Submission ID #: 62177
Scriptwriter Name: Madhulika Pathak
Supervisor Name: Anastasia Gomez
Project Page Link: https://www.jove.com/account/file-uploader?src=18964033

Title: Fluorescence-based Measurements of Phosphatidylserine/Phosphatidylinositol 4-Phosphate Exchange Between Membranes

Authors and Affiliations:

Souade Ikhlef*, Nicolas-Frédéric Lipp*, Maud Magdeleine, Guillaume Drin

Université Côte d'Azur, Centre National de la Recherche Scientifique, Institut de Pharmacologie Moléculaire et Cellulaire, Valbonne, France

Corresponding Authors:
Guillaume Drin drin@ipmc.cnrs.fr

Email Addresses for All Authors:
ikhlef@ipmc.cnrs.fr
lipp@ipmc.cnrs.fr
magdeleine@ipmc.cnrs.fr
drin@ipmc.cnrs.fr

Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **NO**
2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **YES, all done**
3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **NO**

Current Protocol Length

Number of Steps: 18
Number of Shots: 27

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Guillaume Drin**: This method uses fluorescence to determine if a protein transfers two biologically-important lipids between synthetic membranes and thus contributes to the distribution of these lipids inside eukaryotic cells.

1.1.0. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Nicolas-Frédéric Lipp**: This technique makes it possible to readily measure the extraction and transport of natural lipids by a protein. It requires standard equipment and fluorescent sensors and liposomes that are easy to make.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.2.1 and 4.3.1*

OPTIONAL:

1.3. **Nicolas-Frédéric Lipp**: This method can be used and modified to get insights into cellular biology by unveiling the proteins behind the distribution of some essential lipids between organelle membranes**.**

1.3.0. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.6.1*

1.4. **Nicolas-Frédéric Lipp**: Visual demonstration is critical to explain how to perform real-time measurements of lipid transfer by fluorescence.

1.4.0. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.5.1*

Introduction of Demonstrator on Camera

1.5. **Guillaume Drin:** Demonstrating the procedure will also be Maud Magdeleine, an engineer from my laboratory.

1.5.0. INTERVIEW: Author saying the above.

1.5.1. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Protocol

2. Measurement of PS or PI(4)P Extraction

2.1. Begin by preparing fresh, filtered, and degassed HEPES-potassium acetate buffer supplemented with 1 millimolar magnesium chloride to form HKM buffer **[1]**. Then, prepare liposomes only made of PC or additionally doped with 2 molar % PS or PI(4)P *(Pronounce: "pi: aɪ 4 pi")* **[2-TXT]**. Now, place the flask on a rotary evaporator to dry the lipid under vacuum **[2.bis]**

2.1.0. WIDE: Talent dissolving components in water

2.1.1. Talent adding lipids to the flask. **TEXT: PC – phosphatidylcholine; PS– phosphatidylserine; PI(4)P – phosphatidylinositol 4-phosphate** *Videographer: This step is important!*

2.1.2.bis Added shot: Talent placing the flask on a rotary evaporator and drying the lipid under vacuum

2.2. In one well, mix liposomes containing 2 molar % PS with lipid sensor NBD-C2 lact to a final concentration of 250 nanomolar in a volume of 100 microliters **[1-TXT]**. Fill a second well with the same amount of liposome and NBD-C2 lact mixed with 3 micromolar lipid transfer protein, or LTP **[2]**.

2.2.0. Talent mixing liposome with lipid sensor in a well **TEXT: 80 µM lipid concentration; NBD-C2: C2 domain of the glycoprotein, lactadherin** *Videographer: This step is important!*

2.2.1. Talent mixing liposome and sensor with LTP in second well *Videographer: This step is important!*

2.3. Fill a third well with 250 nanomolar NBD-C2 lact mixed with pure 80 micromolar PC liposomes and a fourth well with pure 80 micromolar PC liposomes. Repeat these steps to prepare three additional series of four wells **[1]**. Then, place the multi-well plate in the fluorescence reader **[1bis].**

2.3.0. Talent filling the third and fourth wells

2.3.1bis Talent placing the multi-well plate in the fluorescence reader

2.4. For each well, record an NBD spectrum from 505 to 650 nanometers, with a 5-nanometer bandwidth, upon excitation at 490-nanometers at 25 degrees Celsius. For each series, subtract the spectrum recorded with only liposomes from the other spectra **[1-TXT]**.

2.4.0. SCREEN: #62177-Movie1.mp4.0:01-1:45 **TEXT: NBD: 7-nitrobenz-2-oxa-1,3-diazol** *Video editor: Speed up the alignment running*

2.5. For the PI(4)P extraction assay, prepare liposomes doped with 2 molar %

phosphatidylinositol 4-phosphate and carry out measurements with the NBD-PH FAPP *(Pronounce: "FAP)* probe. Perform control experiments and determine the extraction percentage **[1-TXT]**.

2.5.0. Talent mixing the liposome and probe **TEXT: NBD-PH: PH domain of the human four-phosphate-adaptor protein 1**

3. Real-time Measurement of PS Transport

3.1. After finishing extruding liposomes, fill a tube with these extruded liposomes **[0]**. Prepare freshly degassed and filtered HKM buffer and keep the tubes containing extruded liposomes at room temperature **[1]**. Wrap tubes containing liposomes with rhodamine-labelled phosphatidylethanolamine in aluminum foil and store them in an opaque box to prevent any photobleaching **[2]**.

3.1.0. Added Shot: Talent finishing extruding liposomes and filling a tube with these extruded liposomes.

3.1.1. Talent placing the tubes with liposomes at room temperature

3.1.2. Talent wrapping tubes in aluminum foil and keeping it in opaque box

3.2. Set the temperature between 25 and 37 degrees Celsius **[1]** and adjust the excitation monochromators to 460 nanometers, with a short bandwidth of 1 to 3 nanometers, and emission to 530 nanometers with a large bandwidth of greater than 10 nanometers **[2]**. Set the acquisition time to 25 minutes with a time resolution of at most one second **[3]**.

3.2.0. Talent setting the temperature.

3.2.1. SCREEN: #62177-Movie2.mp4.0:00-0:12

3.2.2. SCREEN: #62177-Movie3.mp4.0:00-0:09

3.3. In the quartz cuvette, dilute 30 microliters of the LA liposome suspension and NBD-C2 lact stock solution in prewarmed HKM buffer to prepare a 570-microliter sample that contains 200 micromolar total lipids and 250 nanomolar NBD-C2 lact **[1]**.

3.3.0. Talent diluting LA liposome and NBD-C2 lact in quartz cuvette *Videographer: This step is important!*

3.4. Add a small magnetic stirring bar and position the cuvette in the fluorometer holder **[1]**.

3.4.0. Talent adding magnetic stirring bar and putting cuvette in holder *Videographer: This step is important!*

3.5. Once the sample is thermally equilibrated, trigger the measurement **[1]**. After 1 minute, add 30 microliters of the LB liposome suspension to the sample **[2]**. After 3 minutes, inject LTP into the sample so that the final concentration of the LTP is 200 nanomolar, then acquire the signal for the remaining 21 minutes **[3]**.

3.5.0. SCREEN: #62177-Movie7.mp4.0:00-1:00

3.5.1. SCREEN: #62177-Movie7.mp4.1:10-4:00 *Video editor: Speed up the alignment running!*

3.5.2. SCREEN: #62177-Movie7.mp4.4:10-7:10 *Video editor: Speed up the alignment running!*

3.6. Carry out a parallel experiment to normalize the NBD signal. Mix 30 microliters of LA-Eq *(Pronounce: "LA equilibrium")* liposome suspension with 250 nanomolar NBD-C2 lact in HKM buffer at a final volume of 570 microliters **[1]**. After 1 minute, inject 30 microliters of LB-Eq *(Pronounce: "LB equilibrium")* liposome suspension **[2]**.

3.6.0. Talent mixing LA-Eq to NBD-C2 lact in HKM buffer

3.6.1. Talent injecting LB-Eq

3.7. Convert the kinetic curves measured with an LTP of interest to determine the amount of PS transferred from LA to LB liposomes over time, then normalize each data point of the curve **[1-TXT]**.

3.7.0. #62177-Movie4.mov. 0:00-1:26 **TEXT: F: recorded fluorescence, F_0: signal before LTP addition, F_{Eq}, signal when lipid is equilibrated.** *Video editor: Speed up the alignment running*

4. Real-time Measurement of PI(4)P Transport

4.1. Perform the PI(4)P experiment using the same fluorimeter settings and conditions as for the PS transfer assay **[1]**.

4.1.0. WIDE: Talent setting the fluorimeter

4.2. In the cuvette, mix 30 microliters of LB liposome suspension and NBD-PH FAPP probe with prewarmed HKM buffer to obtain a final volume of 570 microliters **[1-TXT]**.

4.2.0. Talent mixing reagents in the cuvette **TEXT: 200 µM total lipids, 250 nM NBD-PH_{FAPP}**

4.3. Once the thermal equilibration of the sample is reached, start the measurement. After 1 minute, inject 30 microliters of LA liposome suspension **[1]**. After 3 minutes, inject the LTP of interest and record the signal **[2-TXT]**.

4.3.0. Talent injecting LA liposome suspension

4.3.1. Talent injecting LTP **TEXT: final concentration of 200 nM**

4.4. Perform a second experiment to normalize the NBD signal. Mix 30 microliters of LB-Eq liposome suspension with 250 nanomolar NBD-PH FAPP in 570 microliters of HKM buffer **[1]**. After 1 minute, inject 30 microliters of LA-Eq *(Pronounce: "LA equilibrium")* liposome suspension **[2]**.

4.4.0. Talent mixing LB-Eq suspension to the buffer

4.4.1. Talent injecting LA-Eq to the buffer

4.5. Convert the kinetic curves to determine the amount of PI(4)P transferred from LB to LA liposomes over time, then normalize each data point **[1]**.

4.5.0. SCREEN: 62177-Movie5.mov. 0:00-1:23 *Video editor: Speed up the alignment running!*

4.6. To quantify the extent to which an LTP is efficient, perform a linear regression of the first data points of the transfer kinetics to obtain a slope. Divide the slope value by the LTP concentration in the reaction mixture to determine the number of lipid molecules transferred per protein per time unit **[1]**.

4.6.0. SCREEN: 62177-Movie6.mov. 0:00-1:05 *Video editor: Speed up the alignment running!*

Results

5. Results: Membrane-Exchange of Phosphatidylserine/Phosphatidylinositol 4-Phosphate Using Fluorescent Sensors

5.1. Efficient recovery of C2 lact from the beads was verified with SDS-PAGE analysis **[1].**

5.1.0. LAB MEDIA: Figure 2A

5.2. The ultraviolet-visible absorbance spectrum of C2 lact labelled with NBD confirmed that all C2 lact molecules were labelled with an NBD group **[1]**. The purity of NBD-C2 lact and its fluorescence were determined by SDS-PAGE analysis **[2]**.

5.2.0. LAB MEDIA: 2B

5.2.1. LAB MEDIA: 2C

5.3. The fluorescence of NBD-C2 lact or NBD-PH FAPP was maximal when only liposomes containing PS or PI(4)P were used for incubation, indicating that the sensor was membrane-bound. A low fluorescence was seen when Osh6p was also present indicating that this protein efficiently extracted PS or PI(4)P from liposomes **[1].**

5.3.0. LAB MEDIA: 3A and 3B

5.4. The results from a PS transfer assay using Osh6p *(Pronounce: "Osh-six-P")* as an LTP are shown **[1]**. The average kinetic curves of PS transfer from LA liposomes to LB liposomes, doped or not doped with phosphatidylinositol 4-phosphate, were calculated **[2]**. The mean initial PS transport rate was determined from three distinct experiments **[3].**

5.4.0. LAB MEDIA: 4A

5.4.1. LAB MEDIA: 4B

5.4.2. LAB MEDIA: 4C

5.5. Similarly, results from a PI(4)P transfer assay using Osh6p as an LTP are shown here **[1]** along with the average kinetics curves obtained after signal normalization. Mean transfer rates were measured with LA liposomes that were doped or not doped with PS **[2].**

5.5.0. LAB MEDIA: 5A

5.5.1. LAB MEDIA: 5B and 5C

Conclusion

6. Conclusion Interview Statements

6.1. **Nicolas-Frédéric Lipp:** When performing this protocol, use fresh buffer and prepare all liposomes on the same day. Minimize the light exposition of fluorescent liposomes and proteins. Use well-purified NBD-labelled proteins and keep them on ice.

6.1.0. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.1.2, 3.3.1, LAB MEDIA: Figure 2*

6.2. **Guillaume Drin:** In vitro data can be confirmed by measuring PS and PI(4)P transfer between organelles inside eukaryotic cells using genetically-encoded fluorescent lipid sensors.

6.2.0. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 4 and figure 5*

6.3. **Guillaume Drin:** This technique paved the way to a better understanding how PS and PI(4)P are distributed within the cell and the activity and ligand specificity of several LTPs.

6.3.0. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 3*