The fluorescence protease protection (FPP) assay to determine protein localization and membrane topology

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Correct localization and topology are crucial for the cellular function of a protein. To determine the topology of membrane proteins, a new technique, called the fluorescence protease protection (FPP) assay, can be applied. This assay uses the restricted proteolytic digestibility of GFP-tagged transmembrane proteins to indicate their intramembrane orientation. The sole requirements for FPP are the expression of GFP fusion proteins and the selective permeabilization of the plasma membrane, which permits a wide range of cell types and organelles to be investigated. The FPP assay can be carried out in a straightforward manner to obtain reliable results within minutes. Here we provide a step-by-step protocol for the assay. As an example, we use FPP to determine which terminus of an endoplasmic reticulum (ER) transmembrane protein is lumenal and which one is facing the cytosol.

INTRODUCTION

Cellular membranes act as barriers and scaffolds that separate different cellular environments. Consequently, proteins within membranes must adopt appropriate orientations to communicate with their respective interacting partners. To understand the cellular function of a protein, it is therefore crucial to know how a membrane protein is organized with respect to its resident membrane—i.e., its membrane topology. Numerous approaches have been used to determine the topology of transmembrane proteins. Many of these require a substantial investment of time and effort, however, and predictions from different approaches are often inconsistent[1]. Here we describe an alternative method, called the FPP assay[2] (**Fig. 1**), for finding the membrane topology of a protein using GFP chimeras expressed in living cells. This approach provides basic information about the localization of the GFP tag relative to a membrane and requires no additional design beyond the construction of a GFP fusion protein that is expressed either in tissue culture or primary cells. Furthermore, this technique is less invasive than cell fractionation approaches, which can affect organelle integrity and membrane orientation. The assay is based on the accessibility of proteases to exposed polypeptides versus their inaccessibility to polypeptides that are located in 'protected' intracellular regions such as the lumen of organelles. To allow proteases to enter the cell, the FPP assay requires the selective permeabilization of the plasma membrane. We use the cholesterol-binding drug digitonin, a toxin derived from the plant *Digitalis purpurea*, for this purpose. The specific intercalation of the relatively large digitonin molecule into cholesterol-rich membranes leads to the perforation of the lipid bilayer. This permeabilization allows the exchange of the freely diffusible (unbound) portion of the cytosol with the extracellular content across the plasma membrane. The selectivity of this cell surface permeabilization results from the fact that the plasma membrane has the highest concentration of cholesterol, with approximately 65–80% of the free cellular cholesterol[3], which renders the cell surface the prime target for digitonin intercalation with very few effects on intracellular membranes. Therefore, cellular organelles and the cytoskeleton are retained in cells that are permeabilized with digitonin[4,5]. Other approaches can be used to specifically permeabilize the plasma membrane, including staphylococcal α-toxin or streptolysin O treatment or freeze-thawing followed by fixation[6]. We have not, however, tested FPP using these permeabilization techniques.

Using the FPP assay, many questions regarding the topological distribution of a protein in living cells can be addressed in a straightforward and reliable fashion. It becomes possible to determine (i) whether a protein is bound or freely diffusing, (ii) whether an organellar protein is membrane-associated or lumenal and (iii) which portion of a membrane protein faces the lumen or the cytoplasm.

There are a few limitations that need to be considered. If a protein has multiple orientations, the assay will indicate the orientation of the major form and neglect minor forms. Furthermore, protein localization can be affected by the addition of a GFP moiety. The prevalence of GFP fusions clearly demonstrates that most of these proteins do, however, function properly and retain their native conformations.

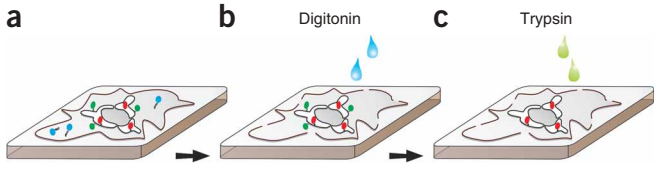

Figure 1 | Cartoon of the FPP assay illustrating a single cell before and after digitonin and trypsin treatment. The blue symbol represents RFP in the cytoplasm. The red and green symbols represent transmembrane proteins with the fluorescent tag facing either the cytoplasm (green) or the lumen of the organelle (red). (**a**–**c**) The situation before (**a**) and after (**b**) permeabilization and the resulting phenotype after protease (trypsin) treatment (**c**) are shown.

MATERIALS

REAGENTS

- Cells to be transfected with GFP fusion protein
- GFP expression vector (Clontech) containing DNA encoding the membrane protein of interest
- FuGENE 6 transfection reagent (Roche) or other standard transfection method
- Standard cell culture medium for cells of interest
- KHM buffer[5]: 110 mM potassium acetate, 20 mM HEPES, 2 mM $MgCl_2$
- Digitonin
- Trypsin or an alternative protease, such as proteinase K, reconstituted in KHM buffer

EQUIPMENT

- Lab-Tek chambered coverglass (Nalge Nunc)
- Fluorescence microscope with image acquisition and capture system

PROCEDURE

1| In preparation for transfection, plate similar numbers of cells in cell chambers that can be used for both DNA transfections and microscopic observations, e.g., Lab-Tek chambered coverglass. Plate similar numbers of cells into the chambers at a concentration that will avoid allowing the cultures to become too confluent at the time of the FPP assay.

2| Using FuGENE 6 transfection reagent or another standard transfection method, transfect cells with DNA coding for the membrane protein of interest tagged with GFP or any other fluorescent protein variant. For single-spanning membrane proteins, two versions of the protein should be created, an amino- and a carboxy-terminal fusion with GFP. For multi-spanning membrane proteins, either fusions with GFP inserted within the protein of interest or truncated fusions should be made. Note the exact position of GFP within the fusion protein amino-acid sequence. As a control for efficient cell permeabilization, transfect cells with DNA encoding GFP alone. For the example shown here, we used FuGENE 6 transfection reagent, but any standard transfection method is suitable. Culture cells under the appropriate conditions until fluorescent signals are detectable (~6–20 h).

3| Remove cell culture medium and wash cells three times for 1 min each in KHM buffer at a temperature that is appropriate for the experiment. In our hands, temperatures of 20–37 °C were suitable for the protocol.

4| Place chamber containing cells in KHM buffer on the fluorescence microscope stage. Set up microscope for fluorescence imaging and record first images, which represent the 'prepermeabilization' situation.

5| To permeabilize the plasma membrane, add the same volume of KHM buffer containing digitonin to the cells. Determine the effective digitonin concentration by applying increasing concentrations of digitonin to cells that express only GFP. GFP diffuses freely in the cytosol and nucleoplasm. Effective permeabilization of the plasma membrane by digitonin results in the disappearance of the GFP signal within 10–60 s.
▲ **CRITICAL STEP** A good starting concentration for most cell lines tested (COS-7, NRK, HeLa, BHK, N2a) is 20 μM digitonin. If 20 μM digitonin is not sufficient to permeabilize the cells, increase the digitonin concentration in 20 μM increments. Use the lowest possible digitonin concentration that provides efficient plasma membrane permeabilization.

6| Incubate cells that express the protein of interest tagged with GFP in KHM buffer containing the (previously determined) effective digitonin concentration. Efficient permeabilization of most cell lines occurs within 1 min after digitonin application.

7| Take images of the cells after digitonin application to capture the 'postpermeabilization' situation.

8| Wash cells in KHM buffer (optional) and then add 4–8 mM of the protease trypsin (in KHM buffer) directly onto the cells. Immediately start taking images on the fluorescence microscope to record whether fluorescent signals persist or disappear. Under special circumstances in which the sensitivity of the protein of interest to trypsin or its accessibility is under question, the use of an alternative protease (e.g., 50 μg ml^{-1} proteinase K) or a combination of proteases is recommended.

9| Assess recorded images and quantify signal intensities to determine the subcellular localization and topology of the protein. Freely available image analysis software (i.e., NIH Image, Image J) or software on existing microscope platforms (e.g., Zeiss LSM Image Examiner, Zeiss) can be used to measure fluorescence intensities.

● TIMING

Steps 1 and 2 require 30 min for cell transfection plus 6–20 h to detect fluorescent protein expression in transfected cells. Steps 3–8 require about 20 min for sample preparation and data acquisition to carry out the FPP assay on one sample. Analyzing the images and quantifying the signal intensities (Step 9) require an additional 10–20 min per sample.

? TROUBLESHOOTING

See **Table 1**

TABLE 1 | Troubleshooting table

PROBLEM	POSSIBLE REASON	SOLUTION
GFP alone does not diffuse out of the cells upon digitonin application.	Inefficient digitonin concentration for plasma membrane permeabilization.	Increase digitonin concentration.
The effective digitonin concentration for cell permeabilization determined in one chamber does not work efficiently for cells in another chamber.	Different chambers have different cell confluencies. Higher confluencies lead to slightly reduced plasma membrane permeabilization by digitonin.	Keep cell numbers and confluencies identical between different cell chambers.
Digitonin permeabilizes intracellular membranes.	Too high a digitonin concentration can permeabilize intracellular membranes.	Reduce digitonin concentration. Determine the concentration for efficient plasma membrane permeabilization without affecting intracellular membranes.
Intracellular organelle morphologies change dramatically upon prolonged digitonin incubation.	Organelle morphologies of different cell types are variably affected by digitonin.	Keep digitonin incubation time as short as possible and wash cells in KHM buffer before protease treatment.
Digitonin permeabilization and protease digestion vary significantly between cells in the same chamber.	The distribution of digitonin or the protease is restricted in the cell chamber.	Ensure efficient blending of digitonin and trypsin by adding sufficient volumes to the chamber.

ANTICIPATED RESULTS

Assuming a successful transfection followed by the expression of fluorescent proteins in cells (Steps 1 and 2), the FPP assay will immediately provide data upon permeabilization of the plasma membrane by digitonin (Steps 3–7). The disappearance of fluorescent signals from the cells indicates that the protein of interest is freely diffusing in the cytosol and nucleoplasm and is neither membrane-associated nor localized in the lumen of an intracellular organelle. Such a complete disappearance of signal from the cells is also observed for GFP alone, which can thus be used as the control molecule to determine the appropriate digitonin concentration (Step 5).

By contrast, fluorescent signals that are retained upon cell permeabilization indicate that the protein of interest is not unbound, i.e., it is not freely diffusing in the cytosol and nucleoplasm. Incubation of permeabilized cells with trypsin (Step 8) then provides further information about the subcellular localization and topology of the protein. At this point, knowing the exact position of the GFP tag within the fusion protein sequence becomes important for topology determinations. If a protein is contained within a protected subcellular environment like the lumen of intracellular organelles, the GFP signal will be unaffected by the addition of protease regardless of the specific placement of the GFP tag within the fusion protein (i.e., N- or C-terminal or central). By contrast, if the protein of interest spans the membrane of an intracellular organelle such that some domains are facing the lumen of the organelle and other domains are exposed to the cytosol, the placement of the GFP tag will dictate whether or not its fluorescence is resistant to protease addition. For instance, the termini of type I and II membrane proteins are on opposite sides of the membrane. In such cases, only the protein terminus that faces the cytoplasm is sensitive to the addition of the protease, whereas the opposite terminus, which is within the lumen of the organelle, is protected 'behind' the membrane.

We show here, as an example, the ER membrane protein CD3δ labeled with two different variants of GFP at either its N (YFP-CD3δ) or C terminus (CD3δ-CFP)[2]. NRK cells that expressed both CD3δ fusion proteins and, as a control for cell permeabilization, red fluorescent protein (RFP), were subjected to the FPP assay. Only YFP-CD3δ showed resistance to both cell permeabilization and protease treatment, which in turn confirmed the known topology of CD3δ (ref. 7) as a type I membrane protein (**Fig. 2** and **Supplementary Video 1**).

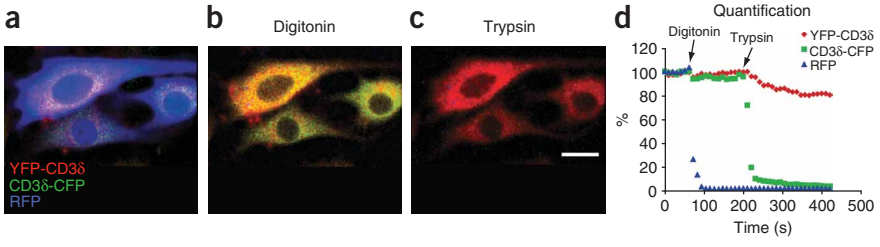

Figure 2 | The FPP assay indicates the topology of the ER membrane protein CD3δ. NRK cells that expressed YFP-CD3δ (shown in red), CD3δ-CFP (green) and RFP (blue) were subjected to the FPP assay. (**a**–**c**) Individual images were taken before (**a**) and after treatment with 20 μM digitonin (**b**) and 4 mM trypsin (**c**). Scale bar, 10 μm. (**d**) The quantification of the fluorescence intensities of the whole time series of the FPP assay (**a**–**c**) is shown.

Note: Supplementary information is available via the HTML version of this article.

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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