Fluorescence protease protection of GFP chimeras to reveal protein topology and subcellular localization

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Understanding the cell biology of many proteins requires knowledge of their *in vivo* topological distribution. Here we describe a new fluorescence-based technique, fluorescence protease protection (FPP), for investigating the topology of proteins and for localizing protein subpopulations within the complex environment of the living cell. In the FPP assay, adapted from biochemical protease protection assays, GFP fusion proteins are used as noninvasive tools to obtain details of protein topology and localization within living cells in a rapid and straightforward manner. To demonstrate the broad applicability of FPP, we used the technique to define the topology of proteins localized to a wide range of organelles including the endoplasmic reticulum (ER), Golgi apparatus, mitochondria, peroxisomes and autophagosomes. The success of the FPP assay in characterizing the topology of the tested proteins within their appropriate compartments suggests this technique has wide applicability in studying protein topology and localization within the cell.

Eukaryotic cells are organized into a complex array of membranes and compartments that are specialized for various biological functions. Within these cellular microenvironments, proteins adopt specific topological distributions: they can be free in the cytoplasm, embedded within membranes, localized within organelle lumens or tightly associated with nonmembranous structures. The specific topological distribution of a protein within these microenvironments dictates its accessibility to interacting and modifying partners and thus underlies its correct functioning within a cell.

Despite the important role of topology in protein function, the techniques now available for characterizing the topological distribution of proteins within the diverse microenvironments of the cell are limited. Computational approaches examining the relative hydrophobicity of different polypeptide regions of a protein can predict membrane-spanning domains that are characteristic of transmembrane proteins, but the predictions are not fully reliable, and different algorithms have led to different results[1]. Moreover, such predictions often do not clarify whether the amino or carboxy terminus of a protein faces the cytoplasm, lumen or cell exterior. Another approach for determining membrane protein topology is to engineer epitopes, such as hemagglutinin (HA) and myc, into the protein of interest and then to assess the immunoreactivity of these epitopes before and after cell permeabilization by antibody labeling. Although useful, this technique has mostly been used to reveal whether the fused epitope is exposed extracellularly or intracellularly. To obtain more detailed topological information about transmembrane proteins located within the membranes of intracellular organelles, more demanding experimental approaches, such as limited proteolysis[2], glycosylation mapping[3] and cysteine substitution[4,5] have been used. Because these approaches often require substantial investments of time and effort, widespread knowledge of the topology of proteins within different cellular microenvironments is lacking.

In this study, we describe a new technique for determining the topological distribution of proteins in living cells. The assay, termed FPP, provides a fluorescence readout in response to trypsin-induced destruction of GFP attached to a protein of interest before and after plasma membrane permeabilization. The method requires the construction of a fusion between the GFP coding sequence and the gene of interest, which is then expressed in cells. Using this method, one can determine whether a protein is membrane-associated, cytoplasmic or lumenal and which part of a membrane protein faces the lumen (or cell exterior) and cytoplasm. We have evaluated the FPP assay using transmembrane and lumenal proteins associated with a wide variety of intracellular compartments and demonstrate its usefulness for uncovering a protein's topological distribution and interactive properties within the cell.

RESULTS

Establishment of the FPP assay

In developing a fluorescence-based assay for determining protein topology within cells, we attached a fluorescent protein to the N or C terminus of a protein of interest. Subsequently, cells expressing the fusion protein were exposed to trypsin either before or after plasma membrane permeabilization by digitonin[6,7]. If the fluorescent protein moiety on the expressed protein faces the environment exposed to trypsin (that is, the cytoplasm), then its fluorescence signal should be lost. Conversely, if the fluorescent protein moiety on the expressed protein faces the environment protected from trypsin (that is, the lumen of a compartment) then

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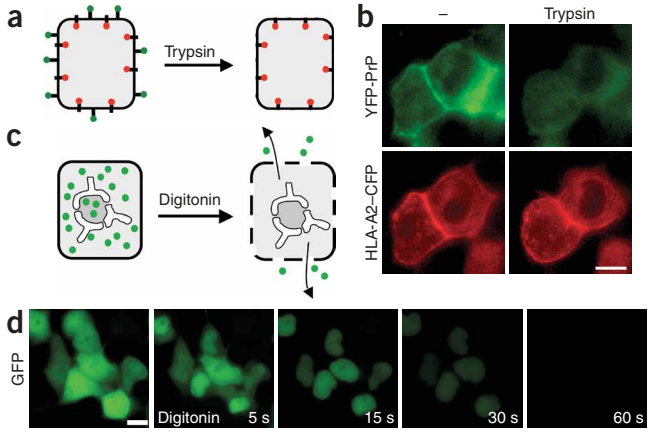

Figure 1 | Extracellular trypsin is unable to enter the interior of intact cells. (**a**) Cartoon of a single cell before and after trypsin treatment showing the localization and topology of YFP-PrP (green) and HLA-A2–CFP (red) on the plasma membrane. (**b**) N2a cells coexpressing YFP-PrP and HLA-A2-CFP were subjected to 6 mM trypsin for 100 s. Images were taken before and after trypsin treatment as indicated. Note that the membrane signal of YFP-PrP was destroyed by the protease, whereas HLA-A2–CFP's membrane signal remained unaffected. (**c**) Cartoon of a single cell before and after digitonin treatment. The green balls represent GFP and its localization in the cytosol and nucleoplasm. (**d**) Digitonin treatment permeabilizes the plasma membrane and releases first cytosolic and later nucleoplasmic GFP. N2a cells expressing GFP were treated with 20 μM digitonin. Images were taken before and after digitonin application at the indicated time points. Scale bars, 10 μm.

its fluorescence should persist. Given these potential outcomes and the fluorescent protein's known engineered position within the protein, we could deduce the orientation of the protein across the lipid bilayer. Additionally, color variants of GFP allowed us to simultaneously image a control protein in one channel and a protein of interest in a second channel.

To evaluate the FPP assay, we first assessed the effect of trypsin on nonpermeabilized cells. Treatment with trypsin should readily extinguish fluorescence associated with fluorescent protein–tagged molecules facing the cell exterior, but should have no effect on fluorescence associated with molecules facing the cell interior. To confirm this, we designed two constructs to express plasma membrane proteins: the glycosylphosphatidylinositol (GPI)-anchored prion protein, PrP, with yellow fluorescent protein (YFP) attached to its N terminus (YFP-PrP)[8], which faces the extracellular space, and HLA-A2 with cyan fluorescent protein (CFP) attached to its C terminus (HLA-A2–CFP), which faces the intracellular space. We imaged cells expressing these chimeras before and after trypsin was added to the medium (**Fig. 1a**,**b**).

Before trypsin treatment, the fluorescent signals from both the cell-surface and intracellular pools of these molecules were bright (**Fig. 1b**). After trypsin treatment, the intracellular signal from YFP-PrP remained, but its plasma membrane signal vanished, whereas both the intracellular and plasma membrane signal from HLA-A2–CFP were preserved (**Fig. 1b**). These results indicate that upon exposure to trypsin, fluorescence associated with GFP-tagged molecules facing the cell exterior is efficiently abolished whereas fluorescence from intracellular molecules persists in nonpermeabilized cells.

We next determined what concentration of digitonin, a small toxin derived from the plant *Digitalis purpurea,* was necessary to permeabilize cells within the assay. When added to cells, digitonin intercalates into cholesterol-rich membranes by forming a complex with hydroxysterols, causing the plasma membrane to become perforated[6]. Cytosolic contents diffuse out of the cell, and small exogenous molecules, like trypsin, can diffuse into the cell and into the nucleus. Notably, the permeabilizing effect of digitonin is limited to the cholesterol-rich plasma membrane. Membranes of intracellular organelles have much lower concentrations of cholesterol and are unaffected when appropriate digitonin concentrations are used[9].

To establish the minimum concentration of digitonin necessary to permeabilize the plasma membrane sufficiently for trypsin to enter, we added digitonin to cells expressing GFP. GFP freely diffuses in the cytosol and nucleoplasm of cells and thus reflects the behavior of the soluble, cytosolic content (**Fig. 1c**,**d**). We identified cells expressing GFP on the microscope stage and washed them in KHM buffer[7]. Increasing digitonin concentrations were then added while GFP fluorescence was monitored. Permeabilization was evident by the complete loss of GFP signal within 1 min of digitonin addition (**Fig. 1d**). Digitonin concentrations ranging from 10 to 50 μM were sufficient to release GFP from the cytosol in many mammalian cell lines, including N2a, NRK, CHO, HeLa, COS-7 and BHK (**Fig. 1d** and data not shown). Because the efficiency of digitonin permeabilization of the plasma membrane decreased slightly with increased confluency of cells, we routinely used 20 μM digitonin to permeabilize subconfluent cells (60–90%). Under these conditions we did not detect leakage of soluble proteins in the lumens of the ER, mitochondria and peroxisomes, indicating that the intracellular membranes remained intact (**Supplementary Fig. 1** online).

To test whether trypsin could enter digitonin-permeabilized cells and extinguish fluorescence from GFP molecules facing the cytosol, we added trypsin in digitonin-containing KHM buffer to cells that expressed red fluorescent protein (RFP) and the ER

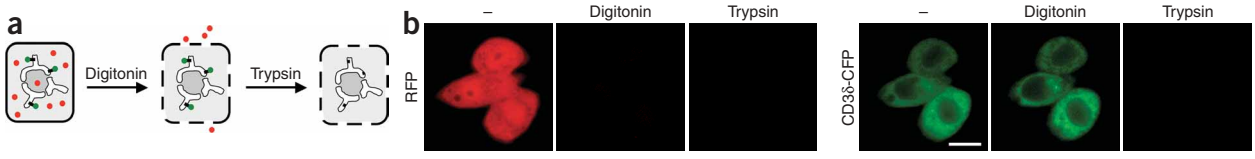

Figure 2 | Digitonin permeabilization releases the freely diffusible cytosolic content and allows trypsin to access the ER membrane protein CD3δ-CFP. (**a**) Cartoon of the FPP assay illustrating a single cell before and after digitonin and trypsin treatment. The red balls represent RFP and its localization in the cytosol and nucleoplasm. The green balls represent CD3δ-CFP in the ER and the position of the fluorescent protein tag relative to its respective membrane. (**b**) FPP assay of N2a cells expressing RFP and the type I membrane protein CD3δ-CFP. Images were taken before and after 1-min treatment with 20 μM digitonin and 4 mM trypsin as indicated. Scale bar, 10 μm.

transmembrane protein CD3δ with CFP attached to its cytoplasmic C terminus (CD3δ-CFP; **Fig. 2a**). Then we monitored the fate of the two different fluorescent signals by fluorescence microscopy. We found that the RFP signal was lost as soon as digitonin was added to cells (**Fig. 2b**), indicating that the cells were permeabilized. The signal from CD3δ-CFP, however, was not affected until trypsin was added to the medium. Then, CD3δ-CFP fluorescence rapidly disappeared from the cells. These results indicate that trypsin readily enters the cytoplasm of cells permeabilized with digitonin and abolishes fluorescence from GFP moieties exposed to the cytoplasm. In all experiments described here, trypsin was applied directly to the cells at concentrations of 4–8 mM at 20–25 °C. These conditions consistently led to an efficient loss of fluorescence from the exposed fluorescent proteins within ~100 s of trypsin addition. A similar rapid loss of fluorescence occurred when trypsin was added to cells that had been washed free of digitonin to avoid prolonged exposure to the permeabilizing agent. Additon of 50 μg/ml proteinase K as an alternative protease led consistently to identical results as seen with trypsin (data not shown).

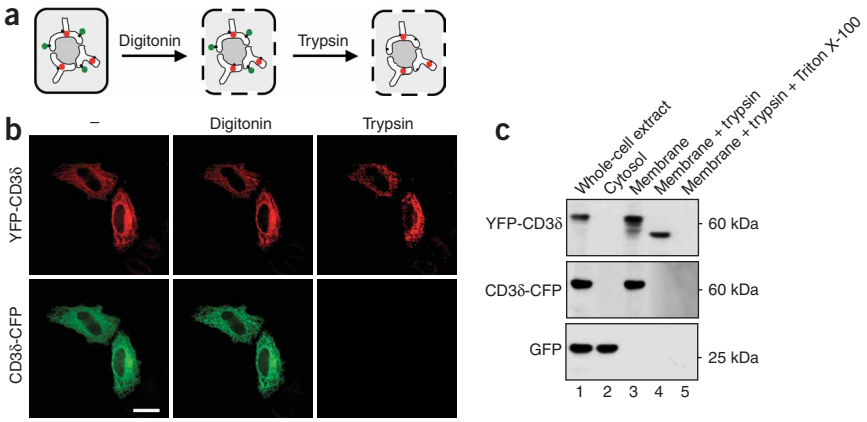

Figure 3 | The FPP assay reveals the topology of the ER membrane protein CD3δ. (**a**) Cartoon of the FPP assay illustrating the cellular localization of ER-bound CD3δ-CFP (green) and YFP-CD3δ (red), and the position of the fluorescent protein tag relative to its respective membrane. (**b**) NRK cells expressing CD3δ-CFP and YFP-CD3δ were subjected to the FPP assay. Images were taken before and after 1-min treatment with 20 μM digitonin and 4 mM trypsin as indicated. Scale bar, 10 μm. (**c**) Biochemical protease protection assay of NRK cells expressing YFP-CD3δ, CD3δ-CFP and GFP. Transfected cells were subjected to SDS-PAGE and Western blot analysis before (lane 1) and after separation of the cytosolic (lane 2) and membrane (lane 3) fractions by ultracentrifugation. Equal amounts of the membrane fraction were treated with trypsin in the absence (lane 4) or presence (lane 5) of Triton X-100. Protein extracts were analyzed by immunoblotting with monoclonal anti-GFP antibody JL-8.

Defining ER membrane protein topology

As proof-of-principle, we used the FPP assay to test whether it could reveal the membrane topology of CD3δ[10], a single-spanning transmembrane protein with its C terminus facing the cytoplasm and its N terminus facing the ER lumen. We constructed two versions of tagged CD3δ and coexpressed them in cells: one with YFP attached to the lumenal N terminus of CD3δ (YFP-CD3δ), and the other with CFP attached to the cytoplasmic C terminus of CD3δ (CD3δ-CFP; **Fig. 3a**).

To determine whether the FPP assay could provide results consistent with the known membrane topology of the constructs, we added 4 mM trypsin to the cells coexpressing CD3δ-CFP and YFP-CD3δ, after permeabilization with 20 μM digitonin for 1 min (**Fig. 3b**). Fluorescence from CD3δ-CFP quickly decreased, suggesting that the CFP moiety on CD3δ facing the cytoplasm was accessible to trypsin. By contrast, fluorescence associated with YFP-CD3δ did not change during the treatment, suggesting the YFP moiety faces the ER lumen where it is protected from trypsin.

We corroborated these results using a biochemical protease protection assay (**Fig. 3c**). We incubated membrane fractions of transfected cells in trypsin-containing buffer, and analyzed them by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. In agreement with the FPP assay, we only observed protection from proteolysis by trypsin for YFP-CD3δ, but not for CD3δ-CFP (**Fig. 3c**). The slight increase in mobility of protected YFP-CD3δ was due to the removal of the cytoplasmic domain of CD3δ by trypsin, leading to a shortened fusion protein whose protected YFP tag was detectable by GFP antibodies. Addition of the detergent Triton X-100 before protease digestion demonstrated the susceptibility of the protected portions of YFP-CD3δ to trypsin digestion

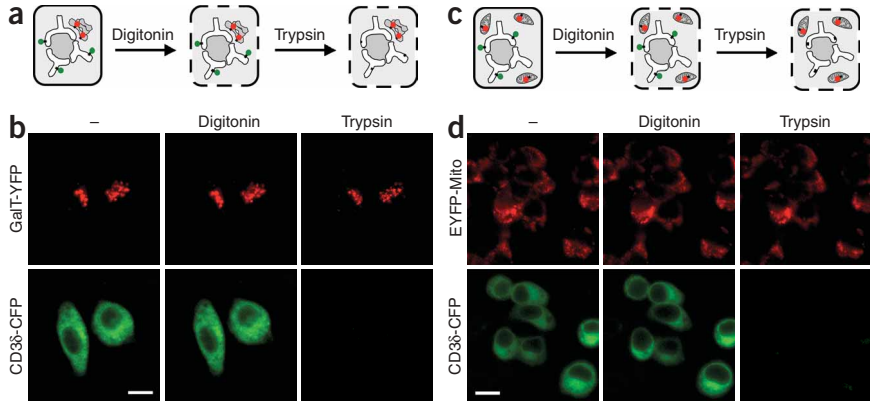

Figure 4 | FPP assays can be used to determine the topology of membrane proteins localized to the Golgi apparatus and mitochondria. (**a**) Cartoon of the FPP assay illustrating the cellular localization of the Golgi marker GalT-YFP (red) and ER-bound CD3δ-CFP (green), and the position of the fluorescent protein tag relative to its respective membrane. (**b**) N2a cells coexpressing GalT-YFP and CD3δ-CFP were subjected to the FPP assay, as in **Figure 2b**. (**c**) Cartoon illustrating localization of mitochondrial EYFP-Mito (red) and Cd3δ-CFP (green). (**d**) N2a cells coexpressing EYFP-Mito and CD3δ-CFP were subjected to the FPP assay, as in **b**. Scale bars, 10 μm.

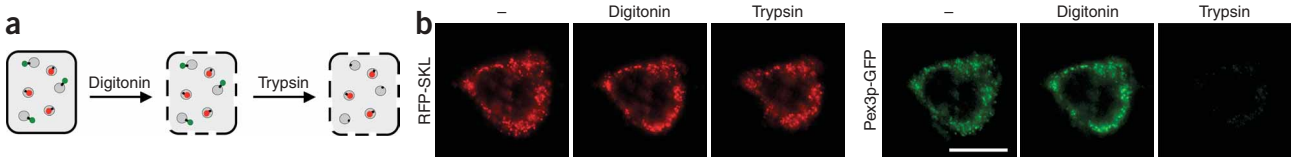

Figure 5 | The FPP assay reveals the localization and orientation of proteins localized to peroxisomes. (**a**) Cartoon of the FPP assay illustrating the cellular localization of RFP-SKL (red) and Pex3p-GFP (green) and the position of the fluorescent protein tag relative to its respective membrane. (**b**) N2a cells coexpressing RFP-SKL and Pex3p-GFP were subjected to the FPP assay. Images were taken before and after 1-min treatment with 20 μM digitonin and 100-s treatment with 4 mM trypsin as indicated. Scale bar, 10 μm.

after membrane solubilization (**Fig. 3c**). The biochemical approach also confirmed our observation (**Fig. 2**) that digitonin permeabilization selectively removes the soluble, cytosolic fraction without affecting the integrity of proteins within membranes. Cell fractionation by ultracentrifugation of digitonin-treated cells revealed that the membrane proteins YFP-CD3δ and CD3δ-CFP remained in the membrane fraction, whereas soluble GFP was exclusively detectable in the supernatant (cytosolic) fraction (**Fig. 3c**).

Based on these results, we concluded the following: (i) the FPP assay functioned properly in that trypsin treatment abolished fluorescence associated with GFP-tagged molecules exposed to the cytoplasm, and (ii) trypsin treatment had no effect on fluorescence associated with GFP-tagged molecules within the lumen of a membrane-bound compartment. Hence, the assay could correctly predict the membrane topology of GFP-tagged CD3δ proteins spanning the ER lipid bilayer.

Applications of FPP using different organelle markers

We further evaluated the applicability of the FPP assay by testing whether it could properly define the topology of other membrane proteins localized to the Golgi apparatus, mitochondria and peroxisomes. We first examined a protein of the Golgi apparatus using a truncated form of galactosyltransferase (GalT), a type II transmembrane protein with its N terminus facing the cytoplasm and C terminus facing the Golgi lumen. Previously, we had designed the fusion protein GalT-YFP, with YFP attached to the C terminus of GalT[11] (**Fig. 4a**). In cells coexpressing GalT-YFP and CD3δ-CFP, the Golgi marker labeled a juxtanuclear structure characteristic of the Golgi apparatus, whereas CD3δ-CFP labeled widely distributed ER membranes (**Fig. 4b**). Upon 20 μM digitonin treatment, the fluorescence associated with both chimeras remained stable. But once we added 4 mM trypsin, the fluorescence from CD3δ-CFP was abolished within 1 min, whereas fluorescence associated with GalT-YFP persisted. Therefore, the data were consistent with the C terminus of GalT facing the Golgi lumen where it is protected from trypsin.

We next examined the mitochondrial marker, Mito, tagged with enhanced yellow fluorescent protein (EYFP). This protein, EYFP-Mito (Clontech) is a fusion of EYFP and the targeting sequence from subunit VIII of human cytochrome *c* oxidase, which targets it to the mitochondrial matrix[12] (**Fig. 4c**). In cells coexpressing EYFP-Mito and CD3δ-CFP, fluorescence from both markers was stable during 20 μM digitonin treatment (**Fig. 4d**). Upon addition of 4 mM trypsin, the signal from CD3δ-CFP was lost, whereas that from EYFP-Mito persisted. Therefore, EYFP-Mito was protected from proteases in the cytoplasm, as expected for a mitochondrial matrix protein.

Next we tested two different classes of peroxisomal proteins in the FPP assay. These were RFP tagged SKL (RFP-SKL), a peroxisomal matrix signal peptide, and Pex3p-GFP, a doubly-spanning membrane protein involved in peroxisomal membrane import[13]. Whereas RFP-SKL should be protected from trypsin (because it resides inside peroxisomes), Pex3p-GFP was expected to be sensitive (since the GFP moiety resides on the cytoplasmic face of peroxisomes; **Fig. 5a**). Consistent with these predicted topological distributions, the FPP assay revealed that RFP-SKL fluorescence was resistant to 4 mM trypsin and Pex3p-GFP was sensitive (**Fig. 5b**). Results from a recent study using TEV protease to determine Pex3p-GFP topology are in agreement with our data[14].

Together, these experiments demonstrate the suitability of protein candidates in many cell organelles for use in the FPP assay. In no case did the protease pass the respective membrane of the tested organelle. This is shown in the graphs quantifying the changes in fluorescence for each type of organelle protein during the FPP assay (**Supplementary Fig. 2** online). The protein domains facing the organelle lumen were efficiently protected from trypsin, whereas domains exposed to the cytosol were digested by the protease.

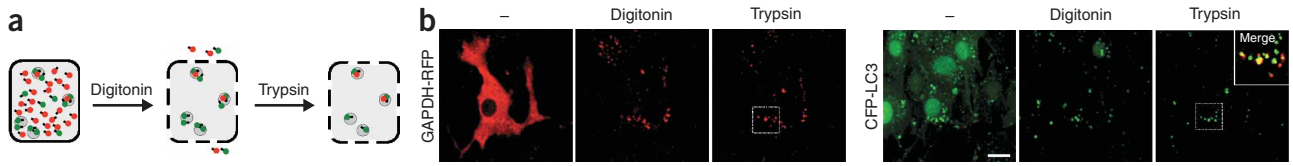

Figure 6 | Detection of the autophagy substrate GAPDH-RFP in autophagosomes by FPP assay. (**a**) Cartoon of the FPP assay illustrating the cellular localization of the proteins and the position of the fluorescent protein tag relative to its respective membrane. GAPDH-RFP is shown in red and CFP-LC3 is shown in green. (**b**) NRK cells stably expressing CFP-LC3 were transiently transfected to express GAPDH-RFP and subjected to the FPP assay. Images were taken before and after 1-min treatment with 20 μM digitonin and 100-s treatment with 4 mM trypsin. The inset shows the overlap of the GAPDH-RFP and CFP-LC3 signals (4× magnification of the central area (outlined by a box) of the micrographs. Scale bar, 10 μm.

FPP applied to identify protein subpopulations

Small subpopulations of proteins that are associated with either the lumen or membrane of organelles can be difficult to visualize against a much larger population of the same freely diffusing protein. As the diffusing population masks signals from the bound subpopulation, an approach is needed to separate the population of interest from the masking majority. Therefore, we used the FPP assay to investigate subpopulations of molecules localized within small membranous organelles like autophagosomes[15] and the topological organization of proteins within these organelles.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of several abundant, cytosolic and long-lived proteins removed from the cytoplasm by autophagosomes. GAPDH has been identified in autophagosomes by two-dimensional gel isolation and mass spectrometry analysis, and its half-life is sensitive to inhibitors of the autophagy pathway[16–18]. To determine whether GAPDH could be visualized in autophagosomes, we transfected a plasmid encoding GAPDH-RFP into a clonal cell line stably expressing the autophagosome marker, CFP-LC3 (ref. 19; **Fig. 6a**). Positively transfected cells showed a strong and abundant signal for GAPDH-RFP in the cytosol, making it impossible to detect any subcellular structures (**Fig. 6b**). Upon permeabilization of the cells with digitonin (20 μM), the majority of GAPDH-RFP fluorescence was immediately lost, indicating that most of GAPDH-RFP was free within the cytoplasm (**Fig. 6b**). But small signals of GAPDH-RFP persisted as punctae in the cytosol. These punctae colabeled with CFP-LC3, suggesting that GAPDH-RFP is a *bona fide* substrate for autophagosomes. Furthermore, the subsequent addition of 4 mM trypsin to these cells resulted in the complete protection of the GAPDH-RFP signal and a limited disappearance of the CFP-LC3 fluorescence signal (**Fig. 6b**). This observation is consistent with electron microscopy studies that show LC3 localized to both the outer and inner membranes of autophagosomes[20]. Hence, the FPP assay allowed the detection of the abundant cytosolic protein GAPDH as a substrate of autophagy and substantiated the membrane topology of the autophagosome molecule, LC3.

DISCUSSION

The correct topological distribution within membranes and other microenvironments is essential for proper functioning of many cellular proteins. The FPP assay described here provides a new approach for determining protein topology in cells that offers many advantages over previous methods. First, unlike many biochemical assays, the FPP method does not require large quantities of target proteins. In our hands, only a relatively small number of cells expressing the GFP-tagged protein candidate at detectable levels was needed to unequivocally determine the protein's topology. This may be of particular importance in cell systems that are difficult to transfect or to culture (for example, many primary neuronal cells). Second, the use of GFP chimeras in the FPP technique eliminates the need for appropriate and available antibodies that are required in protein topology assays relying upon site-specific immunoreactivity[21]. In rare cases, the addition of GFP interferes with proper functioning and localization of the fusion protein, which needs to be considered and addressed. Many studies, however, have already demonstrated that the vast majority of GFP chimeras retain the characteristics of the untagged protein. Third, many *in vitro* translation strategies for protein topology determination are limited to ER-derived microsomes as the source of cellular membranes[22,23]. As the FPP assay surveys proteins that are delivered by the cellular machinery to their correct destination, these restrictions no longer exist.

The FPP assay is limited in its ability to discriminate slightly different membrane topologies of the same protein. For example, as long as the fluorescent protein tag is protected by a lipid bilayer, the assay will not distinguish between a fully translocated and a transmembrane form of the same protein. Therefore, more elaborate techniques that allow the separation of different protein sizes in gels after proteolysis may still be preferable. For many experimental systems, however, the FPP assay's straightforwardness and reliability make it a valuable technique for obtaining data about the general orientation of a protein within a membrane.

Regarding its applicability, the FPP assay can be used in many different cell biological systems. The assay has only two requirements: expression of a GFP fusion protein and selective permeabilization of the plasma membrane. GFP fusion proteins can be exogenously expressed in eukaryotic cell types ranging from yeast to immortalized mammalian cell lines[24]. Additionally, GFP expressing primary cells can be isolated from transgenic organisms or organisms infected with viral expression vectors encoding GFP fusion proteins. The use of digitonin as a permeabilization reagent is also well established for a wide range of eukaryotic cell types from *Saccharomyces cerevisiae* to *Homo sapiens*[6,7,25,26]. Furthermore, because GFP fusion proteins exist for many of these systems already, the assay can be applied with minimal additional effort to extend information about proteins of interest.

In addition to small-scale studies involving specific proteins of interest, FPP is applicable to large-scale analysis. The number of fully sequenced genomes is steadily growing and comprehensive projects are now underway to characterize large numbers of encoded proteins using high throughput approaches. Large portions of the yeast and other proteomes are already GFP-tagged[27–32]. Because the FPP assay has a fluorescence-based readout, the assay can be applied *en masse* to obtain additional important information about large GFP-tagged protein arrays.

Finally, the FPP technique is likely to become an important new tool for cell biology research and pharmacological development. These areas of study are critically dependent on knowledge of protein topology for understanding protein function. Over 25% of all sequenced open reading frames encode membrane proteins, and these molecules constitute the majority of the known drug targets[5]. Given the ability of the FPP assay to easily and reliably establish the orientation of a transmembrane protein within an intracellular compartment, this technique should be considered as a method of choice to study membrane protein topology and sterical protein arrangement within the cell.

METHODS

Cell culture and transfection. We obtained NRK and N2a cells from the American Type Culture Collection. We grew the cells in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Inc.) containing 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 5% CO_2, 95% air at 37 °C. We performed transient transfections of cells using the Fugene 6 reagent (Roche) according to the manufacturer's instructions. For imaging, we grew cells on Lab-Tek chambered coverglass (Nalge Nunc) and analyzed them 24 h after transfection.

Biochemical protease protection assay. For biochemical proteolysis studies, we grew equal amounts of NRK cells and transfected them in 24-well plates (Corning). Twenty-four hours after transfection, we detached the cells by trypsin and washed them twice in serum-containing DMEM and three times in KHM buffer (110 mM potassium acetate, 20 mM HEPES, 2 mM $MgCl_2$). We prepared whole-cell extracts from one quarter of the cells of each sample and transferred the remaining three quarters into three reaction tubes. Cells in the three tubes were pemeabilized in KHM buffer containing 20 μM digitonin on ice for 10 min. After permeabilization, we immediately ultracentrifuged the cells in one tube at 100,000*g*, 4 °C for 30 min. We separated the supernatant and the pellet and boiled them in SDS-Laemmli buffer to obtain protein extracts for the cytosolic and the membrane fraction. We incubated the cells in the other two tubes with 4 mM trypsin in the absence or presence of 1% (vol/vol) Triton X-100 (Sigma) on ice for 30 min, and then ultracentrifuged them at 100,000*g*, 4 °C for 30 min. We discarded the supernatants and boiled the pellets in SDS-Laemmli buffer. We subjected equal aliquots of the samples to SDS–PAGE and western blot analysis using the monoclonal GFP antibody JL-8 (Clontech).

Fluorescence microscopy and imaging. We performed confocal laser scanning microscopy on Zeiss LSM 510 confocal systems. All images were taken with a 40× 1.4 numerical aperture (NA) oil differential interference contrast Plan-Neofluar or 63× 1.4 NA Apochromat objective (Zeiss). For quantification of fluorescence intensities, nonsaturated images were taken with a full-open pinhole. For multichannel imaging, each fluorescent dye was imaged sequentially in the frame-interlace mode to eliminate cross-talk between the channels. All image processing was performed using the Zeiss LSM 5 image examiner software.

Additional methods. Description of plasmid construction is available in the **Supplementary Methods** online.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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