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Determine membrane protein topologies in single cells and high-throughput screening applications

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Abstract

Correct localization and topology are crucial for a protein's cellular function. To determine topologies of membrane proteins, a new technique, called fluorescence protease protection (FPP) assay, has recently been described (Lorenz, 2006). The sole requirements for FPP are the expression of fluorescent-protein fusion proteins and the selective permeabilization of the plasma membrane, permitting a wide range of cell types and organelles to be investigated. Proteins topologies in organelles like endoplasmatic reticulum, Golgi apparatus, mitochondria, peroxisomes and autophagosomes have already been determined by FPP. Here, two different step-by-step protocols of the FPP assay are provided. First, we describe the FPP assay using fluorescence microscopy for single adherent cells, and second, we outline the FPP assay for high-throughput screening applications.

Keywords

protein topology; fluorescence microscopy; high-throughput screening

Unit Introduction

This unit describes a fluorescence based imaging technology, which combines live cell microscopy with a biochemical approach to assess transmembrane protein topology. The technique uses fluorescent-protein (FP) - fusion proteins. Our approach provides information about the position of a FP tag relative to a lipid membrane. The FPP assay requires no additional design beyond the construction of a FP fusion expressed either in tissue culture or primary cells. The assay is based upon the inaccessibility of proteases to protected intracellular regions of permeabilized cells. To permeabilize cells, we take advantage of the sharp gradient of cholesterol in the lipid bilayers of cells. Relative to other intracellular membranes, the lipid bilayer of the plasma membrane contains by far the most cholesterol, with approximately 65-80 % of the free cellular cholesterol (Liscum and Munn, 1999). In this assay we use the cholesterol binding drug digitonin to selectively permeabilize the plasma membrane.

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Permeabilization allows the protease trypsin to enter the cell from the extracellular environment. Trypsin meditates the destruction of FPs facing the cytosol. Creating different FP fusion proteins with your protein-of-interest (*e.g.*, N-, C-terminal tag) will shed light on the orientation of the protein (Figure 1).

In this unit, two FPP protocols are described to visualize and quantitate protein topology: FPP for fluorescence microscopes, see **Basic Protocol 1**, and FPP for high-throughput screening using a fluorescence reader, see **Basic Protocol 2**.

Strategic Planning

Fluorescent-Protein Tag

The method requires the construction of a fusion between the coding sequence of a FP and the gene-of-interest. We recommend creating different sets of expression plasmids encoding FP-fusion proteins. For single-spanning membrane proteins, at least two versions of the protein should be created, an amino- and a carboxy-terminal fusion with a FP. For multi-spanning membrane proteins, either a fusion with a FP inserted within the protein of interest or a truncated fusion should be made. Special care must be taken when genes of transmembrane proteins with a targeting sequence or signal sequences are fused to a FP at the amino-terminus. The targeting sequence or signal sequence is often removed by proteolytic cleavage following protein translocation. In the case of an existing targeting sequence or signal sequences, the FP must be inserted right after the cleavage site of the signal sequence to generate a mature fusion protein-of-interest harboring an amino-terminal FP tag.

Basic Protocol 1

FPP-assay for confocal laser scanning microscopes

This protocol describes, first, the plating and transfection of cells in chamber slides, which is required for live-cell imaging, and second, the use of digitonin and trypsin to determine membrane topology of your protein-of-interest.

To optimize the FPP assay with untested proteins-of-interest, please pay special attention to **Support Protocol 1**.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile techniques should be applied accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

CAUTION: Digitonin is toxic by inhalation, by skin contact and if swallowed. Wear suitable protective clothing and gloves.

Materials

Solutions and reagents

- FP expression plasmid (*e.g.*, Clontech) encoding the fluorescent protein-of-interest

  Optional: FP (soluble) expression plasmid, e.g., mCFP, EGFP (Clontech). Whereby soluble and FP-tagged protein-of-interest should have non-overlapping spectral properties.

- Adherent cells to be transfected with a soluble FP and/or a FP fusion plasmid

- Cell culture medium for cells of interest (*e.g.*, RPMI 1640 or DMEM, supplemented with 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin)
- Tissue culture flasks with air-filter cap
- 0.05% (w/v) trypsin/0.53 mM EDTA
- Sterile 15 mL conical tube
- Hemocytometer
- 8-well Lab-Tek chamber slide (Nalge Nunc, cat. no. 155411)
- Transfection reagent (*e.g.*, FuGENE 6 (Roche))
- KHM buffer or cell culture medium without FCS and without phenol red
- Digitonin (Calbiochem, cat. no. 300410)
- Trypsin or an alternative protease, such as proteinase K

Special equipment

- Sterile tissue culture hood
- Inverted fluorescence microscope, capable of time lapse recording

Set up the imaging chamber

1. To image adherent cells on chambered slides, seed cells on Lab-Tek chambered coverglass.

The cell density for imaging depends on cell type, cell size, and experimental design. Confluence of cells should be 60-90% at the time of the FPP assay.

These chambers consist of wells with a cover glass bottom, which permits the use of high-numerical-aperture oil objectives for viewing.

Transfect cells

2. To enhance the probability of having multiple cells to choose from, transfect cells with FP chimeric protein vector using a high-efficiency transfection method, such as a lipid transfection reagent with low toxicity—*e.g.* FuGENE6 (Roche).

Before performing a FPP assay protocol, the investigator must ensure that there is sufficient FP fluorescence in the expressing cell to maintain a significant fluorescent signal relative to background noise during image acquisition. Most standard transfection protocols are sufficient to provide bright specimens. Stable transfectants express lower levels of protein. Transient transfectants usually express higher levels of proteins; this sometimes results in overexpression artifacts, such as protein aggregation or saturation of protein targeting machinery, which lead to inappropriate localization. Immunofluorescence staining of the endogenous protein with specific antibodies should always confirm proper localization of your overexpressed FP-tagged protein.

Adherent cells should be transiently transfected 6 to 24 hours prior to the experiment. Most commercially available FP expression vectors are under the control of a very strong promoter, e.g. CMV promoter.

NOTE: To control plasma membrane permeabilization, it is advised to use double transfected cells. Cells co-expressing your FP-tagged protein-of-interest together with a spectral different soluble FP.

Set up imaging system

3. Set up the fluorescence microscope and its associated hardware.

It is assumed that the investigator is familiar with the basic operation of the microscope.

Use a high-NA objective for maximal signal collection and spatial resolution.

Configure the light path for optimal excitation and emission detection of the fluorophores expressed in your transfected cells.

We recommend that the investigator closely examines the spectral profiles of the FPs of interest to ensure an optimal excitation and emission filter combination.

Most software packages provide the user with a list of preset light path configurations for combinations of common fluorophores. However, the investigator should determine if the preset configuration is indeed optimal for the specific FP of interest and modify the configuration, if needed.

The choice of filters is critical for achieving high signal-to-noise levels and minimizing spectral bleed-through at the same time.

Optional: pre-trypsinisation for plasma membrane localized FP

If you are investigating the topology of a plasma membrane protein, these steps are required, otherwise continue with step 10.

4. Remove cell culture medium from cells co-expressing the FP-tagged protein-of-interest and a soluble FP. Wash cells three times for 1 min each in KHM buffer (or alternatively with serum-free medium) at a temperature that is appropriate for the experiment. In our hands, temperatures of 20–37°C were suitable for the protocol.

5. Place chamber containing cells in KHM buffer on the fluorescence microscope stage.

6. Record images, which represent the ‘pre-trypsinisation and pre-permeabilization’ situation.

7. Add 4–8 mM of the protease trypsin (in KHM buffer) directly onto the cells.

Use effective trypsin concentration according to **Support Protocol 1**.

8. Immediately start taking images on the fluorescence microscope to record how fast fluorescent signals of your protein-of-interest tagged with a FP disappear or persist.

9. Wash cells three times for 1 min each in cell culture medium containing serum at a temperature that is appropriate for the experiment. In our hands, temperatures of 20–37°C were suitable for the protocol.

This step is required to inhibit the protease activity of trypsin before permeabilizing the plasma membrane.

Permeabilize the plasma membrane

10. Remove cell culture medium from cells co-expressing the FP-tagged protein-of-interest and a soluble FP. Wash cells three times for 1 min each in KHM buffer (or alternatively with serum-free medium) at a temperature that is appropriate for the experiment. In our hands, temperatures of 20–37°C were suitable for the protocol.

11. Place chamber containing cells in KHM buffer on the fluorescence microscope stage.

12. Record your first images, which represent the 'pre-permeabilization' situation.

13. To permeabilize the plasma membrane, add the same volume of KHM buffer containing the (previously determined) effective digitonin concentration to the cells (see **Support Protocol 1**).

    The overexpressed soluble FP diffuses freely in the cytosol and nucleoplasm. Effective permeabilization of the plasma membrane by digitonin results in the disappearance of the FP-signal within 10–60 s.

14. Take images of the cells after digitonin application to capture the 'post-permeabilization' situation.

    The fluorescence signal of your membrane-anchored FP will stay and data acquisition should be reduced to few frames to prevent bleaching of your FP.

Disrupt fluorescence signal

15. Wash cells in KHM buffer (optional) and then add 4–8 mM of the protease trypsin (in KHM buffer) directly onto the cells.

    Use effective trypsin concentration according to **Support Protocol 1**.

16. Immediately start taking images on the fluorescence microscope to record how fast fluorescent signals of your protein-of-interest tagged with a FP disappear or persist.

    Duration of imaging should be adapted according to **Support Protocol 1**.

Quantify fluorescence signal-intensities

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

Support Protocol 1

Establish conditions for optimal plasma membrane permeabilization and destruction of FPs

This protocol describes how to optimize plasma-membrane permeabilization by digitonin, followed by the determination of effective trypsin concentration.

Additional Materials

- FP (soluble) expression plasmid, *e.g.*, mCFP, EGFP (Clontech)

- Membrane anchored, but cytosol-facing FP, *e.g.*, Caveolin 1-YFP (Tagawa, 2005), VSVG-YFP (Presley, 1997)

Whereby soluble and membrane anchored FPs must have non-overlapping spectral properties.

Set up the imaging chamber

1. To visualize adherent cells on chambered slides, seed cells on Lab-Tek chambered coverglass.

Transfect cells

2. To enhance the probability of having multiple cells to choose from, transfect cells with soluble and membrane anchored-FP vector using a high-efficiency transfection method, such as a lipid transfection reagent with low toxicity—*e.g.*, FuGENE6 (Roche).

3. Determine the optimal expression level of your FPs and your time requirements for imaging empirically for each sample and condition.

Set up imaging system

4. Set up the fluorescence microscope and its associated hardware.

Permeabilize the plasma membrane

5. Remove cell culture medium from cells expressing a soluble FP only. Wash cells three times for 1 min each in KHM buffer (or alternatively with serum-free medium) at a temperature that is appropriate for the experiment. In our hands, temperatures of 20–37°C were suitable for the protocol.

6. Place chamber containing cells in KHM buffer on the fluorescence microscope stage.

7. Record first images, which represent the ‘pre-permeabilization’ situation.

8. 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. Effective permeabilization of the plasma membrane by digitonin results in the complete disappearance of the fluorescence signal within 10–60 s.

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.

Permeabilize the plasma membrane and disrupt FP

9. To permeabilize the plasma membrane of cells that express a soluble FP and a membrane-anchored FP wash cells in KHM buffer. Record your first images, which represent the ‘pre-permeabilization’ situation.

10. Incubate cells with KHM buffer containing the (previously determined) effective digitonin concentration. Take images of the cells after digitonin application to capture the ‘post-permeabilization’ situation.

11. Wash cells in KHM buffer (optional) and 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 how fast fluorescent signals disappear or persist.

    The effective protease concentration results in the disappearance of the FP-signal within 30-90 s.

    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 g m^{-1} proteinase K) or a combination of proteases is recommended.

Basic Protocol 2

FPP-assay for high-throughput screening (HTS)

This protocol describes, first, the preparation of cells for the HTS FPP-assay, and second, provides a step-by-step guide to determine the membrane localization of your protein-of-choice under a variety of conditions.

It is recommended to determine required concentrations of digitonin and trypsin, timing of the permeabilization step, and FP destruction according to **Basic Protocol 1** and **Support Protocol 1** prior to the following protocol.

Materials

Solutions and reagents

- FP (soluble) expression plasmid, *e.g.*, mCherry (Clontech)
- FP expression plasmid containing DNA encoding the protein-of-interest
- Adherent cells to be transfected with a soluble FP and/or a FP fusion plasmid
- Cell culture medium for cells of interest (*e.g.*, RPMI 1640 or DMEM, supplemented with 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin)
- Tissue culture flasks with air-filter cap
- 0.05% (w/v) trypsin/0.53 mM EDTA
- Sterile 15 ml conical tube
- Hemocytometer
- 10 cm cell culture dish
- Transfection reagent (*e.g.*, FuGENE 6, Roche)
- 96-well tissue culture plates (Special Optics Low Fluorescence Assay Plates (Sigma-Aldrich: Corning® CLS3720)
- KHM buffer or cell culture medium without FCS and without phenol red
- Digitonin (Calbiochem, cat. no. 300410)
- Trypsin or an alternative protease, such as proteinase K

Special equipment

- Sterile tissue culture hood

- Microplate washer and dispenser (*e.g.*, EL406, BioTek)

  Multichannel pipettes may be used for initial trial experiments

- Microplate fluorescence reader (*e.g.*, from Varian (Cary Eclipse), BioTek, Tecan)

  Fluorescence readers with a bottom-reading mode are preferred.

Transfect cells

1. To facilitate robust expression levels of your FPs in cells, transfect cells in a big tissue culture dish (*i.e.* 10 cm tissue culture dish) using a high-efficiency, non-toxic transfection method, such as a lipid transfection reagent with low toxicity —*e.g.*, FuGENE6 (Roche).

Adherent cells should be transiently transfected 18 to 24 hours prior to the FPP assay.

To reduce standard deviation of your HTS use stable transfectants of your protein-of-interest. However, stable transfectants express lower levels of protein in comparison to transient transfected cells. Low protein expression could lead to difficulties in fluorescence detection. For this reason, special care must be taken by choosing the right clone of your stable transfected population of cells.

To control each step of your experiment, it is advised to use four different cells. First, cells transfected with a soluble FP only; second, cells transfected with your protein-of-interest tagged with a FP; third, cells transfected with both plasmids; and fourth, non-transfected cells. Both FPs should have non-overlapping spectral properties.

Set up the imaging chamber

2. Trypsinize the transfected cells, pipet up and down several times to break up cell clumps. Count cells with a hemocytometer and transfer them 5-6 hours after transfection into 96-well tissue culture plates equipped with transparent bottoms.

These chambers consist of white or black wells with a cover glass bottom, which permits the use of fluorescence reader.

The cell density for fluorescence scanning depends on cell type, cell size and experimental design. Confluence of cells should be 70-80% at the time of the FPP assay.

3. Leave cells for at least 12 hours in tissue culture incubator.

Modulate cell culture conditions

4. Add certain drugs or inhibitors, infect with viruses or bacteria etc. according to your protocol.

Set up fluorescence reader

5. Set up the fluorescence reader and the acquisition software.

We recommend that the investigator closely examine the spectral profiles of the FPs of interest to ensure an optimal excitation and emission filter combination.

Most software packages provide the user with a list of preset light path configurations for combinations of common fluorophores. However, the

investigator should determine if the preset configuration is indeed optimal for the specific FP of interest and modify the configuration, if needed.

The choice of filters (bandwidth of fluorescence detection) and time of acquisition is critical for achieving high signal-to-noise levels and minimizing spectral bleed-through at the same time.

The bottom-reading mode should be preferred over top reading, as it provides better signal-to-noise ratios for adherent cells.

Permeabilize the plasma membrane

6. Place 96-well plate with transfected cells in microplate washer and dispenser.
7. Remove cell culture medium from cells and wash cells three times for 1 min each in KHM buffer (or alternatively with serum-free and phenol-red free medium) at a temperature that is appropriate for the experiment. In our hands, temperatures of 20–37°C were suitable for the protocol.
8. Place 96-well plates with cells in KHM buffer on the fluorescence reader.
9. Record your first dataset, which represents the ‘pre-permeabilization’ situation.
10. Place 96-well plate in microplate washer and dispenser.
11. Add the same volume of KHM buffer containing the (previously determined) effective digitonin concentration to the cells (see **Support Protocol 1**).

    The soluble FP diffuses freely in the cytosol and nucleoplasm. Effective permeabilization of the plasma membrane by digitonin results in the disappearance of the FP-signal within 10–60 s.
12. Place 96-well plates in digitonin/KHM buffer on the fluorescence reader.
13. Measure fluorescence of the cells after digitonin application to capture the ‘post-permeabilization’ situation. Use end-point or single-point acquisition mode to prevent bleaching of your fluorescence signal.

Destruction of fluorescence signal

14. Place the 96-well plate with your permeabilized cells in the microplate washer and dispenser.
15. Wash cells in KHM buffer (optional) and then add 4–8 mM of the protease trypsin (in KHM buffer) directly onto the cells.
16. Place the 96-well plate with your cells in trypsin/KHM buffer on the fluorescence reader.
17. Use kinetic-fluorescence acquisition mode to record how fast fluorescent signals of your protein-of-interest tagged with a FP disappear or persist. Measure in intervals to prevent bleaching of your fluorescence signal; *e.g.*, every 1-minute, dependent on your previously determined timing of trypsin mediated disruption of the fluorescence signal (see **Support Protocol 1**).

Quantify fluorescence signal intensities

18. Assess acquired fluorescence data. Normalize your data using the fluorescence signal of non-transfected cells (‘blank’). Use signal intensities of replicates to calculate the median and standard deviation of your samples, and determine loss of fluorescence signal dependent on different treatments or concentration.

Reagents and Solutions

Use deionized, distilled water in all recipes and protocol steps.

Digitonin stock solution

Dissolve digitonin in water (24.58 mg/mL, 20 mM) by shaking in a thermomixer at 100°C until a clear solution is obtained. Commercial digitonin has sometimes very low solubility because of purity problems. Choose always highest-purity or purify digitonin according to a protocol by Janski and Cornell (1980). Solubilized digitonin remains stable in solution for at least one week at room temperature. The capability of digitonin to permeabilize membranes may differ from batch to batch from all suppliers because of differences in purity.

KHM buffer

110 mM potassium acetate, 20 mM HEPES pH 7.4, 2 mM $MgCl_2$

Protease solution

Dissolve trypsin in KHM buffer to get an 8 mM solution. Alternative, *50 $\mu g\ ml^{-1}$* proteinase K can be used for the FPP assay. Make aliquots and store at -20°C. Don't storealiquots longer than 12 months.

Commentary

Background Information

The FPP assay utilizes the specific permeabilization of the cholesterol-rich plasma membrane by digitonin and the resulting accessibility of intracellular compartments by unspecific proteases.

Cholesterol is the prevalent sterol in vertebrates, and the intercalation of digitonin into cholesterol-rich membranes leads to their leakiness. Digitonin forms a complex with unesterified 3-β-hydroxysterols (Takagi, 1982).

The extent of permeabilization is sufficient to allow cytosolic contents to diffuse across the plasma membrane. However, intracellular organelles and the cytoskeletal system are retained in cells permeabilized with digitonin (Plutner 1992; Wilson 1995). In addition to movement of cytosolic contents out of the cell, permeabilization allows relatively small molecules like the protease trypsin to enter the cell from the extracellular environment.

Digitonin is unable to permeabilize lipid membranes with low cholesterol content efficiently (endoplasmatic reticulum, Golgi, peroxisomes, mitochondria and autophagosomes). Hence, lipid membrane enclosed organelles are not accessible to trypsin. Trypsin will exclusively destruct the FP, if it is facing the cytosol or exoplasmic environment. Therefore, the FPP assay provides information about the localization and membrane orientation of a fluorescence tagged protein.

Critical Parameters

The critical parameters for setting up time-lapse imaging (**Basic Protocol 1**) and high-throughput screening (**Basic Protocol 2**) experiments are described in great detail in the respective protocols. There are several considerations for microscopy imaging and high-throughput screening experiments in living cells that require additional emphasis.

Choice of fluorescent protein

The investigator should use the latest generation of spectral variants, which have been improved for folding, chromophore maturation, brightness and photostability. In all cases, the investigator needs to minimize undesired photobleaching of the FPs during data acquisition. Bright and photostable FPs are preferable. For multifluorophore imaging, the investigator should closely examine the spectral profiles of the FPs of interest, as well as the excitation and emission filters available in the microscope and fluorescence reader setup, in order to optimize the light path and obtain maximal signal without spectral bleed-through. A further problem is the potential aggregation of some fluorescent proteins, which impedes any cellular application and the FPP assay especially.

Choice of objectives

High-numerical aperture (NA) oil-immersion objectives corrected for coverglass thickness, field curvature, and chromatic aberrations (*e.g.*, Zeiss Plan-Apochromat 63×/1.4 NA oil lens or the Olympus PLAN APO 60×/1.4 NA oil lens) are good choices for high-resolution imaging. However, high magnification and high numerical aperture objectives may increase the risk of photobleaching.

Troubleshooting

Table 1 lists some problems that may be encountered in fluorescence imaging and HTS of your FPP assay along with some possible causes and solutions.

Anticipated Results

By following the suggestions given in every protocol, the investigator should be able to select cells expressing the fluorescent-tagged protein of interest. Plasma membrane permeabilization upon digitonin treatment will immediately provides data whether the protein of interest is stably bound to any cellular structure or is freely diffusing in the cytosol or nucleoplasm (the fluorescence signal disappears). Such a complete disappearance of signal from the cells is also observed for soluble FP alone. As outlined in **Support Protocol 1**, a soluble FP alone can be used as the control molecule to determine the appropriate digitonin concentration to permeabilize the plasma membrane.

Addition of trypsin to digitonin-permeabilized cells will provide further information about the subcellular localization and topology of the protein-of-interest. The exact position of the FP tag within the fusion protein sequence is important for topology determination using the FPP assay. If the FP tag is fused to the terminus of a protein, which is enclosed within a protected subcellular environment like the lumen of intracellular organelles, the FP signal will be resistant to protease addition. By contrast, if the protein of interest spans the membrane of an intracellular organelle and the FP tagged domain is exposed to the cytosol, the FP tag will be affected by the addition of protease.

Time Considerations

Basic Protocol 1

Steps 1 and 2 require 30 min each for cell plating and transfection, plus 6–24 h to detect fluorescent protein expression in transfected cells. Steps 3–10 require about 20 to 30 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 17) require an additional 10–20 min per sample.

Basic Protocol 2

Steps 1 and 2 require 30 min each for cell plating and transfection, plus 12–24 h to detect fluorescent protein expression in transfected cells (step 3). Steps 5–17 require about 20 to 30 min for sample preparation and data acquisition to carry out the FPP assay on one 96-well plate. Analyzing the images and quantifying the signal intensities (step 18) require an additional 10-20 min per plate.

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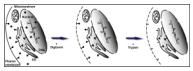

Figure 1. Mechanism and possible applications of the FPP assay

Cartoon of the FPP assay illustrates localization of different fluorescent-protein tagged proteins, soluble or membrane bound. Addition of digitonin will permeabilize the plasma membrane and causes release of unbound cytosolic and nuclear molecules (star). Membrane (disc) or DNA bound (spiral) molecules will stay. Incubation with the protease trypsin digests all fluorescent-proteins, which are not protected by a lipid membrane. Luminal proteins of the ER (rectangle) or mitochondrium (triangle) are protected from proteases by a lipid membrane. These shielded fluorescence signals will remain.

Table 1

Troubleshooting Guide for Fluorescence Protease Protection (FPP) Assay

PROBLEM	POSSIBLE CAUSE	SOLUTION
Fluorescence signal of soluble FP does not disappear after digitonin application.	Inefficient digitonin concentration for plasma membrane permeabilization.	Increase digitonin concentration.
The effective digitonin concentration for cell permeabilization does not work in every cell culture well.	Difference in cell confluencies lead to altered plasma membrane permeabilization.	Keep cell confluencies constant in different cell chambers.
	Remaining media with FCS (contains cholesterol) in the cell culture chamber capture digitonin.	Be sure to wash 3-times your samples with KHM buffer before application of digitonin.
Digitonin permeabilizes intracellular membranes.	Too high digitonin concentration may permeabilize membranes with low cholesterol content.	Reduce and optimize digitonin concentration.
Fluorophore signal on either side of a organelle membrane is gone after addition of trypsin.	Both fluorophores were bleached.	Decrease excitation light intensity or reduce acquisition time per frame.
	Too high digitonin concentration.	Reduce and optimize digitonin concentration.
Intracellular organelle morphology change dramatically upon prolonged digitonin incubation.	Organelle morphology of different cell types are variable affected by digitonin.	Keep digitonin incubation time as short as possible and wash cells in KHM buffer immediately after permeabilization.
	Change of milieu for very sensitive organelles, like mitochondria.	Replace KHM buffer with a more complex, cytosol like buffer (Kuznetsov, 2008).
Digitonin permeabilization and protease digestion vary significantly between cells in the same chamber.	The distribution of digitonin and protease is restricted in the cell chamber.	Ensure efficient blending of digitonin and protease by adding sufficient volumes to the chamber.
Cells are mostly on the curb of the tissue culture well.		Use a small volume for cell seeding and add more medium later.
Cells do not adhere to tissue culture well with glass bottom for imaging or HTS.	Cells adhere better to plastic than to glass.	Try fibronectin or laminin coating before seeding.
No difference in fluorescence signal before and after digitonin treatment with a soluble FP in HTS-protocol.	Bottom reader detects FP, which was released from cells into supernatant.	Add one additional wash step between digitonin treatment and scanning with fluorescence reader.
Very low signal with fluorescence reader.	You are using a top reader.	Use bottom reader mode. Use a well-established FP tagged protein to verify sensitivity of your fluorescence reader.
	FP expression is very low.	Choose a higher expressing clone of your stable transfected cell line.