Chapter 19

In Vitro Strategy to Measure Sterol/Phosphatidylinositol-4-Phosphate Exchange Between Membranes

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Abstract

Recent findings unveiled that Oxysterol-binding protein-related proteins (ORP)/Oxysterol-binding homology (Osh) proteins, which constitute a major family of lipid transfer proteins (LTPs), conserved among eukaryotes, are not all mere sterol transporters or sensors. Indeed, some of them are able to exchange sterol for phosphatidylinositol-4-phosphate (PI4P) or phosphatidylserine (PS) for PI4P between membranes and thereby to use PI4P metabolism to generate sterol or PS gradients in the cell, respectively. Here, we describe a full strategy to measure in vitro a sterol/PI4P exchange process between artificial membranes using Förster resonance energy transfer (FRET)-based assays and a standard spectrofluorometer. Such an approach can serve to better characterize the activity of known sterol/PI4P exchangers, but also to reveal whether ill-defined ORP/Osh proteins or LTPs belonging to other families have such an exchange activity. Besides, this protocol is amenable to test whether molecules can act as Orphilins, which have been found to inhibit the sterol/PI4P exchange activity of certain ORPs. Last, our strategy to measure in real-time PI4P transport using a known lipid-binding domain can serve as a basis for the design of novel in vitro protocols aiming to detect other lipid species.

Key words ORP/Osh proteins, Sterol, Dehydroergosterol, PI4P, FRET, Liposome, Recombinant protein

1 Introduction

Lipids are precisely distributed within and between the membranes of eukaryotic cells [1, 2]. Such a distribution relies on lipid metabolism as well as lipid transport processes that are mostly mediated by cytosolic proteins called LTPs. These proteins belong to various families but share a common feature: they have a domain with a lipid-pocket, generally to host only one lipid molecule at a time. Decrypting the mode of action of these LTPs is an important issue in cell biology [3–5]. It is commonly assumed that a LTP is able to extract a lipid from a donor organelle membrane, shield this highly hydrophobic molecule from the aqueous medium, diffuse through the cytosol, and deposit this lipid in a target organelle

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[6–9]. These LTPs work either across long distances or confined in membrane contact sites where two organelles are in close apposition. In vitro approaches are invaluable to decipher at the molecular level how these LTPs work (e.g., some recent references [10–14] and several chapters in this book). Recently, we devised protocols to establish that LTPs, Osh4p, and OSBP (Oxysterol-binding protein), belonging to the ORP/Osh family, have the ability to bind alternately PI4P and sterol as well as to exchange these lipids between membranes [15]. We demonstrated with OSBP that a sterol/PI4P exchanger can use PI4P metabolism to transport vectorially sterol from the endoplasmic reticulum (ER) to the Golgi [16]. In vitro, we showed that Osh4p is able to dissipate a preexisting PI4P gradient to generate in return a sterol gradient between two distinct membranes [17]. This mechanism of counterexchange fueled by PI4P metabolism is used by other ORP/Osh proteins to transport a lipid that is not sterol. Indeed, Osh6p/Osh7p in yeast and ORP5/ORP8 in humans use a PI4P gradient to transport PS from the ER to the plasma membrane [18, 19]. Along with this, it has been revealed that the sterol/PI4P exchange activity of OSBP has a pivotal role in the replication of many human viruses, including Hepatitis C virus [20] or in cancer [21]. Consequently, OSBP appears as a potential pharmaceutical target and small molecules called Orphilins have been found to inhibit its exchange activity [21–23]. In that respect, the availability of our in vitro approach to measure a sterol/PI4P exchange activity with high temporal resolution proved to be useful to characterize some of these compounds [22, 23]. Herein, we describe in detail how to set up this approach and kinetic measurements which simply require a standard L-format spectrofluorometer.

Our strategy combines two FRET-based assays to accurately measure (1) sterol transport from L_A to L_B liposomes, which are present in an equimolar amount and mimic each an organelle membrane (ER and *trans*-Golgi, respectively, in reference to our studies on Osh4p and OSBP [16, 17]) and (2) PI4P transport in the opposite direction. It is noteworthy that these two experiments are performed under the same conditions (i.e., identical buffer, temperature, total lipid concentration, sterol, and PI4P concentration) such as to observe whether these two transport processes are coupled, and thus the occurrence of sterol/PI4P exchange (Fig. 1) [17]. In the first assay (Fig. 1a) we use $L_{A\text{-}1}$ and $L_{B\text{-}1}$ liposomes that are essentially made of phosphatidylcholine (*see* **Note 1**). $L_{A\text{-}1}$ liposomes contain specifically both dehydroergosterol (DHE, 5 mol%), a natural fluorescent analogue of ergosterol and cholesterol (major sterol in yeast and human, respectively) and a second fluorescent lipid called 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(5-dimethylamino-1-naphthalenesulfonyl) (DNS-PE, 2.5 mol%). DHE molecules excited at 310 nm transfer resonance energy to the dansyl group of DNS-PE [24]. The level of FRET

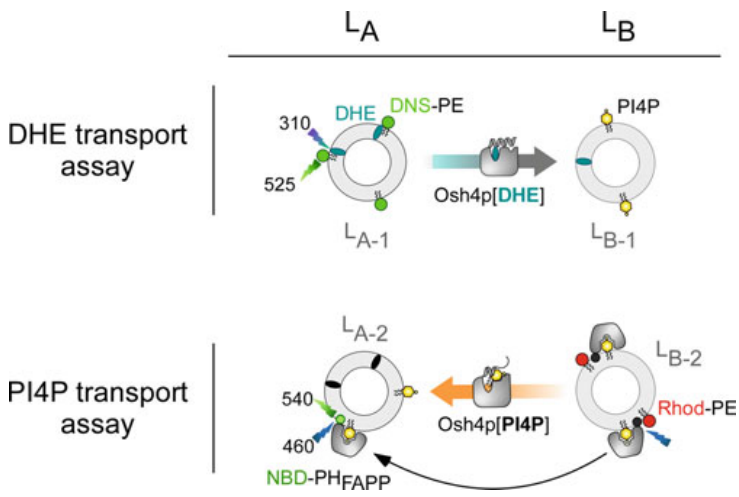

Fig. 1 Description of the two transport assays. In the DHE transport assay, L_{A-1} liposomes (DOPC/DHE/DNS-PE, 92.5/5/2.5 mol/mol) are mixed with L_{B-1} liposomes (DOPC/PI4P 96/4 mol/mol). FRET between DHE and DNS-PE in the L_{A-1} liposomes diminishes if DHE is transported to the L_{B-1} liposomes. In the PI4P transport assay, DOPC/PI4P/Rhod-PE liposomes (94/4/2 mol/mol, L_{B-2}) are incubated with 250 nM NBD-PH_{FAPP}. DOPC liposomes (L_{A-2}) doped with 5 mol % DHE are added. If PI4P transport occurs, this provokes a dequenching of the NBD signal corresponding to the translocation of NBD-PH_{FAPP} from L_{B-2} to L_{A-2} liposomes

depends on the amount of DHE in the L_{A-1} membranes. If DHE is transferred from L_{A-1} to L_{B-1} liposomes, this elicits a decrease in the FRET signal because DHE is not anymore in close proximity to DNS-PE. The normalization of this signal allows quantifying the transport of DHE from L_{A-1} to L_{B-1} liposomes. Using this assay, Osh4p was found to efficiently transport DHE and this, in a proportional manner to the amount of PI4P initially present in the L_{B-1} liposomes [17]. Here, we describe a protocol in which L_{B-1} liposomes incorporate 4 mol% PI4P.

To measure the transport of PI4P in the reverse direction, from L_B to L_A liposomes, we designed a PI4P-probe, derived from the Pleckstrin Homology (PH) domain of the human phosphoinositol-4-phosphate adapter protein (FAPP-1, Uniprot: Q9HB20). This domain has a binding site for the PI4P headgroup and a hydrophobic wedge that inserts into the membrane [25]. We reengineered this domain to include a single solvent-exposed cysteine (C13), localized in the hydrophobic wedge, to which is covalently linked a polarity-sensitive NBD (7-nitrobenz-2-oxa-1,3-diazol) fluorophore [17]. Its ability to recognize PI4P at the liposome surface is unaffected by the level of sterol [17]. In the transport assay (Fig. 1b), NBD-PH_{FAPP} is mixed with L_{B-2} liposomes, which contain 4 mol% PI4P and 2 mol% Rhod-PE, and L_{A-2} liposomes, which incorporate 5 mol% DHE. At time zero, the NBD fluorescence is quenched due to a FRET process with Rhod-PE. If PI4P is

transported from $L_{B\text{-}2}$ to $L_{A\text{-}2}$ liposomes (e.g., upon injecting Osh4p), a fast dequenching occurs, due to the translocation of NBD-PH_{FAPP} onto $L_{A\text{-}2}$ liposomes (Fig. 1b). In our assay, NBD-PH_{FAPP} is completely bound to the membranes which contain an accessible amount of PI4P between 2 and 4 μM [17]. As a consequence, the NBD signal directly reflects the distribution of NBD-PH_{FAPP} between L_A and L_B liposomes and can thus be easily normalized and converted into an amount of transported PI4P. This tracking method, albeit indirect, has at least two advantages. First it does not rely on a fluorescently labeled PI4P that is unlikely to be properly accommodated by the binding pocket of ORP/Osh proteins, as it bears an extra bulky moiety. Second, this approach offers a far higher time resolution than methods based on radiolabeled PI4P and liposome separation. In addition, such a PI4P is not commercially available and remains uneasy to produce. Along with this, we have indications that the ability of a protein to transport PI4P is not impacted by the presence of NBD-PH_{FAPP}. Given the amount of NBD-PH_{FAPP} and of accessible PI4P in our assays, only 6.25% of PI4P can be monopolized by the probe during the measurement. In addition, only 0.9% of the membrane surface is covered by the probe, considering the maximal membrane surface that one PH molecule can cover (≈4.8 nm^2, estimated from references [26, 27]) and the total surface of liposomes (L_A + L_B liposomes; 5.1 × 10^{16} nm^2 with an area of 0.7 nm^2 per lipid). Experimentally, we observed that doubling or dividing by two the amount of NBD-PH_{FAPP} in the sample did not change the transport kinetics that we recorded (unpublished results).

Our strategy, which is detailed step by step below, allows for measuring the transport of sterol along its concentration gradient between two membranes by Osh4p via the dissipation of a preexisting PI4P gradient. It can be modified to measure the transport of sterol against its concentration gradient simply by changing the initial amount of DHE in the L_A and L_B liposomes as done in [17]. Our strategy is also potent to further characterize known sterol/PI4P exchangers via structure–function analysis or to screen novel drugs for their ability to block an exchange activity. Likewise, it can help to define in the future whether other members of the ORP/Osh family are sterol/PI4P exchangers. Besides, it can serve to explore the ability of LTPs, belonging to other families, to transport sterol and/or PI4P, as done recently with START-like proteins [13]. NBD-PH_{FAPP} has been used to follow the transport of phosphatidylinositol-4,5-bisphosphate (PIP2) between membranes, as it can recognize this phosphoinositide [13, 28]. We also developed a NBD-$C2_{Lact}$ domain to specifically follow PS transport between liposomes and thereby unveiled the capacity of Osh6p and Osh7p to serve as PS/PI4P exchangers [19]. This exemplifies that our FRET-based strategy can be adapted to measure in vitro other transport processes by using lipid-binding

domains whose tridimensional structure and lipid-binding site are known.

2 Materials

2.1 Protein Purification and Labeling

1. pGEX-PH_{FAPP} and pGEX-Osh4p plasmids (available on request from our lab).
2. Sterilized water.
3. Electro-competent bacteria: BL21 Gold Competent Cells (Agilent).
4. Ampicillin: Prepare a 50 mg/mL stock solution with filtered and sterilized water and store it at −20 °C.
5. LB medium: Lennox LB Broth medium without glucose (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) prepared with deionized water and autoclaved.
6. LB/Ampicillin: Dilute ampicillin stock solution to 1/1000 in LB medium (50 μg/mL final concentration).
7. 1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG) stock solution in water.
8. 1 M Dithiothreitol (DTT) stock solution in water. Prepare 1 mL aliquots and store them at −20 °C.
9. PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM NaH_2PO_4, 1.8 mM KH_2PO_4, autoclaved and stored at 4 °C.
10. TN buffer: 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, filtered with a Stericup filtration unit (Merck) or with a membrane vacuum filtration equipment. Store at 4 °C.
11. TND buffer: Dilute 1 M DTT solution to 1/500 in TN buffer (final concentration 2 mM DTT).
12. 200 mM PMSF (Phenylmethylsulfonyl fluoride) stock solution in isopropanol. Store at 4 °C.
13. Lysis buffer. To prepare 50 mL, dissolve one tablet of complete EDTA-free protease inhibitor cocktail (Roche) in TN buffer by vortexing (*see* **Note 2**). Add 250 μL of 200 mM PMSF stock solution. Complete with other antiproteases, i.e., 50 μL of 1 mM bestatin stock solution, 50 μL of 1 mg/mL pepstatin A in MeOH, and 50 μL of 1 mM phosphoramidon solution (these solutions are stored at −20 °C). Add 100 μL of 1 M DTT stock solution to obtain a final concentration at 2 mM.
14. DNAse I Recombinant, RNAse free, in powder.
15. 2 M $MgCl_2$ solution. Filter the solution using a 0.45 μm filter.

16. Thrombin stock solution: Dissolve 20 units in 1 mL of double-deionized water and prepare 25 µL aliquots in 0.5 mL Eppendorf tubes. Then freeze and store at −80 °C.
17. 10 mM $CaCl_2$ stock solution in water.
18. Dimethylformamide (DMF), anhydrous, >99% pure.
19. IANBD Amide (*N*,*N*′-Dimethyl-*N*-(Iodoacetyl)-*N*′-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)Ethylenediamine). Dissolve 25 mg of IANBD in 2.5 mL of dimethylsulfoxide (DMSO) and prepare 25 aliquots of 100 µL in 1.5 mL screw cap tubes. Do not completely screw the cap. Then, remove DMSO in a freeze-dryer to obtain 1 mg of dry IANBD per tube. Tubes are closed and stored at −20 °C in the dark.
20. 10 mM L-cysteine solution in degassed water stored at −20 °C.
21. Glycerol (99% pure).
22. 50 mL polypropylene tube (e.g., Falcon).
23. Eppendorf tubes (0.5, 1 and 2 mL).
24. Electroporator (e.g., Eppendorf 2510) and electroporation cuvette (e.g., Cell Projects, 50 × 2 mm gap).
25. Glutathione Sepharose 4B beads stored in 20% (v/v) ethanol at 4 °C.
26. French pressure cell press (French press).
27. Fixed-angle rotor and tubes for ultracentrifuge (e.g., Ti45, Beckman).
28. Ultracentrifuge (e.g., Beckman).
29. Amicon Ultra-4 and Ultra-15 centrifugal filter units, each with a molecular weight cut-off (MWCO) of 3 and 10 kDa (Millipore).
30. UV/Visible absorbance spectrometer with quartz cuvettes.
31. Poly-Prep® chromatography column (with a 0–2 mL bed volume and a 10 mL reservoir).
32. Econo-Pac® chromatography columns (1.5 × 12 cm).
33. Illustra NAP 10 desalting column (GE healthcare).
34. Thermomixer.
35. XK 16/70 column packed with Sephacryl S200HR for FPLC system (GE healthcare).
36. FPLC system: ÄKTA purifier (GE healthcare) or similar.
37. TG-SDS running buffer: 25 mM Tris, pH 8.6, 192 mM glycine, 0.1% SDS.
38. 4× highly denaturating Laemmli buffer: 62.5 mM Tris–HCl, pH 6.8, 10% (v/v) glycerol, 8% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.005% (w/v) bromophenol blue.

39. Precast 15% acrylamide gel SDS-PAGE.
40. SDS-PAGE standards, low range (Biorad).

2.2 Liposome Preparation and Fluorescent Assays

1. Chloroform ($CHCl_3$) and methanol (MeOH).
2. Phospholipid stock solutions (usually in $CHCl_3$) are aliquoted in 2 mL amber glass vials filled with argon and tightly sealed with a Teflon cap. Vials are stored at −20 °C. DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine, 25 mg/mL in $CHCl_3$), brain PI4P (L-α-phosphatidylinositol-4-phosphate, 5 mg/mL in $CHCl_3$/MeOH/H_2O (20:9:1)) and DNS-PE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(5-dimethylamino-1-naphthalenesulfonyl), 1 mg/mL) and Rhod-PE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl), 1 mg/mL) are from Avanti Polar Lipids.
3. DHE stock solution: Dissolve 5 mg of DHE powder (Ergosta-5,7,9(11),22-tetraen-3β-ol E2634, purity ~96%, Sigma-Aldrich) in 1 mL of MeOH. Dilute 10–20 μL of this solution to 1/30 in MeOH and record an absorbance spectrum of this solution against a blank using two quartz cells (e.g., Hellma 104-002B-QS with a chamber of 2 mm in width and 1 cm optical path) and a standard double beam UV/visible spectrometer. Determine the DHE concentration by measuring the DHE absorbance peak at 325 nm, considering a molar extinction coefficient of 13,000 M^{-1} cm^{-1}. If necessary, adjust the volume of the stock solution with MeOH to set the DHE concentration at 2.5 mM. Store 500 μL aliquots in 2 mL glass vials at −20 °C, as done with phospholipids.
4. C16:0/C16:0-PI4P stock solution: Dissolve 1 mg of C16:0/C16:0-PI4P powder (Echelon Lipids) in 250 μL of MeOH and 250 μL of $CHCl_3$. Then complete with methanol to 1 mL. The solution must become clear.
5. HK buffer: 50 mM HEPES-KOH, pH 7.4, 120 mM K-Acetate. Dissolve 23.8 g of HEPES and 23.6 g of K-Acetate in 2 L of deionized water at room temperature. Adjust the pH to 7.4. Prepare frozen aliquots of 50 mL.
6. HKM buffer: Add 25 μL of a 2 M $MgCl_2$ stock solution to 50 mL of HK buffer.
7. Rotary evaporator (Buchi B-100 or similar).
8. Pear-shaped glass flasks (25 mL, 14/23, Duran).
9. Aluminum foil.
10. 4 mm-diameter glass beads (Sigma-Aldrich).
11. Avanti Polar Mini-Extruder with two 1 mL gas-tight Hamilton syringes.

12. Prefilters (10 mm in diameter, Avanti Polar Lipids).
13. Polycarbonate filters (19 mm in diameter, Avanti Polar Lipids) with pore size of 0.2 μm.
14. Hemolysis tubes with a cap.
15. Water bath.
16. Liquid nitrogen.
17. UV/visible spectrofluorometer with a temperature-controlled cell holder and stirring device.
18. Quartz cuvette (e.g., Hellma) for UV/visible fluorescence (minimum volume of 600 μL).
19. Small magnetic PFTE stirring bar (5 × 2 mm).
20. Hamilton syringes (10, 25, and 50 μL).

3 Methods

3.1 Purification and Fluorescent Labeling of NBD-PH_{FAPP}

The PH domain of the FAPP-1 protein was mutated to introduce a unique cysteine within its membrane-binding interface (at position 13 of the protein) and label it with a NBD fluorophore. PH_{FAPP} (T13C/C45S/C57S/C94S) construct is cloned into a pGEX-4T3 vector (GE healthcare) to be expressed in fusion to GST. A thrombin cleavage site is located between the GST construct and the N-terminal end of the PH domain. We describe here a purification protocol based on the use of a French press and a final gel filtration step using an ÄKTA FPLC system. This protocol can be modified to include other methods for disrupting bacteria and other FPLC chromatography devices. It is important to use very freshly degassed and filtered lysis buffer supplemented with 2 mM DTT to avoid any oxidation of cysteine. In contrast, to label the protein, it is mandatory to perfectly remove DTT using a desalting column and a freshly degassed DTT-free buffer. All the steps must be done on ice at 4 °C, to avoid any degradation of the protein.

3.1.1 Expression of PH_{FAPP} in E. coli

1. Mix 2 μL of pGEX-PH_{FAPP} plasmid (at ~65 ng/μL) with 20 μL of electro-competent bacteria and 18 μL of sterilized water. Transform the bacteria by electroporation with a 1500 V pulse for 5 ms. Then, resuspend the bacteria with 150 μL of LB medium and let them recover for 1 h at 37 °C.
2. Dilute the 150 μL culture into 25 mL of LB/Amp medium in a 200 mL sterilized Erlenmeyer flask.
3. Incubate the flask in a shaker at 37 °C at 185 rpm overnight. The preculture can be used the next day or stored one day long at 4 °C before induction procedure.

4. Inoculate 4 × 500 mL of LB/Amp medium with 5 mL of preculture suspension and let bacteria growth at 37 °C under agitation at 220 rpm in 2 L Erlenmeyer flasks.
5. Once the optical density of the suspension, measured at a wavelength (λ) of 600 nm, reaches a value between 0.6 and 0.7, add 500 µL of 1 M IPTG stock solution in each flask to induce protein expression. Maintain the flasks for 4 h at 37 °C under constant shaking at 185 rpm.
6. To harvest the bacteria, fill four polypropylene centrifuge bottles each with 500 mL of culture and centrifuge for 30 min at 3000 × *g* at 4 °C.
7. Remove the supernatant and resuspend each pellet with 25 mL of cold PBS buffer. Then, pool the bacterial suspension and fill two 50 mL falcon tubes with the suspension.
8. Centrifuge the tubes for 30 min at 3500 × *g* at 4 °C. Discard the supernatant, let drain the residual buffer upon absorbent paper, and store the pellets at −20 °C (*see* **Note 3**).

3.1.2 Purification and Labeling of PH_{FAPP}

To check the proper progress of the purification, it is requested to collect 30 µL aliquots at different steps of the protocol to perform an analysis on a 15% SDS-PAGE gel (Fig. 2). Add to each aliquot 30 µL of 4× highly denaturating Laemmli buffer, vortex, spin down, and heat the aliquot at 95 °C in a dry bath heater. Store the tubes at −20 °C until analysis. Generally, we run a first gel to check the first part of the purification process prior to the gel filtration (Fig. 2a). At **step 28**, for the SDS-PAGE analysis of the protein content of the collected fractions after gel filtration (Fig. 2b, c), we mix 25 µL aliquot of each interesting fraction with 15 µL of Laemmli buffer prior to heating and loading onto a gel.

1. On ice, prepare two 50 mL falcon tubes that each contain 50 mL of lysis buffer.
2. Add up to 30 mL of lysis buffer to the two tubes, prepared at **step 8** of Subheading 3.1.1, which contain a bacterial pellet. Let defreeze the pellets on ice for 5–10 min. Crush the pellets with a stainless spatula, vortex, and resuspend well by pipetting back and forth the suspension with a 25 mL pipette until it is homogeneous.
3. Lysis is performed using a prechilled French press. Load 30 mL of sample inside the press cell and apply a pressure to reach 1000 psi. Collect the lysate in the same tube, keep the tube on ice, and continue this first round of lysis with the second sample.
4. Repeat **step 3** one more time to obtain a smoothed lysate (*see* **Note 4**).

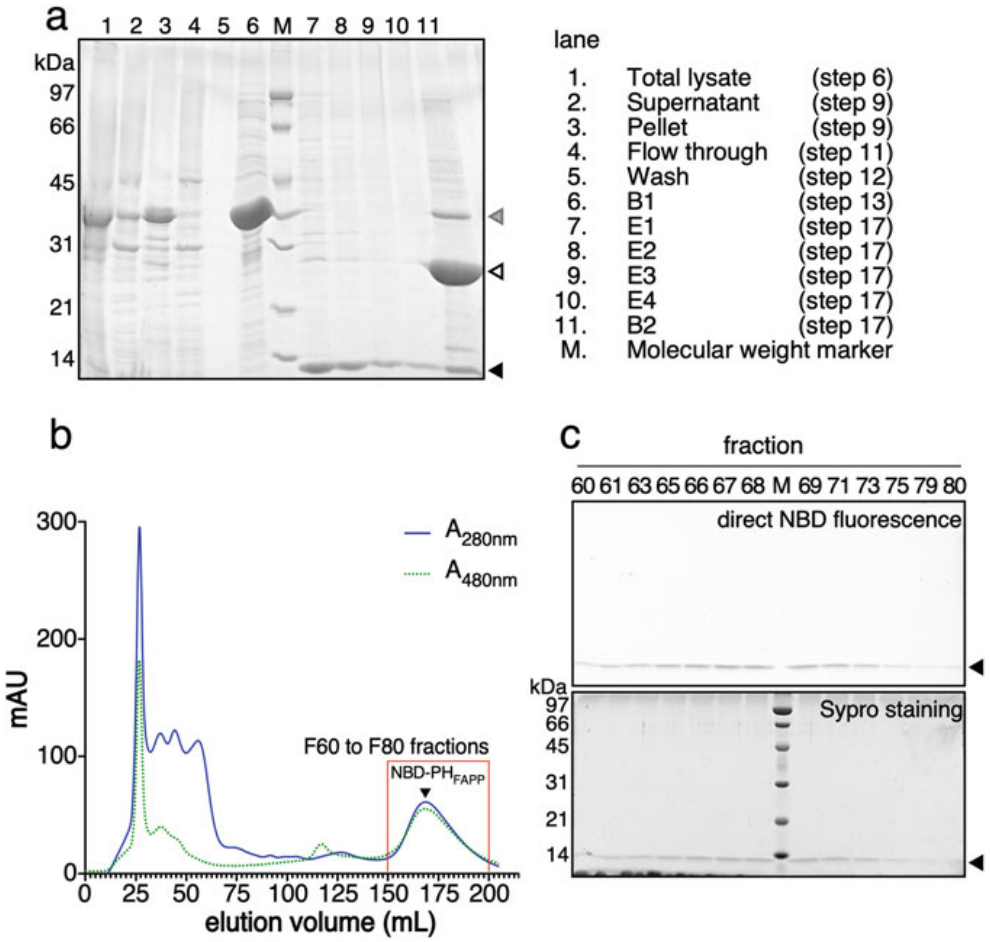

Fig. 2 NBD-PH_{FAPP} purification (**a**) SDS-PAGE analysis is used to check the first part of the purification protocol before labeling. It is used to verify the presence of the protein at different steps of the procedure. The arrowheads indicate the position of the PH_{FAPP} domain (black arrowhead), of the GST alone (open arrowhead) and of the GST-PH construct (gray arrowhead). An equivalent of 2.5 μL of each aliquot is loaded onto the gel (**b**) Gel filtration step. The chromatogram shows the evolution of the tryptophan and NBD absorbances monitored, respectively, at a wavelength of 280 and 480 nm as a function of the volume of buffer passed on the column. The peak corresponding to the protein (after 150–200 mL of elution) and the 2.5 mL fractions that are collected are framed in red. (**c**) SDS-PAGE analysis of collected fractions. The first picture is acquired under UV illumination without staining and reveals the presence of the NBD-PH_{FAPP} construct since it emits fluorescence. The second picture shows the same gel after a Sypro staining procedure (Note that the gel was protected from light during migration to avoid any bleaching of the NBD fluorophore)

5. Use the remaining 40 mL of lysis buffer to rinse the French press cell immediately after lysate collection and to adjust each volume of lysate (~30 mL) to a final volume of 50 mL.
6. Add 125 μL of 2 M $MgCl_2$ solution (final concentration 2 mM) and a pinch of DNAse I (using a spatula) to each 50 mL lysate to cut DNA in small fragments, thereby limiting the viscosity of the sample. Incubate on ice for 30 min. Take an aliquot for gel analysis.

7. Transfer each 50 mL lysate in a precooled polycarbonate ultracentrifuge tube (two in total) and run a centrifugation for 1 h at 186,000 × *g* at 4 °C with an appropriate rotor and ultracentrifuge.
8. During centrifugation, dispense 1.4 mL of Glutathione Sepharose 4B bead slurry into two 50 mL tubes. Wash the beads twice by centrifugation at 500 × *g* for 5 min with 20 mL of TND buffer.
9. Once the centrifugation is over, take an 30 μL aliquot from the supernatant and transfer the supernatant from each ultracentrifuge tube in the two 50 mL tubes containing washed beads. For gel analysis, resuspend the debris pellet in one of the ultracentrifuge tube with 50 mL of TND buffer and collect a 30 μL aliquot.
10. Place the tubes on a rotator to constantly homogenize the solution and the beads for 3–4 h at 4 °C.
11. Pool the bead suspensions in a unique Econo-Pac® chromatography column. Let decant the beads by gravity flow to remove buffer and unbound proteins. Take an aliquot from the eluate for analysis.
12. Wash the beads by resuspending them with 20 mL of TND buffer followed by decantation. Repeat this step twice. Collect and pool the eluates and take an aliquot for analysis. After decantation, a volume of ~2 mL of a suspension of bead, to which GST-PH_{FAPP} is bound, lies at the bottom of the column.
13. Fill two 2 mL snap-cap microcentrifuge tubes each with 1 mL of bead suspension. Complete each tube with TND buffer up to 1.970 mL. Take a 30 μL aliquot from one tube for analysis (aliquot B1). Add 10 μL of 10 mM $CaCl_2$ solution and 25 μL of human thrombin protease to each tube for cleaving the PH_{FAPP} construct off the GST domain.
14. Close and place the tubes overnight on a rotator at 4 °C for an optimal cleavage.
15. Add 10 μL of 200 mM PMSF solution to the 2 mL bead suspension for irreversibly stopping the thrombin activity.
16. Centrifuge the tubes at 700 × *g* for 3 min 30 s. Collect out of each tube up to 1 mL of supernatant, which contains soluble PH_{FAPP} domain, pool, and transfer it in 2 mL snap-cap microcentrifuge tube (E1 eluate) which is maintained on ice. Avoid pipetting beads during this step.
17. Resuspend the beads with 1 mL of TN buffer to wash them and repeat three times **step 16** to recover most of the protein. Each time, pool and transfer the supernatants into a new 2 mL tube (E2, E3, and E4 eluates). For SDS-PAGE analysis take an

aliquot from each eluate and from the bead suspension (aliquot B2) at the end of the wash.

18. Pool the supernatants (i.e., the content of 4 tubes, ~8 mL) collected during **steps 16** and **17** into a 10 mL Poly-Prep® chromatography column. Retrieve by gravity flow the eluate that is free of any contaminating beads as those latter are retained on the bed support.
19. Concentrate the solution to ~1 mL in an Ultra-4 centrifugal filter unit with a MWCO of 3 kDa using a centrifugation speed of 1500 × *g*.
20. Equilibrate an Illustra NAP 10 desalting column with TN buffer. Load 1 mL of the PH_{FAPP} concentrated sample inside the column and let it fully penetrate into the Sephadex gel. Then, elute the sample by adding 1.5 mL of freshly degassed TN buffer devoid of DTT to the gel and collect the eluate by gravity flow into a 2 mL snap-cap microcentrifuge tube.
21. Dilute 50–100 μL of the eluate to 1/6 in TN buffer and record an absorbance spectrum from 230 to 450 nm against a blank using two quartz cuvettes. Determine the PH_{FAPP} concentration using the maximal absorbance at 280 nm and considering an extinction coefficient $\varepsilon = 29{,}450\ M^{-1}\ cm^{-1}$.
22. To guarantee a complete labeling of the PH_{FAPP} domain, it is preferred to add IANBD to the protein with a ten-times molar excess. Dissolve 1 mg of IANBD powder in DMF considering that the final volume of DMF used for labeling must not exceed 5% (v/v) of PH_{FAPP} sample volume. Thus adjust the volume of DMF (V_{DMF}) to dissolve IANBD following these formulas.

$$\begin{aligned} V_{DMF} &= V_{max} \times (m_0/m) \text{ with } V_{max} \\ &= 0.05 \times V \text{ and } m \\ &= 10{,}000 \times C \times V \times MW_{IANBD} \end{aligned}$$

C is the deduced concentration of PH_{FAPP} based on absorbance measurement as described in **step 21**, V is the volume of PH_{FAPP} solution, MW_{IANBD} is the molecular weight of IANBD (420 g/mol), m is the required amount of IANBD in mg, V_{max} is the maximum volume of DMF usable for PH_{FAPP} labeling, m_0 corresponds to 1 mg of dry IANDB powder (*see* **Note 5**).
23. Add the IANBD solution to the PH_{FAPP} protein sample and shake thoroughly the reaction mixture at 800–900 rpm for 30 min at 25 °C using a thermomixer protected from light. Let the reaction continue for 90 min on ice to reach its completion.

24. Add L-cysteine to the reaction mixture to quench unreacted IANBD. L-cysteine must be in a tenfold molar excess relative to IANBD.
25. Transfer the NBD-PH_{FAPP} solution into an Ultra-4 centrifugal filter unit with a MWCO of 3 kDa. Add 5 mL of TN buffer and concentrate the sample until 2 mL to remove a large part of free NBD from the protein by centrifugation at 1500 × *g*. Repeat this washing step twice more.
26. Store the 2 mL protein sample at 4 °C before the gel filtration procedure. Storage shall not exceed a day long.
27. Prior to gel filtration, check that no orange deposit is present at the bottom of the tube. Aggregation may occur during the concentration step due to an excessive amount of protein. In that case, centrifuge the sample for 10 min at 540,000 × *g*. Collect and inject the clarified supernatant on the column for gel filtration.
28. Gel filtration is performed on a Sephacryl S200HR column. Column is fully equilibrated with TND buffer. Flow rate is set at 1 mL/min. Tryptophan and NBD absorbances are followed at $\lambda = 280$ and 480 nm, respectively.
29. Load the protein sample in a 2 mL clean injection loop. Inject the sample on the column and immediately collect the eluate in hemolysis tubes using a fractionation volume of 2.5 mL. A major peak, which is concomitantly detected at $\lambda = 280$ and 480 nm, appears after an elution volume of ~150 mL (Fig. 2b). All the fractions that correspond to this peak are analyzed on a 15% SDS-PAGE gel (Fig. 2c).
30. Pool all the fractions that only contain NBD-PH_{FAPP} protein (~13 kDa). Add glycerol to the sample at a final concentration of 10% (v/v) for the cryo-protection of the protein. Concentrate the sample using an Ultra-15 centrifugal filter unit with a MWCO of 3 kDa to a final volume of 2 mL by centrifugation at 1500 × *g*.
31. Record an absorbance spectrum from 230 to 650 nm against a blank (*see* **Note 6**) using two quartz cuvettes. Determine the NBD-PH_{FAPP} concentration using the maximal absorbance at 280 and 495 nm, and considering an extinction coefficient $\varepsilon = 29{,}450\ M^{-1}\ cm^{-1}$ and $25{,}000\ M^{-1}\ cm^{-1}$ for the protein and the NBD moiety, respectively. If the two concentration values are alike, this means that the protein is labeled at 1:1 ratio with the NBD group (*see* **Note 7**).
32. Make 50 µL aliquots of protein in 0.5 mL Eppendorf tubes. Flash-freeze the tubes in liquid nitrogen and store them at −80 °C for several months.

3.2 Osh4p Purification

We express Osh4p in fusion in GST without any labeling step. The plasmid (pGEX-Osh4p) with the coding sequence of the full-length Osh4p (1–434 residues) is used to transform electro-competent bacteria. The purification protocol, except a few differences, is similar to the one used to obtain NBD-PH_{FAPP}. Follow **steps 1–8** described in Subheading 3.1.1 but let the bacteria grow overnight at 30 °C after IPTG induction. Thereafter, follow all the steps described in Subheading 3.1.2, but at **step 19**, concentrate the protein sample to 2 mL for gel filtration and skip **steps 20–27**. Use ultracentrifugal filter units with a MWCO of 10 kDa. The protein is usually collected once 60–90 mL of buffer are passed on the gel filtration column.

3.3 Preparation of Liposomes

Perform all the steps at room temperature otherwise specified. Handle organic solvent, rotavapor, and liquid nitrogen with care. Use fresh, filtered, and degassed HK buffer.

1. To make the liposomes for the transport assays, mix volumes of lipid stock solutions in a pear-shaped glass according to Table 1 (also *see* **Notes 8** and **9**). Adjust the volume of each mixture to 1 mL with pure $CHCl_3$.
2. Write down the name of each liposome type (L_A, L_B, . . .) on the neck of the respective flask. Wrap the flasks containing a mixture with fluorescent lipids (i.e., DHE, DNS-PE, or Rhod-PE) with aluminum foil.
3. Connect the flask to a rotary evaporator. Remove the solvent under vacuum while heating the flask at 25 °C for at least 1 h using a rotation speed of 100 rpm (*see* **Note 10**). A lipid film appears on the glass surface. Fill the evaporator and the flask with argon. Remove the flask and place it in a vacuum chamber for 45 min to remove any solvent traces.
4. Hydrate the film with 2 mL of HK buffer. Add a half-dozen of 4 mm-diameter glass beads to the flask to optimize the resuspension. Then, gently vortex the flask for at least 2 min in order to obtain a suspension of multilamellar lipid vesicles (MLVs) with a 4 mM lipid concentration. Divide the suspension into 1 mL aliquots stored in screw cap micro tubes. Properly label each tube cap with the liposome name.
5. Freeze and thaw the tubes five times (using liquid nitrogen and a water bath at 37 °C, respectively). Liposomes can then be directly extruded or stored at −20 °C.
6. Wet two prefilters with HK buffer, and place each of them over the orifice, inside the O-ring inner diameter of each membrane support of the mini-extruder. Insert one of the membrane supports, with the prefilter, into the extruder outer casing with the O-ring facing up. Place one 19 mm polycarbonate filter over the prefilter support and O-ring. Carefully place the

Table 1
Volume of lipid stock solutions to be mixed for liposome preparation

Liposome	Lipid composition (mol/mol)	Lipid stock solution				
		DOPC (25 mg/mL)	DHE (2.5 mM)	DNS-PE (1 mg/mL)	Brain PI4P (5 mg/mL)	Rhod-PE (1 mg/mL)
L_{A-1}	**DOPC/DHE/DNS-PE** 92.5/5/2.5	233 µL	160 µL	199 µL		
$L_{A-no\ DHE}$	**DOPC/DNS-PE** 97.5/2.5	245 µL		199 µL		
L_{B-1}	**DOPC/PI4P** 96/4	241 µL			62 µL[a]	
L_{A-2}	**DOPC/DHE** 95/5	239 µL	160 µL			
L_{B-2}	**DOPC/PI4P/Rhod-PE** 94/4/2	236 µL			62 µL[a]	200 µL
L_{A-Eq}	**DOPC/DHE/PI4P** 95.5/2.5/2	240 µL	80 µL		31 µL[b]	
L_{B-Eq}	**DOPC/DHE/PI4P/Rhod-PE** 93.5/2.5/2/2	235 µL	80 µL		31 µL[b]	200 µL

[a]Alternatively, add 306 µL of C16:0/C16:0-PI4P at 1 mg/mL
[b]Alternatively, add 153 µL of C16:0/C16:0-PI4P at 1 mg/mL

second membrane support into the casing (O-ring facing down). Place the retainer nut on the threaded end of the extruder outer casing and screw the retainer nut by hand just until it is sufficiently tight.

7. Prepare two hemolysis tubes: one filled with 1 mL of MLVs suspension and a second one to store the liposomes once they are extruded (*see* **Note 11**).
8. Load the MLVs sample into one of the 1 mL gas-tight Hamilton syringes and carefully connect the syringe at one end of the mini-extruder.
9. Connect the second syringe, which is empty, to the other end of the mini-extruder. The syringe plunger must be set to zero.
10. Gently push the plunger of the first syringe until the lipid solution is completely transferred to the second syringe. Gently push the plunger of the second syringe to transfer the solution back to the first syringe. Repeat these operations to pass the suspension 21 times through the filter and fragment the MLVs

into smaller and unilamellar liposomes of ~200 nm in diameter. Fill the second syringe with the final liposomes suspension and transfer this suspension into a hemolysis tube. The final suspension should be less milky and more opalescent than the suspension prior to extrusion.

11. Store extruded liposomes at 4 °C and in the dark if they contain fluorescent lipids. Liposomes should be used within 2 days.

3.4 Real-Time Measurement of Sterol Transport

Measurements are performed using a standard fluorimeter (90° format; e.g., Shimadzu RF5301PC or JASCO FP-8300) with a temperature-controlled cell holder. To accurately measure kinetics, it is mandatory to continuously stir the sample at a constant temperature (30 °C or 37 °C depending whether it is a yeast or mammalian protein, respectively). The protocol described thereafter is for measuring transport in a 600 μL sample contained in a cylindrical quartz cell (*see* **Note 12**) and the use of a Shimadzu RF5301PC apparatus. Use fresh, filtered, and degassed HKM buffer (*see* **Note 13**). Maintain the tubes filled with the suspension of extruded liposomes at room temperature during the experiment. Wrap the tubes containing fluorescent liposomes with aluminum foil and/or store them in an opaque box to protect them from light.

1. To measure the DHE-to-dansyl FRET, tune the monochromator such as to excite the sample with a wavelength (λ) equal to 310 nanometer (nm) along with a short bandwidth (1–3 nm) to prevent any DHE photobleaching (*see* **Note 14**). Record the dansyl emission at λ = 525 nm with a larger bandwidth ($\geq$5 nm) to maximize the signal-to-noise ratio. Set up the acquisition time at 24 min and, if possible, with a time resolution <1 s to facilitate any subsequent kinetics analysis (*see* Subheading 3.6).
2. Mix 30 μL of $L_{B\text{-}1}$ liposome suspension with 540 μL of pre-warmed (*see* **Note 15**) and filtered (either at 30 °C or 37 °C) HKM buffer in the cell to obtain a final concentration of 200 μM total lipids. Put the small magnetic stirring bar into the sample using a clean small Millipore tweezer and place the cell in the spectrofluorometer holder.
3. Wait a few minutes to allow for the thermal equilibration of the sample.
4. Start the recording, and after 1 min, inject 30 μL of $L_{A\text{-}1}$ liposome suspension (200 μM total lipids, final concentration). A signal jump must be seen as $L_{A\text{-}1}$ liposomes contain DHE and DNS-PE and consequently light is emitted at 525 nm due to DHE-to-dansyl FRET.
5. After 3 min, inject the protein to obtain a final concentration of 200 nM (*see* **Note 16**) and record the signal during the next

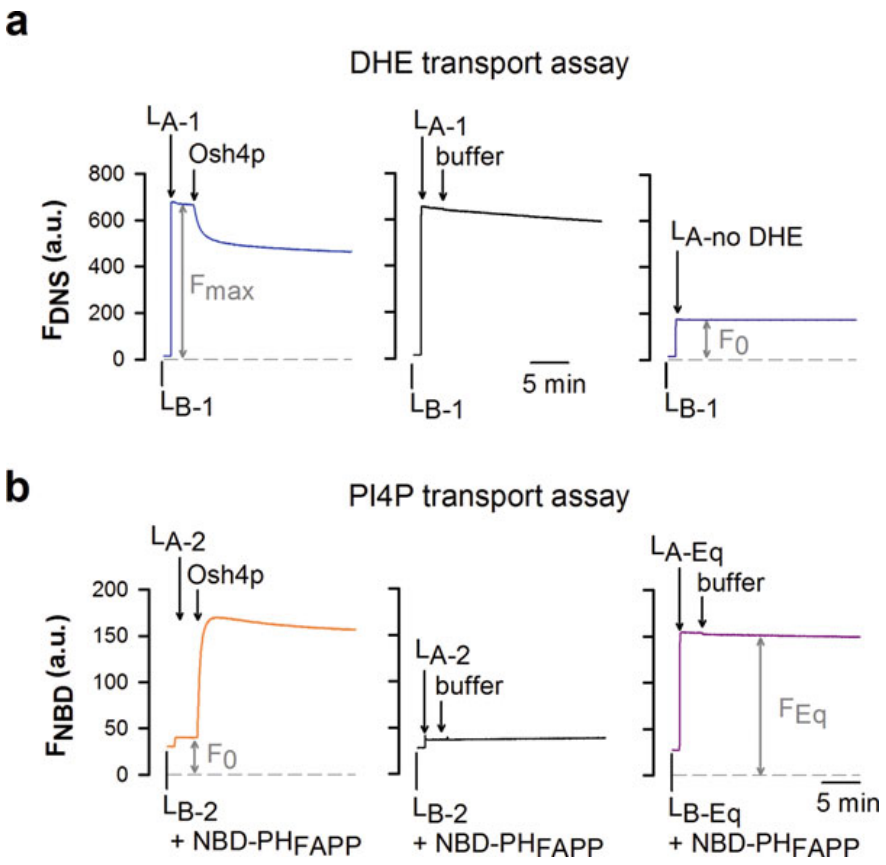

Fig. 3 Typical DHE and PI4P transport kinetics measured with Osh4p and control curves. (**a**) DHE transport assay. $L_{B\text{-}1}$ liposomes (200 μM total lipids) and a sterol/PI4P exchanger (Osh4p, 200 nM) are sequentially added to a quartz cell containing $L_{A\text{-}1}$ liposomes (200 μM), resulting in a diminution of FRET indicative of the transport of DHE from $L_{A\text{-}1}$ to $L_{B\text{-}1}$ liposomes (left panel). F_{max} is the fluorescence measured before adding LTP. A control experiment is done by adding buffer instead of protein (middle panel). Liposomes, with 2.5 mol% DNS-PE but no DHE ($L_{A\text{-no DHE}}$), are added to $L_{B\text{-}1}$ liposomes (right panel) in order to determine F_0. (**b**) PI4P transport assays. $L_{A\text{-}2}$ liposomes (200 μM lipids) and Osh4p (200 nM) are sequentially added to a cuvette containing $L_{B\text{-}2}$ liposomes (200 μM) and NBD-PH_{FAPP} (250 nM) resulting in an increase of NBD signal indicative of the transport of PI4P from $L_{B\text{-}2}$ to $L_{A\text{-}2}$ liposomes (left panel). A control experiment is done by adding buffer instead of protein (middle panel). To determinate F_{Eq}, $L_{A\text{-Eq}}$ liposomes are added to $L_{B\text{-Eq}}$ liposomes in the presence of 250 nM NBD-PH_{FAPP}. Buffer is added instead of the protein (right panel)

20 min. If the protein of interest transports sterol from $L_{A\text{-}1}$ to $L_{B\text{-}1}$ liposomes, a decrease in FRET signal must be seen (Fig. 3a, left panel).

6. As a control, repeat the experiment by adding, instead of protein solution, an equivalent volume of HKM buffer in order to record the spontaneous DHE transport from $L_{A\text{-}1}$ to $L_{B\text{-}1}$ liposomes (Fig. 3a, middle panel).

7. Record a third experiment to measure the signal F_0 that would be obtained if the entire pool of DHE molecules in $L_{A\text{-}1}$ liposomes was transferred to $L_{B\text{-}1}$ liposomes. To do this, mix 30 µL of $L_{B\text{-}1}$ liposomes with 540 µL of pre-warmed and filtered HKM buffer. Start the recording and inject 30 µL of $L_{A\text{-no DHE}}$ liposomes after 1 min. A prompt increase in signal must be seen and the signal must remain stable until the end of the recording (Fig. 3a, right panel). It corresponds to the basal fluorescence of DNS-PE upon excitation at 310 nm in the absence of FRET (F_0, *see* **Note 17**).
8. Normalize the kinetic curves measured with the LTP of interest or Osh4p as a control to determine the amount of DHE (in µM) transferred from L_A to L_B liposomes over time by transforming each data point of the curve (F value) using the formula $10 \times [1 - ((F - F_0)/(F_{max} - F_0))]$. F_{max} is the F value just before injecting LTP (*see* **Note 18**) and F_0 is the signal measured with the mix of $L_{B\text{-}1}$ and $L_{A\text{-no DHE}}$ liposomes. The volumic DHE concentration in the assay is equal to 10 µM since the DHE pool accounts for 5% of 200 µM lipids and is quickly equilibrated between the inner and outer leaflet of liposomes ($t_{1/2}$ of transbilayer movement <1 min [24]) and hence entirely accessible to the protein. Apply the same normalization procedure to the control curve measured by injecting only buffer.

3.5 Real-Time Measurement of PI4P Transport

1. Set the excitation monochromator at $\lambda = 460$ nm with a short bandwidth (1–3 nm) and emission monochromator at $\lambda = 540$ nm with a larger bandwidth (≥10 nm). Set up the acquisition time and the time resolution as for the DHE transport assay.
2. Mix 30 µL of $L_{B\text{-}2}$ liposome suspension and NBD-PH_{FAPP} with pre-warmed, filtered, and degassed HKM buffer to a final volume of 570 µL in the quartz cuvette to obtain a final concentration of 200 µM total lipid and 250 nM NBD-PH_{FAPP} (*see* **Note 19**). Add a magnetic stirring bar and place the cuvette in the spectrofluorometer holder. Allow the thermal equilibration of the sample.
3. Start the recording. The signal must be low since the NBD-PH_{FAPP} is bound to $L_{B\text{-}2}$ liposomes and the NBD fluorescence emission is quenched by Rhod-PE. After 1 min, inject 30 µL of $L_{A\text{-}2}$ liposome suspension (200 µM total lipids, final concentration). A small jump in the signal must be seen due to light scattered by this second population of liposomes (Fig. 3b, left panel).
4. After 3 min, inject Osh4p or LTP to obtain a final concentration of 200 nM and record the signal during the next 20 min. If

the protein of interest transports PI4P from $L_{B\text{-}2}$ to $L_{A\text{-}2}$ liposomes, the NBD signal must increase and possibly reaches a plateau at the end of the measure as seen with Osh4p (Fig. 3b, left panel).

5. As a negative control, repeat the experiment by adding, instead of LTP solution, an equivalent volume of HKM buffer. No change in the NBD signal must be seen (Fig. 3b, middle panel).
6. Perform a third experiment to normalize the NBD signal. Mix 30 μL of $L_{B\text{-}eq}$ liposomes with NBD-PH_{FAPP} (250 nM) in a final volume of 570 μL of HKM buffer. Sequentially inject $L_{A\text{-}Eq}$ liposomes (at $t = 1$ min) and a volume of buffer similar to the volume of injected LTP (at $t = 4$ min). Liposomes have a size and lipid composition similar to that of the liposomes used in the transport assay, except that each population contains initially 2 mol% PI4P. Thus, the NBD signal termed F_{eq} corresponds to a situation where PI4P is already fully equilibrated between the two liposome populations and remains stable over 20 min (Fig. 3b, right panel).
7. Convert the kinetics curve measured with Osh4p or the LTP of interest to determine the amount of PI4P (in μM) transferred from $L_{B\text{-}2}$ to $L_{A\text{-}2}$ liposomes as a function of time. Normalize each data point of the curve by applying the formula $F_{Norm} = 0.5 \times ((F - F_0)/(F_{eq} - F_0))$ in which F corresponds to the value of the data point, F_0 corresponds to the NBD signal prior to the addition of Osh4p (*see* **Note 20**), and F_{Eq} is the signal measured with $L_{A\text{-}Eq}$ and $L_{B\text{-}Eq}$ liposomes. The amount of PI4P (in μM) transferred from $L_{B\text{-}2}$ to $L_{A\text{-}2}$ liposomes corresponds to $4\times F_{Norm}$ considering that the protein has only access to PI4P molecules present in the outer leaflet of the $L_{B\text{-}2}$ liposomes, i.e., a pool of 4 μM (corresponding to 4% of 0.5 × 200 μM total lipids).

3.6 Kinetics Analysis

When studying Osh4p, we recorded complex DHE and PI4P transport kinetics whose amplitude and shape could only be interpreted using a multistep kinetic model. Those curves could not be fitted for instance to exponential functions that are often used to describe simple lipid equilibration processes between two membranes akin to first-order reactions. So we recommend, as a first approach, to calculate the initial transport velocities which are representative of the efficiency of a LTP. At this end, we consider that the injection of the LTP sets the time zero of the transport process. The first data points of the curve, after injection, are then fitted with a linear equation by regression analysis. The slope is divided by the LTP concentration in the cuvette such as to determine the number of lipid molecules transported per protein per time unit (min or s). Here an interesting aspect to examine is whether or not the DHE and PI4P transport rates are similar or

equal. If yes, this might be indicative of the coupling between the two transport processes and thus of a sterol/PI4P exchange activity (*see* **Note 21**).

4 Notes

1. We describe here the preparation of liposomes with simple lipid compositions and a diameter of ~200 nm. These features are well adapted to measure the activity of Osh4p but can also be suitable for other LTPs. DOPC is the major lipid in our liposome preparation. It is better to use this lipid to make L_A liposomes because DOPC, with two mono-unsaturated C18 chains (C18:1), forms lipid bilayers with a low lipid-packing, thereby facilitating sterol extraction. Substituting DOPC by POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), which has one saturated C16 chain (C16:0) and one mono-unsaturated C18 chain (C18:1), to make L_A liposomes, results in slower kinetics of transport by Osh4p because sterol is less extractable. It is yet possible to make L_A liposomes with POPC and measure fast kinetics by making liposomes with small radii and thus with more packing defects, which are known to facilitate sterol extraction. In contrast, our experiences with Osh4p indicated that the use of L_B liposomes made of POPC with a diameter of 200 nm does not impact the kinetic of sterol/PI4P exchange. However, using L_B liposomes made of DOPC and with a low diameter provokes a massive slowdown of the transport as Osh4p tends to remain strongly associated to these liposomes that combine a low lipid-packing, due to the high curvature, and negative charges, due to the presence of PI4P (unpublished data).
2. For faster and better solubilization of complete EDTA-free tablets, plunge the 50 mL falcon tubes in an ultrasonic cleaner bath.
3. Bacterial pellets can be stored for weeks to months.
4. From a lumpy and thick suspension, one should obtain a smoothed and fluid lysate.
5. Typically, considering that a 50 μL aliquot was used for the dosage, the volume of IANBD in DMF should not exceed 72.5 μL for 1450 μL of protein sample and should contain the required mass of IANBD.
6. We recommend using the eluate as a blank.
7. Even if the fluorophore/protein ratio is lower than 1, the PH_{FAPP} probe can be used for PI4P transport assay. In this case consider only the tryptophan absorbance to determine the concentration of the protein.

8. DHE crystallizes in methanol when it is stored at −20 °C. Consequently, it is mandatory to warm the vial with hot water to fully re-solubilize DHE. Make sure that the vial is well closed during this operation.
9. The major PI4P species in commercial brain PI4P solution has a mono-unsaturated chain (C18:1) and a poly-unsaturated chain (C20:4) and is quite different from a yeast PI4P. Yet it is well transported and exchanged for sterol by Osh4p. However, we observed with other LTPs such as Osh6p, that this PI4P species is hardly exchangeable with the second lipid counterligand (PS, unpublished data). In that case, it was preferable to use C16:0/C16:0-PI4P which is close in term of its acyl-chains to the most abundant yeast PI4P species [29]. As a guideline, one can thus recommend to use brain PI4P to work with mammalian LTPs and C16:0/C16:0-PI4P with yeast proteins. It is noteworthy that a third PI4P species with long acyl chains (C18:1/C18:1-PI4P) can be purchased from Avanti Polar Lipids.
10. For lipid films including PI4P, it is key to pre-warm the mix. Put the flask under a gentle rotation in the water bath with a temperature between 32 and 34 °C for 5 min prior to drying the lipid mixture under vacuum. This step is necessary to properly mix PI4P with the other lipids.
11. MLV suspensions in 1.5 mL tubes cannot be collected with the mini-extruder syringes.
12. It is ideal to inject reactants with 10, 25, or 50 μL Hamilton syringes through a guide in the cover of the fluorimeter. The guide is set up to position the tip of the needle in contact with the meniscus of the sample. Because the needle does not cross the light beam, the measurement is not interrupted by injections: kinetics can be correctly recorded with a <1 s time resolution.
13. If necessary, it is possible to add 1 mM DTT to the buffer to avoid any oxidation of the LTP.
14. The use of a neutral filter can be useful to reduce even more the intensity of the excitation beam, thus limiting the occurrence of photobleaching.
15. If the spectrofluorometer is connected to a water bath to regulate the temperature of the cell holder, one can place a Falcon 50 tube containing HKM buffer in the bath such as to pre-warm buffer at the appropriate temperature.
16. Using a stock solution of lipid transfer protein at 12–40 μM is optimal as only 3–10 μL of sample are added with a 10 μL Hamilton syringe to the reaction mix to obtain a final

concentration of 200 nM, thus avoiding any strong dilution effect and thus a signal modification.

17. Alternatively, it is possible to do this measure without using $L_{A\text{-}no\ DHE}$ liposomes. To do so, mix $L_{A\text{-}1}$ and $L_{B\text{-}1}$ liposomes as done with the experiment with the LTP. Then, instead of adding LTP, add methyl-β-cyclodextrin to obtain a final concentration of 10 mM in the sample. This compound at this concentration is able to extract all the DHE molecules from the $L_{A\text{-}1}$ liposomes within 1–2 min. So the DNS signal decreases and reaches a plateau whose intensity corresponds to F_0 (basal dansyl signal in the absence of FRET). Please also note that at 1 mM, methyl-β-cyclodextrin acts as a LTP, able to equilibrate DHE between $L_{A\text{-}1}$ and $L_{B\text{-}1}$ liposomes. So the fluorescence measured at the end of the kinetic trace corresponds to the signal that would be obtained if DHE was found in equal amount between the two liposome populations.
18. Calculate the average of the *F* values measured during 1 min prior to the addition of the LTP.
19. We could substitute DHE by ergosterol to make $L_{A\text{-}2}$ liposomes used in the PI4P transport assays. Indeed, the fluorescence of DHE is not measured in that assay and Osh4p, at least, has the same affinity for DHE or ergosterol. However, in our hands, ergosterol reveals itself particularly instable over time compared to DHE likely due to sensitivity to light. Likewise, the use of cholesterol might be potentially interesting yet we noted that Osh4p does not recognize cholesterol as well as DHE or ergosterol. Based on these observations, in order to compare the DHE and PI4P transport measurements and thus establish a sterol/PI4P exchange process, we recommend using the same source of sterol in the two assays. It is noteworthy that the presence of DHE in the $L_{A\text{-}2}$ liposomes does not contribute to the NBD signal measured in the PI4P transport assay.
20. Like for the DHE transport assay, average the *F* values measured during 1 min prior to the addition of the LTP.
21. Two additional sets of experiments can be done to confirm this point. First it is interesting to measure the DHE transport activity of the LTP with $L_{B\text{-}1}$ liposomes devoid of PI4P and to measure the transport of PI4P with $L_{A\text{-}2}$ liposomes that do not contain DHE. In both cases, if the LTP is a sterol/PI4P exchanger, one should see a reduction of the transport activity of the protein in the two cases. Second, it is also very informative to identify specific mutations able to simultaneously change the DHE and PI4P transport activity of the LTP under investigation.

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