli</i> DH5 α -competent cells	TAKARA	9057
Chaperone-competent cells pGro7/BL21	TAKARA	9122
Chemicals, peptides, and recombinant proteins		
18:1-06:0 NBD-PS	Avanti Polar Lipids	810194
18:1-06:0 NBD-PE	Avanti Polar Lipids	810155
18:1-06:0 NBD-PC	Avanti Polar Lipids	810132
C6-NBD Sphingomyelin (NBD-SM)	Avanti Polar Lipids	810218
BSA, essentially free of fatty acids, $\geq 96\%$	Sigma-Aldrich	A6003
Annexin V-Cy5	BioVision	#1013-1000
Bovine MFG-E8-FITC labeled	Haematologic Technologies	BLAC-FITC
His-NT-Lys-mRFP	Segawa et al. ¹	N/A
His-mCherry-D4	Segawa et al. ¹	N/A
SYTOX Blue Dead Cell Stain	Thermo Fisher Scientific	S34857
BamHI-HF	NEB	R3136
HindIII-HF	NEB	R3104
KpnI-HF	NEB	R3142
XhoI	NEB	R0146
Ampicillin sodium salt	Nacalai	19769-64
Chloramphenicol	Nacalai	06285-94
LB broth, Miller	Nacalai	20068-04
LB agar, Miller	Nacalai	20069-65
Isopropyl- β -D-thiogalactopyranoside (IPTG)	Nacalai	19742-81
L-Arabinose	Nacalai	03306-04
Protease inhibitor cocktail (EDTA free) (100 \times)	Nacalai	03969-34
Imidazole	Nacalai	19004-22
RPMI1640	Nacalai	30264-56
HBSS (-)	Nacalai	17461-05
Penicillin-streptomycin mixed solution	Nacalai	26253-84
Trypan Blue solution	Nacalai	20577-34
Propidium iodide	Nacalai	29037-92
DMSO	Nacalai	13408-64
A23187	Abcam	ab120287
Ionomycin	FUJIFILM Wako	095-05831
Experimental model: Cell line		
WR19L	ATCC	TIB-52

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
pCold I DNA	TAKARA	3361
pQE30/His6-mRFP-NT-Lys	RIKEN BRC	RDB13960
pCold I-His6-mRFP-NT-Lys	Segawa et al. ¹	N/A
pET28/His6-mCherry-D4	RIKEN BRC	RDB14300
pCold I-His6-mCherry-D4	Segawa et al. ¹	N/A
Software and algorithms		
FlowJo™v10	BD Bioscience	https://www.flowjo.com
BD FACSDiva software	BD Bioscience	https://www.bdbiosciences.com/en-us/products/software/instrument-software/bd-facsdiva-software
Other		
Ni-NTA Agarose	QIAGEN	30210
Poly-Prep Chromatography Columns	Bio-Rad	Cat. #731-1550
Amicon Ultra-15 (10 kDa MWCO)	MERCK	REF: UFC901024
PAGEBlue Protein Staining Solution	Thermo Fisher Scientific	24620
QIAprep Spin Miniprep Kit	QIAGEN	27104
Wizard SV Gel and PCR Clean-Up System	Promega	A9281
LigaFast Rapid DNA Ligation System	Promega	M8221
Millex-GV Syringe Filter Unit, 0.22 μm, PVDF	MERCK	SLGVR33RS
Xpress Micro Dialyzer MD300 12–14 kDa	Scienova	40790
5 mL Polystyrene Round-Bottom Tube with Cell-Strainer Cap	FALCON	352235
Extra PAGE One Precast Gel 5%–15%	Nacalai	13062-84

STEP-BY-STEP METHOD DETAILS

Assay for flippase activity at the plasma membrane

⌚ Timing: 1 h

Using a floating lymphoma cell line, we describe protocols to detect flippase activity that translocates phospholipids from the outer to the inner leaflet of plasma membranes.^{12,13} Animal cells typically exhibit flippase activity for PtdSer and PtdEtn, and this activity depends on the expression of plasma membrane flippases, such as ATP11A and ATP11C, which are members of the type IV P-type ATPase family, in most cases.¹⁴ Flippase activity towards other phospholipids, such as PtdCho and SM, can be evaluated using similar procedures. The ATP11A mutant (Q84E) that aberrantly translocates PtdCho was analyzed using this protocol.¹

1. WR19L or their transformant cells are expanded in RPMI1640-10% FCS containing 100 U/mL penicillin and 100 μg/mL streptomycin in a 10-cm culture dish.

Note: Culture conditions may affect or compromise flippase activity. We generally maintain WR19L-derived cells between 1×10^4 and 2×10^6 cells/mL and do not exceed 2×10^6 cells/mL during the cell culture. For the assay, we typically use cells at 5×10^5 – 2×10^6 cells/mL.

2. Count cells using a hemocytometer or cell counter (TC20, Bio-Rad) with trypan blue staining.
3. Transfer 1 – 2×10^6 of viable cells to a 1.5- or 2-mL tube.
4. Centrifuge at $300 \times g$ at 20°C – 25°C for 3 min.
5. Aspirate the supernatant and add 1 mL of phosphate-buffered saline (PBS).
6. Resuspend the cells and centrifuge at $300 \times g$ at 20°C – 25°C for 3 min.
7. Aspirate the supernatant and resuspend the cells in 300 μL of HBSS containing 2 mM CaCl_2 and 1 mM MgCl_2 .

8. Stand the tube in a dry block incubator set at 15 or 20°C for 7 min.

Note: The incubation temperature needs to be optimized for the respective cell types within 10°C–20°C.

△ **CRITICAL:** Do not incubate cells at >20°C unless necessary. An incubation at 37°C or >20°C induces endocytosis and membrane trafficking, and NBD-lipids in the plasma membrane may be incorporated independent of flippase activity.

9. Add 2 µL of 1 mM NBD-PS or NBD-PE to 1 mL of HBSS with 2 mM CaCl₂ and 1 mM MgCl₂ (final concentration of 2 µM).
10. Immediately add 300 µL of HBSS containing NBD-lipids (step 9) to the cell suspension (final concentration of 1 µM).

Note: NBD-lipid concentration needs to be optimized for the respective cell types within 0.1–1 µM.

Note: During the assay, NBD-lipids are kept in DMSO on ice and thawed before adding the solution. Diluted NBD-lipids in water or HBSS may be unstable.

11. Incubate cells at 15 or 20°C in the dry block incubator.

Note: We generally incubate cells with NBD-lipids within 40 min because a prolonged incubation may decrease the fluorescence signals of internalized NBD-lipids. Incubation times need to be optimized for the respective cell types and NBD-lipids. To prevent the cell precipitation, we pipet the cell suspension every 5–10 min during the assay.

12. At several time points, mix the cell suspension by pipetting and then transfer 100- to 150-µL aliquots quickly to a 1.5-mL tube with 150 µL of pre-chilled HBSS containing 5 mg/mL fatty acid-free BSA on ice to extract NBD-lipids from the outer leaflet.
13. Resuspend the cells quickly and incubate on ice for 1 min.

Note: Fatty acid-free BSA efficiently extracts lysophospholipids or artificial NBD-lipids from the outer leaflet of the plasma membrane without inducing cell lysis.^{15,16} NBD-conjugation alters the phospholipid structure, and the hydrophilicity of the NBD moiety causes the acyl chain to tend toward to the lipid-water interface.^{17,18} This characteristic of NBD-lipids enables the BSA-mediated extraction.

Note: Incubation with 5 mg/mL fatty acid-free BSA on ice for 1 min was sufficient to fully extract NBD-lipids in the outer leaflet of plasma membranes of WR19L or a human myeloma cell line that we used previously.¹⁹ The fluorescence signals should be compared in both the presence and absence of BSA to assess whether the BSA-mediated lipid extraction works. Treatment with BSA should quickly reduce the fluorescence signals of NBD-PC, which is not flipped to the inner leaflet at 15°C. Nevertheless, optimization is required for other cell types.

△ **CRITICAL:** A prolonged incubation in this step at 20°C–25°C or a higher temperature may reduce fluorescence signals, regardless of the BSA-mediated extraction.

14. Transfer 300 µL of the cell suspension to a 5-mL round-bottomed test tube with a cell strainer snap cap (FALCON, ref. 352235).
15. Analyze cells immediately with FACSCanto II or FACSAria III (BD) (Figure 1).

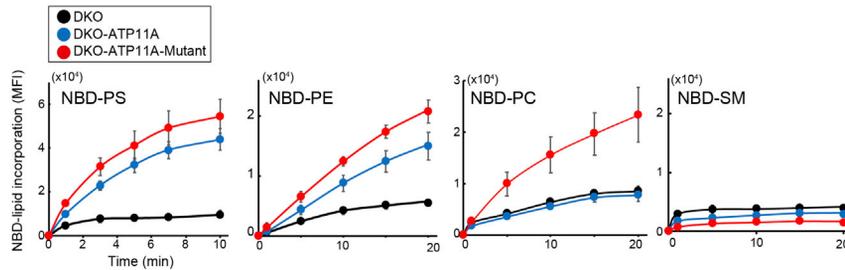


Figure 1. Flippase activity at the plasma membrane

The flippase, ATP11A, and its mutant (Q84E) translocate NBD-lipids at the plasma membrane. The cells were incubated with 1 μ M NBD-lipids at 20°C for the indicated times. *Atp11a-Atp11c* double-null (DKO) WR19L-derived cells exhibit weak flippase activity towards NBD-PS, NBD-PE, NBD-PC, and NBD-SM on the cell surface (black line). Expression of wild-type human ATP11A (blue line) and its mutant (Q84E) (red line) conferred flippase activity for NBD-PS and NBD-PE in DKO cells, whereas only the ATP11A mutant aberrantly translocated NBD-PC on the cell surface. Experiments were performed three times, and average values were plotted with S.D. (bar). Figure 1 is adapted from the data in¹ with modifications.

Note: The analysis by flow cytometry needs to be quickly performed. We generally use FACSCanto II (BD) in the high-speed mode and record 2,000 or 3,000 cells within 1 min.

Note: We gate cells by standard forward and side scatter (FSC/SSC) gating on the BD FACSDiva software to exclude dead cells from the population. Dead cell staining reagents, such as SYTOX Blue, are also available for the assay.

Note: *Atp11a-Atp11c* double-deficient mutant (DKO) cells specifically lose flippase activity for PtdSer and PtdEtn,^{1,14} and they are valid as host cells for analyzing the flippase activity of type IV P-type ATPase family members localized at the plasma membrane (Figure 1). The expression of ATP11A or the ATP11A mutant (Q84E) confers the flippase activity toward NBD-PS and NBD-PE in DKO cells. In contrast, basal activity toward NBD-PC and NBD-SM is low, and only cells expressing the Q84E mutant aberrantly flip NBD-PC (Figure 1). To obtain the best signal-to-noise ratio, the NBD-lipid concentration and incubation temperature should be optimized depending on the cell type. Using mouse and human lymphoma cell lines, we typically titrate from 0.1 to 1 μ M at 15 or 20°C.

△ CRITICAL: This assay detects the unidirectional translocation of phospholipids from the outside to the inside of the plasma membrane. Accordingly, it cannot detect floppase or scramblase activity in the opposite direction on the plasma membrane or flippase activity on the intracellular membranes.

Assay for detection of PtdSer on the cell surface

⌚ Timing: 1 h

PtdSer is confined to the inner leaflet of plasma membranes under steady-state conditions, but is exposed on the cell surface in several biological processes, such as apoptosis, blood clotting, and lymphocyte activation. The externalization of PtdSer can be detected with PtdSer-binding proteins, such as Annexin V,²⁰ MFG-E8,^{21,22} and Evectin-2.²³ Annexin V, but not MFG-E8 nor Evectin-2, requires calcium at a concentration of 1–3 mM to bind to PtdSer. MFG-E8 facilitates efferocytosis of apoptotic cells in a PtdSer-dependent manner, whereas Evectin-2 plays an essential role in retrograde transport from recycling endosomes to the trans-Golgi network.²⁴ Annexin V and MFG-E8 or its PtdSer-binding (C2) domain are used to detect PtdSer and are commercially available.

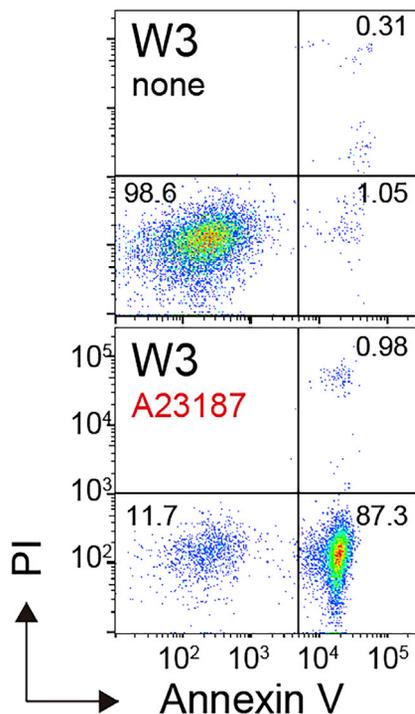


Figure 2. Asymmetrical distribution of PtdSer and its exposure on the cell surface

W3 cells, a derivative of WR19L, confine PtdSer under steady-state conditions, and these cells are not stained with Annexin V, exhibiting PtdSer asymmetry. PtdSer is externalized to the outer leaflet by a calcium ionophore stimulation, and it binds Annexin V. Percentages in the population are shown in dot plots.

16. WR19L or their transformant cells are expanded in RPMI1640-10% FCS containing 100 U/mL penicillin and 100 μ g/mL streptomycin in a 10-cm culture dish.
17. Count cells using a hemocytometer or cell counter (TC20, Bio-Rad) with trypan blue staining.
18. Transfer 1×10^6 of viable cells to a 1.5- or 2-mL tube.
19. Centrifuge at $300 \times g$ at 20°C – 25°C for 3 min.
20. Aspirate the supernatant and add 1 mL of Annexin staining buffer (10 mM HEPES-KOH, pH=7.4, 140 mM NaCl, and 2.5 mM CaCl_2).

Note: HEPES-NaOH is also available for Annexin staining buffer.

21. Resuspend cells and centrifuge at $300 \times g$ at 20°C – 25°C for 3 min.
22. Aspirate the supernatant and add 1 mL of Annexin staining buffer containing 1,000-fold diluted Annexin V-Cy5 (BioVision) and 5 μ g/mL PI.

Note: MFG-E8-FITC can be used at a final concentration of 10–50 nM instead of Annexin V.

23. Incubate at 20°C for 3 min in a dry block incubator.

Note: Alternatively, incubate at 4°C or on ice for 30 min.

24. Transfer 500 μ L of the cell suspension to a 5-mL round-bottomed test tube with a cell strainer snap cap, followed by an analysis with FACSCanto II or FACSAria III (BD) (Figure 2).

Optional: When intracellular calcium levels increase, cells expose PtdSer through the action of a calcium-dependent scramblase, TMEM16F.²⁵ As a positive control for PtdSer exposure, the remaining 500 μ L of the cell suspension in step 24 can be stimulated by the calcium ionophore, A23187, at a final concentration of 1–3 μ M. We generally incubate cells at 25 or 30°C for 5 min to induce PtdSer exposure. Most cells are not stained by Annexin V under steady-state

conditions but bound firmly to PtdSer on the cell surface upon A23187 treatment without inducing PI-positive cells (Figure 2).

Note: A23187 has a fluorescence spectrum similar to that of SYTOX Blue. Therefore, PI may be preferred to exclude dead cells in the presence of A23187.

Optional: Ionomycin, another calcium ionophore, can be used to induce PtdSer exposure instead of A23187. In this case, SYTOX Blue can be used at a final concentration of 100 nM instead of PI.

△ CRITICAL: A high concentration of A23187 or ionomycin and prolonged incubation may cause cell death. Accordingly, the concentration and incubation time must be optimized for each cell type.

Assay for the detection of SM- or cholesterol-rich domains in the outer leaflet of plasma membranes

The worm toxin, Lysenin, exclusively binds to SM-rich membrane domains and induces pore formation in the plasma membrane. Kiyokawa et al. generated a truncated Lysenin tagged with RFP that binds SM without pore formation or cell toxicity.⁹ Perfringolysin O (PFO) is a toxin secreted by *Clostridium perfringens* that binds to cholesterol in the outer leaflet of plasma membranes.²⁶ Cholesterol on the cell surface may be classified into two classes, “accessible” and “inaccessible” pools, based on its ability to bind PFO. Cholesterol associated with SM becomes an “inaccessible” form.³ D4 of PFO is sufficient for cholesterol binding without cell toxicity, and it fuses with the fluorescent protein, mCherry, to generate mCherry-D4 for monitoring the membrane properties of cholesterol.^{10,11} To increase the bacterial expression levels of NT-Lys-mRFP and mCherry-D4, we employed a cold-shock expression system using the pCold I DNA vector, which included the cold-shock protein A (*cspA*) promoter for the high-yield production of recombinant proteins in *E. coli*.²⁷

Expression of His-tagged NT-Lys-mRFP or mCherry-D4 in *E. coli*

⌚ **Timing:** ~3 days

25. Transform 50 μ L of chaperone-competent cells pGro7/BL21 with 1–10 ng of pCold I-His6-mRFP-NT-Lys or pCold I-His6-mCherry-D4 in a 1.5-mL tube.
26. Stand the tube on ice for 10–15 min.
27. Stand the tube at 42°C for 45 s in a dry block incubator.
28. Stand the tube on ice for 1–2 min.
29. Add 1 mL LB medium without antibiotics.
30. Shake the tube at 180–200 rpm at 37°C for 60 min.
31. Ten- to one hundred-microliter aliquots are plated onto an LB agar plate containing 100 μ g/mL ampicillin and 20 μ g/mL chloramphenicol.
32. Incubate the plate at 37°C for 12–16 h.
33. A colony is selected and transferred to a 50-mL tube with 10 mL LB medium containing 100 μ g/mL ampicillin and 20 μ g/mL chloramphenicol.
34. Shake the tube at 180–200 rpm at 37°C for 12–16 h.
35. Five-milliliter aliquots are transferred to a 1-L flask with 250 mL of LB medium containing 100 μ g/mL ampicillin, 20 μ g/mL chloramphenicol, and 0.8 mg/mL L-arabinose.

Note: L-arabinose may be added at step 39.

36. Grow cells under shaking at 180–200 rpm at 37°C for 3 h.
37. Measure optical density at 600 nm (OD_{600}) every 30 min.

38. When OD_{600} reaches approximately 0.5, stand the flask in an incubator at 15°C for 30 min without shaking.
39. Add IPTG at a final concentration of 0.1 mM (mRFP-NT-Lys) and 0.2 mM (mCherry-D4), respectively.

Note: If L-arabinose is not added at step 35, it may be added at a final concentration of 0.8 mg/mL at this step together with IPTG.

40. Shake the flask at 180–200 rpm at 15°C for 24 h.

Note: If expressed, the color of the culture becomes magenta by RFP or mCherry in *E. coli*.

41. Collect cells, transfer them to a 250-mL tube, and centrifuge at $2,500 \times g$ at 4°C for 10 min.
42. Remove the supernatant and wash the pellets with PBS.
43. Centrifuge at $2,500 \times g$ at 4°C for 10 min.
44. Repeat steps 42 and 43.
45. Remove the supernatant, and the bacteria pellet is snap-frozen in liquid nitrogen.
46. Store the pellet at –80°C.

▣▣ **Pause point:** Store the pellet at –80°C for up to one month.

Purification of His-tagged NT-Lys-mRFP or mCherry-D4

⌚ **Timing:** ~3 days

47. Thaw the frozen pellet and stand the tube on ice.
48. Add 10 mL of buffer A (50 mM Tris-HCl, pH=8.0, 150 mM NaCl, and 0.1 mM DTT) supplemented with a protease inhibitor cocktail (Nacalai).
49. Suspend the pellets and transfer the cell suspension to a 15-mL tube.
50. Stand the tube on ice.
51. Disrupt the cells by sonication (Branson digital Sonifer SFX 250, EMERSON) at an amplitude of 35% (3-s on and 15-s off) and repeat the on-off procedure 20–30 times.
52. Centrifuge at $12,000 \times g$ at 4°C for 20 min and transfer the supernatant to a new 50-mL tube.
53. Add 20 mL buffer A supplemented with a protease inhibitor cocktail.
54. Add 1 mL of buffer A-equilibrated Ni-NTA Agarose (QIAGEN).
55. Rotate the tube at approximately 10 rpm at 4°C for 12–16 h.
56. Centrifuge at $3,000 \times g$ at 4°C for 10 min.

Note: If NT-Lys-mRFP or mCherry-D4 binds Ni-Agarose, the precipitated agarose becomes magenta after centrifugation, and the color of the supernatant becomes clear.

57. Remove the supernatant and suspend agarose in 5 mL of buffer A.
58. Beads are loaded on a Poly-Prep Chromatography Column (Bio-Rad) at 4°C.
59. The column is sealed with a silicone stopper and connected with a three-way stock cock and tube (Figure 3).
60. Load pre-chilled PBS into the column over 12–16 h by adjusting the three-way stopcock (the total wash volume is approximately 2 L).
61. Bound proteins are sequentially eluted with 4 mL elution buffer (50 mM Tris-HCl, pH=7.5, 150 mM NaCl) by increasing imidazole in a stepwise manner from 10 to 500 mM (10, 100, 200, 300, and 500 mM). The total volume of elution buffer is 20 mL.

Note: An elution of His-tagged NT-Lys-mRFP or mCherry-D4 may be chased in real-time by the color of eluates. Typically, the recombinant proteins are eluted at 100–200 mM of

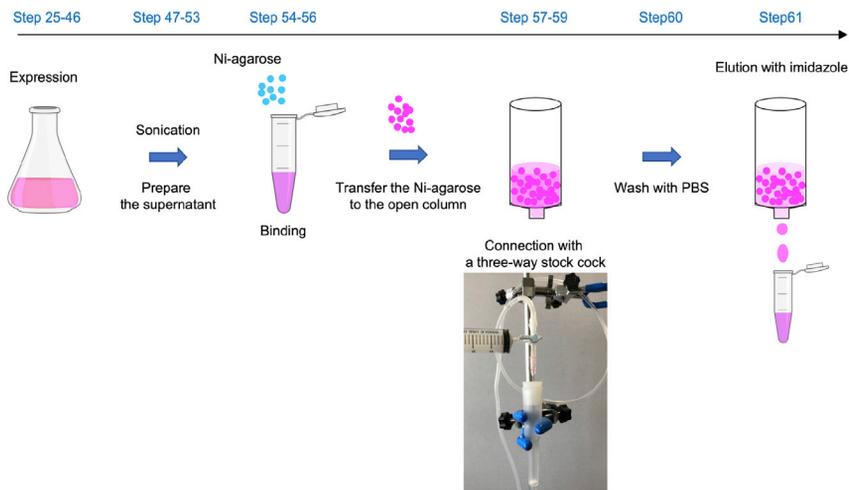


Figure 3. Schematic of the purification procedures

The purification procedure is outlined. A photograph depicts the connection of a three-way stock cock to the column at step 59.

imidazole. However, we recommend analyzing all fractions by SDS-PAGE and then pooling appropriate fractions.

62. Eluates at appropriate fractions are mixed and loaded to pre-equilibrated Amicon Ultra -15 Centrifugal Filters (10 K cut-off, MERCK).
63. Centrifuge at $4,000 \times g$ at 4°C for 1–2 h to concentrate the solutions.

Note: We generally concentrate until the volume is less than 1 mL.

64. Transfer concentrated eluates to a micro dialyzer (Xpress Micro Dialyzer MD300 12–14 kDa, Scienova) and dialyze against 2 L of PBS at 4°C with stirring for 12–16 h.
65. Change PBS to another 2 L of PBS and dialyze with stirring at 4°C .
66. Purified proteins are aliquoted, snap-frozen in liquid nitrogen, and stored at -80°C .

▣ **Pause point:** Store recombinant proteins at -80°C for at least one year.

Measurement of NT-Lys-mRFP or mCherry-D4 concentrations

⌚ **Timing:** 1 day

67. Purified proteins and BSA proteins in a series of concentrations (1 μg - 31.25 ng) as a standard are separated with SDS-PAGE using a 5%–15% gradient gel (Extra PAGE One, Nacalai).
68. The gel is stained by Coomassie Brilliant Blue (CBB) (PAGEBlue Protein Staining Solution, Thermo Fisher Scientific) and analyzed with the ChemiDoc MP Imaging system and Image Lab software (Bio-Rad) (Figure 4).

Note: CBB signals are quantified by infrared fluorescence with the ChemiDoc MP imaging system.

Analysis of SM- and cholesterol-rich domains in the outer leaflet of plasma membranes using NT-Lys-mRFP and mCherry-D4, respectively

⌚ **Timing:** 1 h

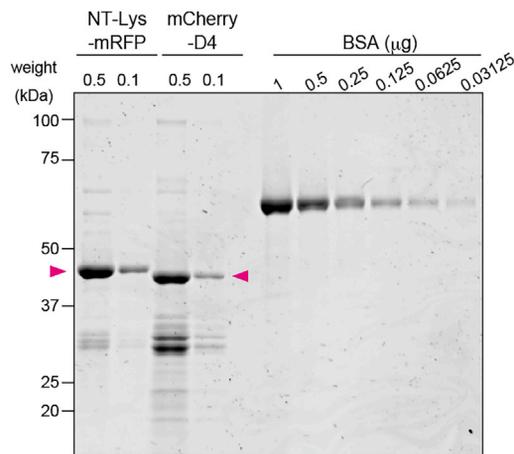


Figure 4. Purified NT-Lys-mRFP and mCherry-D4 proteins

Purified proteins (μL) were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue staining. The red arrows indicate NT-Lys-mRFP and mCherry-D4. BSA: Bovine serum albumin. The expected molecular weights of His-NT-Lys-mRFP and His-mCherry-D4 are approximately 45,000 and 41,000, respectively.

69. WR19L or their transformant cells are expanded in RPMI1640-10% FCS containing 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin in a 10-cm culture dish.
70. Cells (2×10^5) are transferred to a 1.5-mL tube and centrifuged at $300 \times g$ at 20°C – 25°C for 3 min.
71. Remove the supernatant and add 1 mL of PBS.
72. Centrifuge at $300 \times g$ at 20°C – 25°C for 3 min.
73. Add 300 μL of pre-chilled HBSS containing 10 $\mu\text{g}/\text{mL}$ NT-Lys-mRFP or mCherry-D4.
74. Resuspend cells and incubate on ice for 30 min.
75. Add SYTOX Blue at a final concentration of 100 nM.
76. Transfer 300 μL of the cell suspension to a 5-mL round-bottomed test tube with a cell strainer snap cap, followed by an analysis with FACSCanto II or FACS Aria III (BD) (Figure 5).

Note: The NT-Lys-mRFP or mCherry-D4 concentration needs to be optimized for the respective cell types within 0.5–20 $\mu\text{g}/\text{mL}$.

Note: Washing procedures after staining in step 73 may reduce the fluorescence signals of NT-Lys-mRFP or mCherry-D4 on the cell surface.

Note: Cells at step 76 can be observed using confocal microscopy with a glass-bottomed dish.

EXPECTED OUTCOMES

The asymmetrical distribution of PtdSer in the plasma membrane can be monitored using a PtdSer-binding protein such as Annexin V. Using mouse or human lymphocyte cell lines, the Annexin V binding to the cell surface differs approximately 10- to 20-fold between cells at steady-state conditions and those activated by a calcium ionophore (Figure 2). Flippase activity serves to maintain the PtdSer asymmetry. The *Atp11a-Atp11c* double-null (DKO) cells with low flippase activities may be suitable to assess the flippase activity of members in the type IV P-type ATPase family. The NBD-PS translocation by ATP11A or ATP11C reaches a plateau within 10–15 min, whereas NBD-PE signals increase for at least 30 min in WR19L cells.²⁸ SM and cholesterol embedded in the exofacial leaflet of the plasma membrane can be monitored by NT-Lys and D4, respectively. In this protocol, we generally obtain 2–4 mg of NT-Lys-mRFP or mCherry-D4 proteins from 250 mL of the bacterial culture. SM sequesters cholesterol and makes it unable to bind to D4.³ As shown in Figure 5, the expression of the ATP11A mutant (Q84E) flipping PtdCho alters the lipid organization in the outer leaflet of plasma membranes of DKO cells. The staining intensity of NT-Lys is approximately seven-fold higher in the mutant (Q84E)-expressing cells than in the parental cells, indicating that the mutant cells carry a large number of the SM-rich domains. Accordingly, the “inaccessible” form of cholesterol is

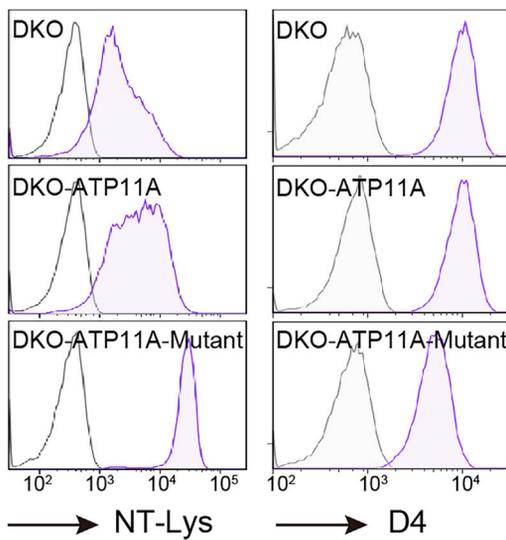


Figure 5. Analysis of SM- or cholesterol-rich domains in the outer leaflet of plasma membranes

Atp11a-Atp11c double-null (DKO) WR19L-derived cells and their transformants expressing wild-type human ATP11A or its mutants (Q84E) were stained with 10 $\mu\text{g}/\text{mL}$ NT-Lys-mRFP (left) or mCherry-D4 (right). Representative histograms of non-stained (grey) and stained (purple) cells are shown. The ATP11A Q84E mutant aberrantly flips PC in the plasma membranes, and the PC content in the outer leaflet decreases. In accord with the decrease of PC, Q84E-expressing cells carry high SM-rich domains on the cell surface, staining intensely with NT-Lys, while it decreases the “accessible” pool of cholesterol, impairing D4 staining. Figure 5 is adapted from the data in¹ with modifications.

increased, leading to a reduction of the D4 binding to the outer leaflet of plasma membranes in the Q84E-mutant cells.

LIMITATIONS

The protocol shown here is based on floating cells *in vitro* and, as such, requires optimization for adherent cells. Staining and activity measurements with NBD-lipids are performed at temperatures below 20°C or 4°C to minimize endocytosis or membrane trafficking systems; therefore, imaging or activity measurements at a physiological temperature of 37°C are not suitable. This may be achieved with different probes, such as TopFluor-lipids (Avanti).¹⁶ Nevertheless, these fluorescence-conjugated analogs are not identical to endogenous phospholipids. Accordingly, the distribution of endogenous phospholipids should be followed by lipid-binding proteins.

TROUBLESHOOTING

Problem 1

Efficacy of BSA-mediated extraction is low (step 12).

Potential solution

The BSA concentration may increase up to 50 mg/mL, or the incubation time may be prolonged to 3 min when the extraction efficacy is low.

Problem 2

Inability to reproduce previous flippase activity; alternatively, flippase activity is lower than that previously obtained (step 15).

Potential solution

The pre-culture conditions of cells for the assay affect or impair flippase activity on the cell surface. We routinely set a range of cell concentrations below 2×10^6 cells/mL in the pre-culture for the assay. Otherwise, NBD-lipids may be degraded during storage for an extended period or in repeated freeze-thaw cycles. If necessary, fresh aliquots are used for the assay.

Problem 3

Inability to detect PtdSer exposure by treatment of a calcium ionophore, A23187 (step 24, Optional).

Potential solution

The A23187 concentration and the incubation temperature may be increased up to 10 μ M and 37°C, respectively. Adherent cells may require a high concentration of A23187 (5–10 μ M) to externalize PtdSer on the cell surface.

Problem 4

Degradation of recombinant proteins during the expression and purification procedures (step 68).

Potential solution

At step 40, shaking time and temperature to produce recombinant proteins in *E. coli* may be shortened and decreased, respectively.

Problem 5

The staining concentration of NT-Lys-RFP or mCherry-D4 for different cell types remains unclear (step 73).

Potential solution

SM contents on the cell surface affect the staining profiles of NT-Lys-RFP and mCherry-D4. Cells with different SM levels, such as the ATP11A-mutant expressing cells, may be a control for staining (Figure 5). Sphingomyelin synthase 1 (Sgms1) is a major SM synthase localized at the Golgi that transfers the phosphocholine moiety to ceramide, generating SM.²⁹ As another control for staining, we establish *Sgms1*-deficient WR19L-derived mutant cells by CRISPR-Cas9 technology and select an optimal concentration of NT-Lys-mRFP for staining. In our cases, a high staining concentration (>20 μ g/mL) of NT-Lys-mRFP results in an increase in background signals.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Katsumori Segawa (segawa.mche@tmd.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

A previously published article partly includes the data shown in this study.¹

Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, K.S.; Methodology, Y.M., K.S.; Investigation, Y.M., K.S.; Writing, Y.M., K.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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