Chapter 7

Investigating Alternative Transport of Integral Plasma Membrane Proteins from the ER to the Golgi: Lessons from the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

Margarida D. Amaral, Carlos M. Farinha, Paulo Matos, and Hugo M. Botelho

Abstract

Secretory traffic became a topical field because many important cell regulators are plasma membrane proteins (transporters, channels, receptors), being thus key targets in biomedicine and drug discovery. Cystic fibrosis (CF), caused by defects in a single gene encoding the CF transmembrane conductance regulator (CFTR), constitutes the most common of rare diseases and certainly a paradigmatic one.

Here we focus on five different approaches that allow biochemical and cellular characterization of CFTR from its co-translational insertion into the ER membrane to its delivery to the plasma membrane.

Key words Secretory traffic, Biochemistry, CFTR, Cystic fibrosis, Endoplasmic reticulum, Golgi, High-throughput microscopy, Plasma membrane, N-glycosylation, ABC transporters

1 Introduction

Secretory traffic became a topical field because many key cell regulators are plasma membrane (PM) proteins (transporters, channels, receptors), thus being key targets in biomedicine and drug discovery. Cystic fibrosis (CF), which affects well over 30,000 sufferers in Europe with a further 50 thousand worldwide, constitutes the most common of rare diseases and certainly a paradigmatic one. CF is caused by defects in a single gene encoding the CF transmembrane conductance regulator (CFTR), a chloride/bicarbonate channel that is also a member of the *A*TP-*b*inding *c*assette ABC transporter superfamily.

Like most membrane proteins, CFTR biogenesis and trafficking follow the secretory pathway, from the endoplasmic reticulum (ER) to the plasma membrane through the Golgi apparatus. After

Andrea Pompa and Francesca De Marchis (eds.), *Unconventional Protein Secretion: Methods and Protocols*, Methods in Molecular Biology, vol. 1459, DOI 10.1007/978-1-4939-3804-9_7,

insertion into the ER membrane, newly synthesized CFTR—both wild type (wt) or bearing a mutation (e.g., F508del, the most common disease-causing variant)—is N-glycosylated through the addition of a 14-unit oligosaccharide on two asparagine residues located in its fourth extracellular loop. This addition generates the immature, core-glycosylated form of CFTR (known as band B). After undergoing correct folding, which is assessed by the ER quality control (ERQC), the mature core-glycosylated form of wt-CFTR is exported in COPII vesicles. When passing through the Golgi, its glycan moieties undergo processing producing the mature form (known as band C). In contrast, CFTR bearing F508del is retained in the ER due to misfolding and prematurely targeted for degradation by the ubiquitin-proteasome pathway (UPP). Thus, this variant never acquires the fully glycosylated pattern [1]. The model that we have proposed to explain the early stages of CFTR trafficking through the secretory pathway [2] involves several checkpoints to assess CFTR folding status, namely (a) two checkpoints involving the cytosolic Hsp70 and ER calnexin chaperones that recognize CFTR structural/glycan cues, respectively [3]; (b) negative selection at the ER exit sites mediated by arginine-framed tripeptides (AFTs) [4, 5]; and (c) positive selection upon exposure of a DAD motif [6, 7].

This movement of CFTR from the ER to its final destination, the PM, involves transport through a series of distinct vesicular compartments. The early CFTR traffic pathways so far described include (a) conventional anterograde traffic from the ER exit sites into COPII vesicles, (b) retrograde recycling from the *cis* Golgi to the ER, and (c) non-conventional trafficking via tubular structures migrating peripherally to the Golgi cisternae. This "unconventional" traffic route for CFTR transport to the PM was described to be insensitive to blocking of conventional ER-to-Golgi traffic and appears to involve the SNARE protein syntaxin 13 [8]. In this pathway however, the protein still travels back to the *cis* Golgi where it undergoes oligosaccharide processing to the complex form.

More recently, another unconventional pathway was described to occur during ER stress involving GRASP (Golgi reassembly stacking proteins). GRASP55/65 were shown to be tethering factors that are involved in the ER stress-induced non-conventional secretion. In this situation, CFTR reaches the PM in its core-glycosylated form [9]—in fact, non-glycosylated (either chemically or genetically) CFTR was also shown to reach the cell surface and to be functional [3].

The complex regulatory processes affecting CFTR traffic cannot be easily rationalized owing to the large number of potential mediators: at least 200 different proteins have been shown to interact with CFTR using a proteomics approach [10]. This figure does not reflect the potentially larger number of indirect players, as we need to determine the functional relationship of these

interactors. Loss-of-function assays employing RNA interference (RNAi) are instrumental for dissecting such complex networks [11]. These assays are typically based on measuring a control CFTR "cellular phenotype" and performing systematic RNAi experiments to identify genes whose knockdown/knockout affects that cellular phenotype. Such genes are then postulated as candidate regulators. In this regard, high-content microscopy (HCM) is a powerful tool as it allows thorough characterization of a biological process [12], overcoming many of the drawbacks of plate reader-based assays. In the context of CF, an HCM protocol enabled identifying diacylglycerol kinase isoform iota (DGKι) and ciliary neurotrophic factor receptor (CNTFR) as novel regulators of the epithelial sodium channel (ENaC), a major contributor to CF morbidity [13]. Because HCM approaches are phenotype based, they can be readily applied to the drug discovery pipeline to identify novel CFTR correctors, as recently demonstrated [14].

In this chapter, we describe a set of biochemical and cell biology methods that allow evaluation of CFTR trafficking to the cell surface—assessing its steady-state levels, turnover and stability, localization at the plasma membrane, glycan processing, and trafficking efficiency.

2 Materials

2.1 Western Blot Detection of CFTR

1. Buffer: 31.25 mM Tris–HCl pH 6.8, 1.5% (w/v) SDS, 5% glycerol, 0.01% (w/v) bromophenol blue, 0.5 mM DTT (*see* **Note 1**).
2. Bradford reagent.
3. Spectrophotometer.
4. Mini sodium dodecyl sulfate-polyacrylamide-gel (SDS-PAGE) gel system.
5. Benzonase: 25 U/ml in sample buffer.
6. 7–9% Laemmli SDS-PAGE gel (either a pre-cast gel, or a in-house prepared with 4% (w/v) stacking and 7–9% (w/v) separating gel, prepared with acrylamide:bisacrylamide mixture 37.5:1).
7. Running buffer: 0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS.
8. Transfer buffer: 0.025 M Tris–HCl pH 8.3, 0.192 M glycine, 20% (v/v) methanol.
9. SDS-PAGE molecular weight standards.
10. PVDF membrane (pore size: 0.45 μm).
11. 0.1% (v/v) Tween 20 in phosphate-buffered saline, PBS (PBST).
12. 5% (w/v) skimmed milk in PBST.
13. Anti-CFTR monoclonal antibody (CFF reference 596): Working solution is 1: 3000 in 5% milk-PBST.

14. Anti-mouse IgG horseradish-peroxidase-conjugated secondary antibody (Bio-Rad): Working solution is 1:3000 in 5% milk-PBST.
15. Western chemiluminescent substrate system.
16. Anti-α-tubulin monoclonal antibody produced in mouse (clone clone B-5-1-2, T5168—Sigma Aldrich).
17. X-ray film.

2.2 Pulse-Chase of CFTR

1. Hanks' balanced salt solution (HBSS).
2. Medium without methionine.
3. Protein G agarose beads.
4. Protease inhibitor cocktail.
5. PBS.
6. L-[^{35}S]/Methionine L-[^{35}S] cysteine protein labeling mix.
7. Methionine: Prepare a 100 mM solution and filter sterilize.
8. Radioimmunoprecipitation assay (RIPA) buffer: 1.0% (w/v) sodium deoxycholate, 1.0% (v/v) Triton X-100, 0.1% (w/v) SDS, 150 mM NaCl, 50 m M Tris pH 7.4 (*see* **Note 2**).
9. Sodium salicylate 1 M.

2.3 Cell Surface Biotinylation and Endocytosis of CFTR

1. EZ-Link Sulfo-NHS-SS-Biotin.
2. Streptavidin-agarose beads.
3. Protease inhibitor cocktail.
4. L-Glutathione reduced.
5. PBS.
6. PBS-CM: PBS, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$.
7. Quenching buffer: 100 mM Tris pH 8.0, 150 mM NaCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glycine, 1% BSA (w/v).
8. Pull-down buffer (PD buffer): 50 mM Tris pH 7.5, 100 mM NaCl, 10% glycerol (v/v), 1% NP-40 (v/v).
9. Wash buffer: 100 mM Tris pH 7.5, 300 mM NaCl, 1% TX-100 (v/v).
10. Stripping buffer: 60 mM L-Glutathione, 90 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 90 mM NaOH, 10% FBS (v/v).

2.4 Assessment of CFTR Glycosylation

1. PNGase F (500,000 U/ml).
2. PNGase F buffer (G7)—supplied—0.5 M sodium phosphate pH 7.5.
3. NP-40 10% (v/v).
4. Endoglycosidase H (500,000 U/ml).
5. Endoglycosidase H buffer (G5)—supplied—0.5 M sodium citrate pH 5.5.

6. Neuraminidase.
7. Neuraminidase buffer—50 mM sodium phosphate buffer pH 6.0.
8. Fucosidase.
9. Fucosidase buffer—50 mM sodium phosphate buffer pH 5.0.
10. Labeling and lysis reagents as in Subheading 2.2.

2.5 Microscopy-Based Assays to Analyze CFTR Traffic

1. CFBE or A549 cell lines expressing a Tet-ON mCherry-Flag-CFTR construct (wt or F508del variants, described in [12]).
2. DMEM high glucose with L-glutamine supplemented with 10% fetal bovine serum, 10 μg/ml blasticidin, and 2 μg/ml puromycin.
3. Doxycycline.
4. VX-809 (*see* **Note 3**).
5. Dulbecco's PBS, supplemented with 0.7 mM $CaCl_2$ and 1.1 mM $MgCl_2$ ($DPBS^{++}$) (*see* **Note 4**).
6. 3% Paraformaldehyde (PFA), freshly diluted in $DPBS^{++}$ (*see* **Note 5**).
7. Bovine serum albumin.
8. Mouse monoclonal anti-FLAG antibody (Sigma F1804): 1:500 (2 μg/ml), prepared in $DPBS^{++}$ supplemented with 1% BSA.
9. Donkey anti-mouse antibody, Alexa Fluor® 647 conjugate (Life Technologies A-31571): 1:500 (2 μg/ml), prepared in $DPBS^{++}$ supplemented with 1% BSA.
10. 0.2 μg/ml Hoechst 33342, in $DPBS^{++}$.
11. 10 cm Cell culture Petri dishes.
12. Chambered cover slips or multiwell plates: Plates can be used as is or after coated with siRNA/lipofectamine for reverse transfection [12, 15] (*see* **Note 6**).
13. Non-targeting scrambled siRNA.
14. COPB1 siRNA.
15. Multidrop™ Combi dispenser.
16. Liquidator™ 96 Manual pipette: Only required for 96- or 384-well plates.
17. Automated wide-field epifluorescence microscope equipped with a 10× objective.
18. Personal computer running CellProfiler (http://www.cellprofiler.org/ [16]).

3 Methods

3.1 Western Blot to Determine Steady-State Levels of Immature and Mature Forms of CFTR

The use of biochemical techniques to study CFTR has been previously described as a workflow [17]. The Western blot technique allows the assessment of steady-state levels of the different forms of CFTR (in general, the immature—with a molecular mass of about 140–150 kDa—and the mature form—with a molecular mass of 170–180 kDa) and, from quantification of these forms, an evaluation of the efficiency of processing.

1. Grow CFTR-expressing cells on 60 mm Petri dishes until confluence.
2. Wash the cells three times in PBS.
3. Make a cell lysate: Solubilize cells in 200 μl sample buffer per dish supplemented with 25 U/ml benzonase.
4. Quantify total protein by Bradford's assay [18] or another appropriate protein quantification method.
5. Load the cell lysate (~30 μg/well) on a mini SDS-PAGE (stacking 4% (w/v), separating 7% (w/v) acrylamide:bisacrylamide 37.5:1) at constant voltage (100–120 V), for 3 h. Use 8–10 μl of an appropriate molecular weight standard (*see* **Note** 7).
6. Transfer proteins to a PVDF membrane using wet blotting (Bio-Rad Mini-PROTEAN) for 1 h 30 min at 400 mA, constant current. The transfer should be done on ice (mixed with water) or with refrigeration (4 °C).
7. Wash the PVDF membrane with PBST for 15 min.
8. Block the PVDF membrane by incubating in 5% (w/v) skimmed milk in PBST for 2 h.
9. Cut the membrane around the 75 kDa molecular weight marker. Use the higher molecular weight part to probe with the anti-CFTR antibody (below). The lower molecular weight segment can be probed with anti-tubulin antibody as an internal loading control.
10. Incubate anti-CFTR 596 monoclonal antibody overnight (diluted 1:3000 in 5% (w/v) skimmed milk in PBST) at 4 °C with gentle mixing.
11. Wash three times for 5 min with PBST.
12. Incubate with anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1:3000 5% (w/v) skimmed milk in PBST) for 1 h.
13. Wash three times for 5 min with PBST.
14. Detect protein bands using the chemiluminescent substrate as per kit instructions by exposing X-ray films for the appropriate time (1–5 min) (Fig. 1a).

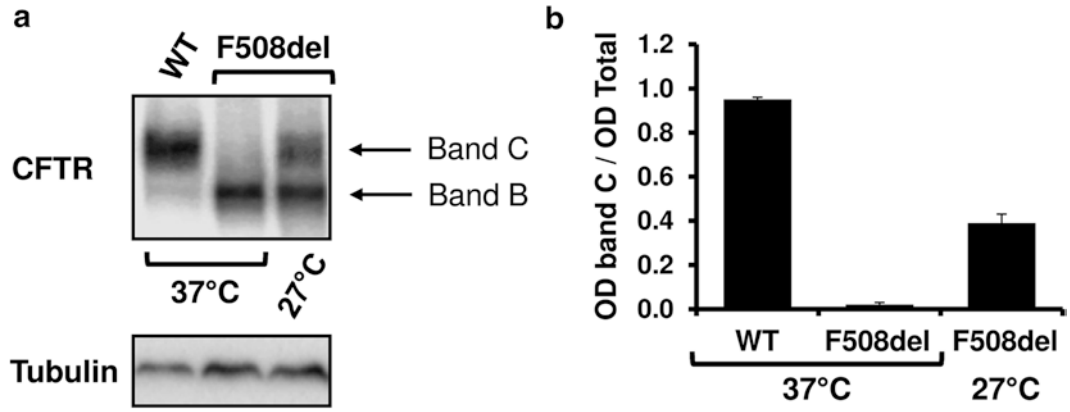

Fig. 1 Western blot detection of CFTR and calculation of processing efficiency. (**a**) BHK cells stably expressing wt- or F508del-CFTR were cultured at 37 °C and either directly lysed or placed for 48 h at 27 °C before lysis, to allow partial rescue of F508del-CFTR maturation. Following electrophoretic separation, proteins were transferred to PVDF membranes and probed with antibodies anti-CFTR and also anti-α-tubulin, as a loading control. Note the partial conversion of immature F508del-CFTR (band B) to mature protein (band C) upon incubation at 27 °C. (**b**) WB films were scanned and the intensity of the bands corresponding to tubulin and CFTR immature and mature forms was quantified using an appropriate software (ImageJ in this example). The graph expresses the efficiency of CFTR processing, calculated as the ratio between the OD values for band C and for the total amount of CFTR (band C + band B). Data are represented as means ± SEM, $n = 7–9$

15. Scan the gel and quantify the bands corresponding to CFTR immature and mature forms using an appropriate software, for example ImageJ, GE ImageQuant 1D, Bio-Rad Quantity One, and Bio-Rad Image Lab.
16. Calculate the efficiency of processing as the ratio between the amount of band C and the total amount of CFTR (Fig. 1b).

3.2 Pulse-Chase Experiments to Determine Turnover Rate of CFTR Immature Form and Efficiency of Maturation

The pulse-chase technique is a well-described and common procedure that, with the use of a labeled amino acid (or amino acids), allows the determination of the turnover of CFTR immature form (band B) and also of the conversion of the immature into the mature form (band C).

Cells expressing the CFTR variant of interest should be grown to loose confluency. After a period of starvation, cells are then incubated, in general, with a mixture of [^{35}S]-methionine/cysteine. This period is called the pulse. After this period, during which the labeled amino acids are incorporated into nascent proteins, the medium containing the radioactive amino acids is removed and replaced by the chase medium that contains an excess of unlabeled methionine. At different times after medium replacement, the cells are lysed and CFTR is immunoprecipitated. Samples are run in an SDS-PAGE gel which after fluorography and drying is exposed to an X-ray film.

Results are then obtained by quantifying CFTR mature and immature forms. The ratio between the amount of labeled immature CFTR at a specific time (P) and the amount of labeled immature

CFTR at the end of pulse (P_0) plotted against the duration of chase corresponds to the turnover of CFTR. The ratio between the amount of labeled mature CFTR at a specific time and the amount of labeled immature CFTR at the end of pulse plotted against the duration of chase corresponds to the efficiency of processing (Fig. 2).

1. Seed BHK cells expressing CFTR on 60 mm dishes 24 h before experiment. The seeding density should be such that cells are at sub-confluency at the time of starting the experiment (*see* **Notes 8** and **9**).
2. Remove media and wash twice with 2 ml of HBSS.
3. Incubate in methionine-free medium for 30 min.
4. Remove media and pulse with 1 ml methionine-free medium containing 150 μCi/ml [^{35}S]-methionine/cysteine for 30 min.
5. After the pulse period, remove the medium containing the labeled amino acids and wash twice with 1 ml HBSS.
6. Feed the cells with their regular medium supplemented with the appropriate amount of fetal bovine serum, 1 mM methionine, and, if needed, 25 μg/ml of cycloheximide (*see* **Note 10**).

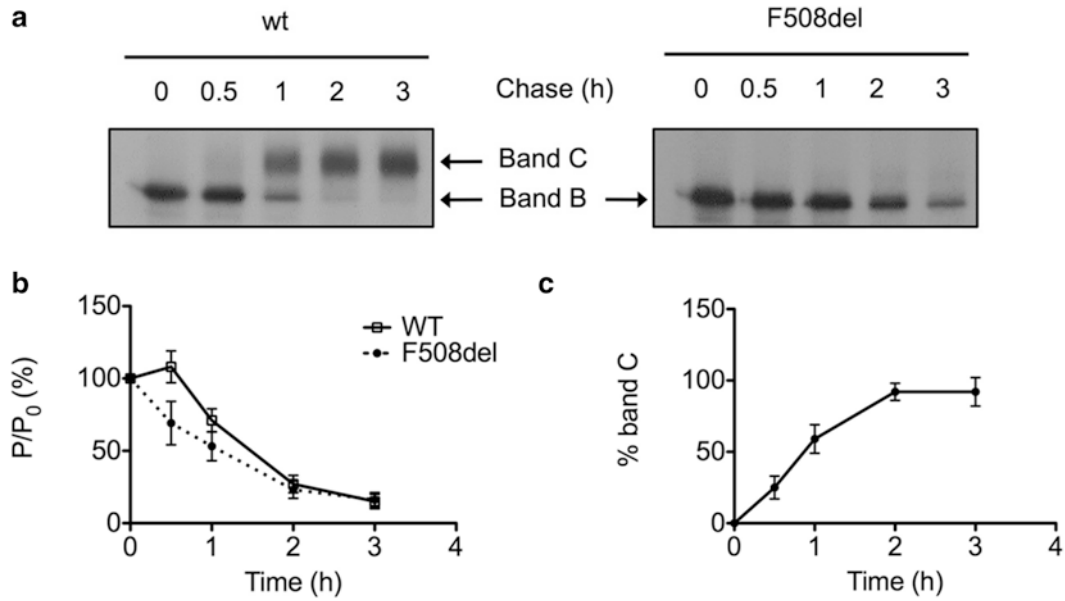

Fig. 2 Turnover and processing of wt- and F508del CFTR. (**a**) BHK cells stably expressing wt- or F508del-CFTR were pulse-labeled with [^{35}S]-methionine and chased for 0, 0.5, 1, 2, and 3 h. Cells were then lysed and immunoprecipitated with an anti-CFTR Ab. Following electrophoretic separation and fluorography, immature (band B) and mature (band C) forms of CFTR were quantified. (**b**) Turnover of the core-glycosylated form (band B) of wt- and F508del-CFTR is shown as the ratio between *P*, the amount of band B at time *t*, and P_0, the amount of band B at the start of the chase (i.e., at the end of pulse). (**c**) The efficiency of conversion of the core-glycosylated form (band B) into the fully glycosylated form of wt-CFTR (band C) is determined as the ratio between the amount of band C at time t and the amount of band B at the start of the chase (P_0)

7. At appropriate intervals of incubation with the chase medium, harvest cells. For this, wash them twice with 1 ml ice-cold PBS, add 1 ml RIPA buffer supplemented with protease inhibitors, and incubate for 30 min at 4 °C.
8. Scrape cells off the plate and pass ten times through a 1 ml pipet tip and then ten additional times through a 200 μl pipet tip.
9. Transfer the cell lysates to a 1.5 ml microcentrifuge tube with O-ring.
10. Centrifuge in a microcentrifuge at 14,000 × *g* for 30 min, 4 °C.
11. Transfer the supernatant to a new microcentrifuge and discard the pellet (*see* **Note 11**).
12. Add the appropriate amount of antibody (1:1000 if using anti-CFTR antibody 596 or 570, provided by CFFT) and 40 μl of Protein G beads (*see* **Note 12**).
13. Incubate overnight in cold room (4 °C) with shaking.
14. Wash the beads three times with 1 ml ice-cold RIPA (*see* **Note 13**).
15. Add 70 μl of sample buffer (*see* Subheading 2.1) and incubate for 30 min with continuous mixing at room temperature.
16. Spin at 14,000 × *g* for 2 min.
17. Collect supernatant (*see* **Note 11**).
18. Load samples onto a 20 cm gel and run overnight at approximately 75 V (*see* **Note 14**).
19. Fix the gel in 30% (v/v) methanol/10% (v/v) acetic acid for 30 min.
20. Wash four times for 15 min in bidistilled H_2O.
21. For fluorography, treat the gel for 1 h with 1 M sodium salicylate.
22. Dry the gel and expose to an X-ray film.
23. Develop in 24/48 h.
24. Scan the film and quantify the bands corresponding to CFTR immature and mature forms using an appropriate software: ImageJ, GE ImageQuant 1D, Bio-Rad Quantity One, or Bio-Rad Image Lab.

3.3 Cell Surface Protein Biotinylation to Assess CFTR Plasma Membrane Abundance and Endocytosis

The end point of the successful processing and trafficking of CFTR is its delivery to and function at the plasma membrane. A number of methods have been proposed to enrich, purify, and quantify the amount of CFTR at the cell surface. Among them, usage of biotinylating reagents and exploitation of the strong interaction between biotin and streptavidin for the purification of biotinylated surface proteins have rapidly gained in popularity and allowed some of the most significant progresses in evaluating and quantifying the efficacy of CFTR trafficking to the plasma membrane and the rate of its turnover from the cell surface.

The most common targets for modifying protein molecules are primary amine groups that are present as lysine side-chain epsilon-amines and N-terminal alpha-amines. Hence, N-hydroxysuccinimide (NHS) esters are among the most widely used amine-reactive biotinylation reagents. Their poor solubility in aqueous solutions has been overcome by the addition of a sulfonate group on the N-hydroxysuccinimide ring, which also made them ideal as surface biotinylation reagents, because sulfo-NHS-esters do not penetrate the cell membrane.

The protocol described herein makes use of another NHS-biotin derivate—the sulfo-NHS-SS-biotin, a reagent that includes a disulfide bond in the spacer arm separating the sulfo-NHS and biotin groups. The S-S bond can be cleaved using reducing agents, enabling the biotin group to be disconnected ("stripped") from the labeled proteins at the plasma membrane. This reagent can thus be used to analyze both the steady-state amount of CFTR at the cell surface and to follow the rate of CFTR internalization from the plasma membrane (Fig. 3). In

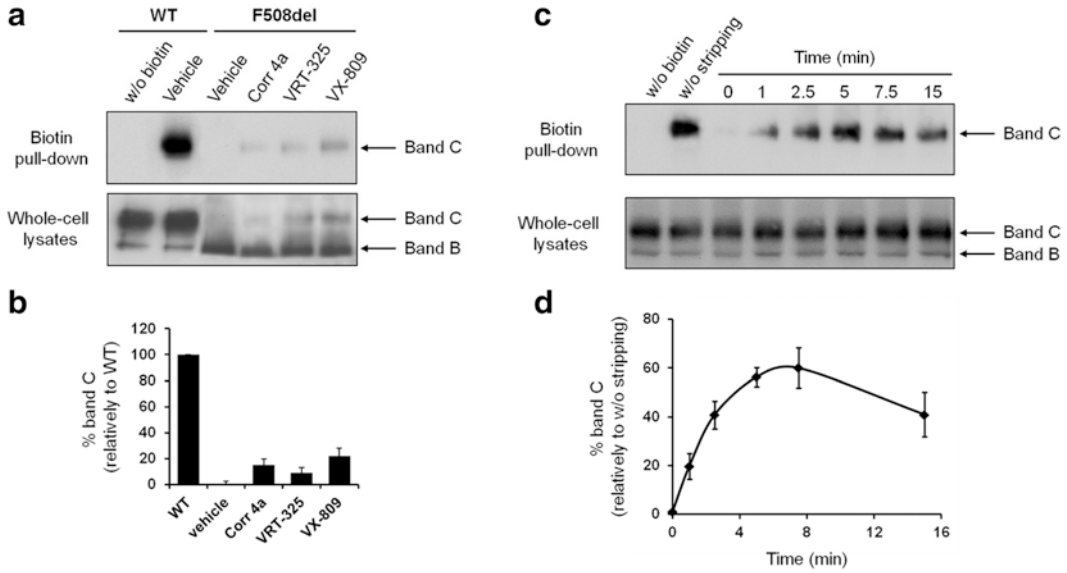

Fig. 3 Abundance and endocytosis of CFTR at the cell surface. (**a**) CFBE cells stably expressing wt- or F508del-CFTR were incubated for 24 h with 5 μM VRT-325, 10 μM Corr-4a, or 3 μM VX-809. CFTR abundance at plasma membrane was analyzed by surface protein biotinylation followed by western blot with an anti-CFTR Ab. Shown are representative images of immunoblots for the biotinylated fraction of mature (band C) CFTR at the plasma membrane (biotin pull-down) and of whole-cell lysates showing the steady-state abundance of immature (band B) and mature (band C) forms of CFTR upon the different treatments. (**b**) Quantification of the abundance of mature F508del-CFTR at the cell surface after treatment with the indicated corrector compounds, relatively to wt-CFTR. Data are mean values ± SEM of $n = 4$ independent experiments. (**c**) Proteins at the surface of BHK cells stably expressing wt-CFTR were labeled with biotin at 0–4 °C and allowed to internalize by placing the cells at 37 °C for 0, 1, 2.5, 5, 7.5, and 15 min. Following striping of the remaining biotin labels at the cell surface, cells were lysed and the internalized, biotin-labeled CFTR proteins were isolated by streptavidin-mediated capture and analyzed by Western blot as in (a). (**d**) Quantification of the amount of CFTR internalized at the different time points, expressed as a percentage of the total amount of CFTR biotinylated at the cell surface (w/o stripping). Data are mean values ± SEM of $n = 6$ independent experiments

the first case cells are placed on ice to stop all exocytic and endocytic trafficking and labeled with a solution of sulfo-NHS-SS-biotin in PBS. After quenching of the reaction with an amine-rich buffer, the cells are thoroughly washed and lysed and biotin-labeled proteins at the cell surface isolated by pull-down with streptavidin-coated agarose beads. To assess the rate of CFTR endocytosis the first protein labeling step is identical but after quenching the reaction the labeled proteins are allowed to internalize by replacing the cells at 37 °C for increasing time intervals, followed by striping the biotin labels from the remaining labeled proteins at the surface with a reducing agent (such as glutathione). In this way, only internalized CFTR proteins, protected inside the cell from the reducing reagent action, will be isolated by streptavidin-mediated capture. Captured proteins are then eluted with dithiothreitol (DDT)-containing Laemmli buffer, allowing the efficient cleavage of S-S bond and their release for analysis by Western blot as described in Subheading 3.1.

The following protocol was successfully used in our lab to analyze CFTR plasma membrane levels and endocytosis in BHK, CFBE, HeLa, and HEK-293 cells [19–22].

1. Seed cells expressing CFTR on 60 mm dishes 24 h before the experiment. The seeding density should be such that cells are at near confluence at the time of starting the experiment.
2. Before starting prepare all required solutions (*see* Subheading 2.3) and pre-block the streptavidin-agarose beads as follows:
   (a) Wash 50 μl/dish of streptavidin bead slurry three times with PBS.
   (b) Remove the supernatant and add at least twice the dry bead volume of PD-buffer (*see* Subheading 2.3) supplemented with 2 % of skimmed milk.
   (c) Rotate for at least 1 h at 4 °C.
   (d) Wash three times with pull-down buffer using 1000 × *g* spins and remove the supernatant.
   (e) Add one volume of PD-buffer supplemented with protease inhibitors to the dry beads.

3.3.1 From Here on Procedures Should Be Carried Out on Ice, Preferably in a Cold Room (4 °C)

1. Wash cells 3–5 times with 2 ml of ice-cold PBS-CM to remove all medium contaminants (washing procedure depends on the cell type used (*see* **Note 15**)).
2. Keep one dish with PBS-CM to function as the "without-biotin" control (to assess the amount of contaminant proteins precipitating with the beads alone) and incubate the remaining dishes with 1.5 ml of ice-cold PBS-CM + 0.5 mg/ml EZ-Link sulfo-NHS-SS-biotin for 30 min (*see* **Notes 16–20**).
3. Aspirate all the labeling buffer and discard. Rinse cells twice with 2 ml of ice-cold quenching buffer.

4. Quench the reaction for 10 min with 2 ml of fresh quenching buffer (ice cold).
5. Wash 3× with 2 ml of ice-cold PBS-CM.
6. Stop here and proceed to subheading 3.3 **(Step 15)** for the endocytosis assay procedure.
7. Lyse cells on ice with 250 μl of ice-cold PD-buffer supplemented with protease inhibitors, scrape cells, and collect whole lysates to 1.5 ml microcentrifuge tubes. Centrifuge for 5 min at 10,000 × *g* at 4 °C.
8. Save 40 μl of cleared lysates to new microcentrifuge tubes containing 40 μl of 2× Laemmli buffer (to assess total CFTR levels in the samples).
9. Pass 200 μl of the cleared lysates to new 1.5 ml microcentrifuge tubes.
10. Add 50 μl of pre-blocked streptavidin bead slurry to each tube and rotate for 1 h at 4 °C.
11. Centrifuge for 1 min at 5000 × *g*, discard supernatant, and wash 3–5 times with wash buffer.
12. Remove the supernatant, dry the beads, and elute captured protein with 25 μl of DTT-containing 2× Laemmli buffer.
13. Analyze samples by Western blot as described in Subheading 3.1.

3.3.2 For Assessing the Rate of CFTR Endocytosis Proceed as Follows

1. Before starting, warm a flask containing the necessary volume of culture medium (~2 ml/dish) to 37 °C.
2. After biotinylation of cell surface proteins (Subheading 3.3.1, **Steps 1–5**, *see* above) place all the 60 mm dishes on ice in a styrofoam box with lid and take them near an incubator at 37 °C.
3. Keep the dishes corresponding to the time, "with out biotin," "without striping," (which will return the total amount of CFTR initially labeled at the cell surface), and "0 min" (where no surface-labeled CFTR is yet internalized) on ice.
4. Replace the buffer in all remaining dishes with 2 ml of warm medium and incubate at 37 °C for the required periods, e.g., 1, 2.5, 5, 7.5, and 15 min.
5. At each time point, quickly discard the buffer, add 2 ml of ice-cold PBS-CM to the dish, and place it back on the ice box. Keep the lid closed and return the dishes to the cold room as soon as possible.
6. Back in the cold room, discard the buffer of all dishes, and wash with 2 ml of fresh, ice-cold PBS-CM.
7. Add 2 ml of stripping buffer to the 0, 1, 2.5, 5, 7.5, and 15 min dishes and incubate on ice for 15 min at 4 °C. Repeat this step three times, discarding the buffer and adding 2 ml of fresh stripping buffer each time to each dish (total stripping time: 45 min divided in three independent washes).

8. Aspirate all the stripping buffer and discard. Rinse cells 3–5 times with 2 ml of ice-cold PBS-CM and proceed with cell lysis and pull-down as above (Subheading 3.3.1, **Steps 7–13**).

3.4 Glycosylation Assessment with Specific Glycosidases to Assess Trafficking Through or Out of the Golgi

CFTR is N-glycosylated at two asparagine residues located in the fourth extracellular loop (N894 and N900). As occurs with membrane and secreted proteins, glycosylation occurs co-translationally at the ER and the 14-unit glycan is then processed through its trafficking in the endoplasmic reticulum and Golgi complex.

Treatment of CFTR with different glycosidases can then be used to assess if the protein passed through different compartments along its secretory trafficking, as shown for wt- and F508del-CFTR (Fig. 4).

Treatment with endoglycosidase H (endoH) assesses protein exit from the ER and reaching the *cis* Golgi. The ER immature forms are sensitive to endoH whereas later/fully processed forms are endoH resistant.

Treatment with PNGase F (N-glycanase) is used to assess the presence of all sorts of N-linked glycans, as the enzyme hydrolyzes the N-glycosidic bond connecting the glycan to the protein's asparagine residues.

Treatment with either neuraminidase or fucosidase will identify if the protein reached the latter cisternae of the Golgi complex where sialic (N-acetylneuraminic) acid or fucose residues are attached to protein-linked glycans. Thus, resistance to treatment with either enzyme will indicate that the protein does not possess such residues, whereas a decrease in molecular weight upon treatment will indicate that they are present in the glycan unit.

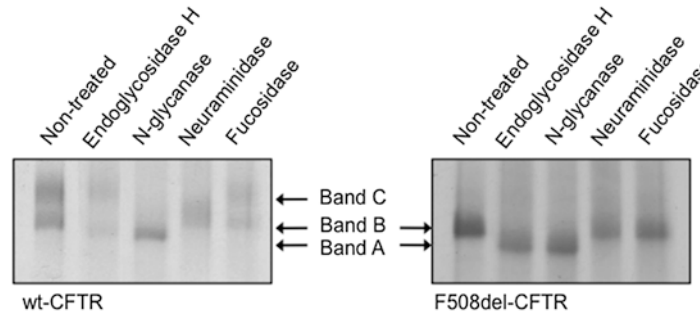

Fig. 4 Analysis of the glycan moieties of wt- and F508del-CFTR with different glycosidases (endoglycosidase H, PNGase F/N-glycanase, neuraminidase, and fucosidase). Following endoH-treatment, a shift is observed in CFTR immature but not in CFTR mature form. Following N-glycanase treatment, all forms are digested, this corresponding to removal of all N-linked glycans, resulting in the de-glycosylated primary CFTR amino acidic chain, also called band A. Following treatment with neuraminidase or fucosidase, there is a shift only in wt-CFTR mature form

1. Seed BHK cells expressing CFTR on 60 mm dishes 24 h before experiment. The seeding density should be such that cells are at sub-confluency at the time of starting the experiment.
2. Label cells for 3 h with 150 μCi of [^{35}S]-methionine/cysteine (for details *see* above Subheading 3.2).
3. After labeling, wash cells twice with ice-cold PBS, lyse them in 1 ml of complete RIPA buffer as above, and add anti-CFTR antibody and protein G-sepharose beads as above (*see* Subheading 3.2).
4. Incubate overnight at 4 °C with rotation.
5. Wash three times with 1 ml of RIPA and elute in 40 μl of sample buffer (*see* Subheading 2.1).
6. Prepare the glycosidase reactions as follows:

Endoglycosidase H	*Neuraminidase*
30 μl of protein sample	30 μl of protein sample
5 μl of buffer G5	5 μl Sodium phosphate buffer 0.5 M pH 6
1 μl of endoglycosidase H	1 μl of neuraminidase
Bidistilled H_2O to 50 μl	Bidistilled H_2O to 50 μl
PNGase F	*Fucosidase*
30 μl of protein sample	30 μl of protein sample
5 μl of buffer G7	5 μl of sodium phosphate buffer 0.5 M pH 5
5 μl of NP-40 10%	1 μl of fucosidase
1 μl of PNGase F	Bidistilled H_2O to 50 μl
Bidistilled H_2O to 50 μl	

7. Incubate the reactions overnight at 37 °C.
8. Run the samples in a 20 cm gel (*see* above Subheading 3.2) (*see* **Note 21**).
9. Treat, dry, and expose gel as above (Subheading 3.2) (*see* **Note 22**).

3.5 Microscopy-Based Assays to Determine Traffic Efficiency and Endocytosis in Inducible Systems

The CFTR traffic assay is based on the quantification of CFTR traffic efficiency in CFBE or A549 cell lines which express a double-tagged CFTR construct: an mCherry-CFTR fusion molecule (wt- or F508del-) harboring a Flag tag insertion at the fourth extracellular loop (Fig. 5), as described in [12]. By performing immunofluorescence labeling in unpermeabilized fixed cells, Flag tags (i.e., CFTR molecules) located at the PM—but not elsewhere—can be detected (Alexa 647) (*see* **Note 23**). Then, the fraction of CFTR molecules delivered to the PM—i.e., the traffic efficiency—is determined in each cell as follows:

$$\text{CFTR traffic efficiency} = \frac{\text{PM CFTR}}{\text{Total CFTR}} = \frac{\text{Alexa Fluor}^{®}\ 647\ \text{Integrated Fluorescence}}{\text{mCherry Integrated Fluorescence}}$$

This assay can be coupled to RNAi or chemical compound treatments. In these cases, the inducible Tet-ON promoter allows initiating CFTR expression only after the onset of knockdown or compound effect (Fig. 5).

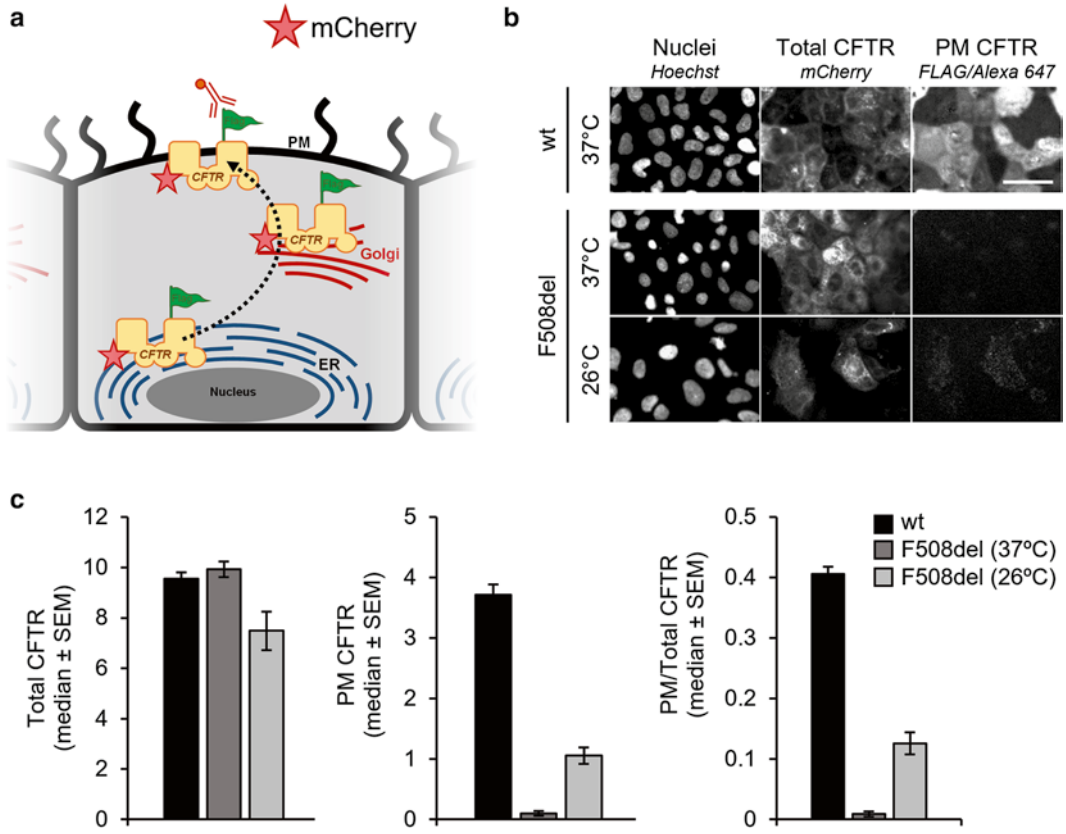

Fig. 5 Overview of the microscopy-based CFTR traffic assay. (**a**) The basis for the traffic assay is a CFTR traffic reporter which is a double-tagged (mCherry and Flag) CFTR molecule (wt- or F508del-variants), whose gene has been stably integrated into A549 or CFBE cell lines. The Flag tag resides at CFTR's fourth extracellular loop and only becomes extracellular if the protein successfully traffics to the PM. By immunostaining the Flag tag in unpermeabilized cells, the fraction of CFTR molecules at the PM can be detected. (**b**) Fluorescence microscopy imaging of the mCherry-Flag-CFTR constructs. wt-CFTR was expressed at 37 °C and F508del-CFTR was expressed at either 37 or 26 °C. No siRNAs or correctors were used. Images were acquired using a metal halide light source (Leica EL6000) at maximum brightness, a 10× N.A. 0.4 objective, a Leica DFC365 FX CCD camera, and the following filter cubes: Leica N.21 (mCherry) and a custom excitation BP 645/30; emission LP 670 filter cube (Alexa 647). Under these conditions, the exposure times for Cherry and Alexa 647 were 1.3 and 8 s, respectively. Scale bar = 50 μm. (**c**) Quantification of the images in panel **b**, showing the rescue of mCherry-Flag-F508del-CFTR at low temperature

3.5.1 CFTR Traffic Assay

1. Culture CFBE/A549 mCherry-Flag-CFTR cells (wt- or F508del-) to confluency in 10 cm dishes using DMEM supplemented with FBS, blasticidin, and puromycin (*see* **Note 24**).
2. Split cells to ~50% confluency.
3. 24 h later, trypsinize and seed cells: 20,000 cells/well (8-well chambered cover slips or 96-well plates), or 3000 cells/well (384-well plates) (*see* **Note 25**). This defines time $t=0$ h (*see* **Note 26**). For the negative controls add scrambled siRNA or DMSO. For the positive control, add COPB1 siRNA (wt-CFTR) or 3 μM VX-809 (F508del-CFTR). Seeding of 96- and 384-well plates must be performed with an automated dispenser (*see* **Note 27**).
4. Grow cells at 37 °C and 5% CO_2.
5. Induce CFTR expression with doxycycline (1 μg/ml) at $t=48$ h (wt-CFTR) or $t=24$ h (F508del-CFTR).
6. Incubate cells until $t=72$ h and immunostain extracellular Flag tags.

3.5.2 Fluorescence Staining of Extracellular Flag Tags

When 96- or 384 well plates are used, all pipetting steps can be significantly expedited by using a 96-channel pipette. In this case, all solutions must be previously dispensed in 96-well source plates. $DPBS^{++}$ can be dispensed from a deep-well reservoir.

1. Wash the plate once with ice-cold $DPBS^{++}$ (*see* **Note 28**).
2. Add mouse anti-Flag antibody: 45 μl/well (8 well), 30 μl/well (96 well), or 15 μl/well (384 well) (*see* **Note 29**).
3. Spin down plates (maximal centrifugal field: ~50×*g*) (*see* **Note 30**).
4. Incubate plate for 1 h at 4 °C.
5. Rinse three times with ice-cold $DPBS^{++}$.
6. Fix the cells with 3% (w/v) paraformaldehyde for 20 min at 4 °C (*see* **Notes 31** and **32**). Typical volumes are 100 μl/well (8 well), 80 μl/well (96 well), and 40 μl/well (384 well). All further steps are performed at room temperature.
7. Wash three times with $DPBS^{++}$.
8. Add goat anti-mouse Alexa 647-conjugated antibody. Use the same volumes as in **step 2**.
9. Spin down plates (maximal centrifugal field: ~50×*g*).
10. Incubate plate for 1 h at room temperature.
11. Wash three times with $DPBS^{++}$.
12. Add Hoechst 33342 for nuclei staining. Use the same volumes as in **step 6**.
13. Incubate plate for 1 h at room temperature.
14. Wash three times with $DPBS^{++}$.
15. Submerge cells in $DPBS^{++}$. Use the same volumes as in **step 6**.

16. Incubate DPBS^{++} overnight at 4 °C in the dark (*see* **Note 33**).
17. Store plates at 4 °C in the dark until imaging (*see* **Note 34**).

3.5.3 Wide-Field Fluorescence Image Acquisition

Imaging of multiwell plates can be performed with any wide-field fluorescence screening microscope (*see* **Note 35**).

1. Input the exact dimensions of the plate's well matrix into the microscope software.
2. Set up multiposition imaging: For statistical reasons, each well should be imaged in at least four sub-positions.
3. Set up autofocus based on the nuclei (Hoechst) staining.
4. Choose exposure time and filter sets for Hoechst, mCherry, and Alexa 647 (visualization of nuclei, total CFTR, and PM CFTR) (*see* **Note 36**).
5. Start automated image acquisition.

3.5.4 Image Analysis

1. Quantify mCherry and Alexa 647 fluorescence as well as the mCherry/Alexa 647 ratio on a cell-by-cell basis using CellProfiler. A previously published analysis pipeline [12] is schematically depicted in Fig. 6.
2. It is convenient to express the overall quantification of a given image by the mean or median value of all cells in an image.
3. The quantification result can be expressed as the absolute value or the deviation to negative controls. HTM Explorer, an excellent software tool for interactive data visualization, quality control, and statistical analysis of HCM data, has been created by Dr. Christian Tischer (EMBL, Heidelberg) and is freely available at https://github.com/tischi/HTM_Explorer. This tool is based on and requires the installation of the R software (https://cran.r-project.org/).

4 Notes

1. Store at −20 °C as single-use vials.
2. When preparing RIPA buffer, be aware that sodium deoxycholate is an irritant, so wear a mask.
3. The compound is stable up to 6 months (DMSO solubilized, −80 °C) or 3 years (powder, −20 °C).
4. Add $CaCl_2$ and $MgCl_2$ just before use.
5. Alternatively, 3% solutions can be stored at −20 °C.
6. This is a lyophilized gelatine/sucrose/lipofectamine/siRNA coating which is quickly rehydrated and uptaken by cells at the moment of seeding. It is stable at room temperature if stored under desiccation conditions [15].

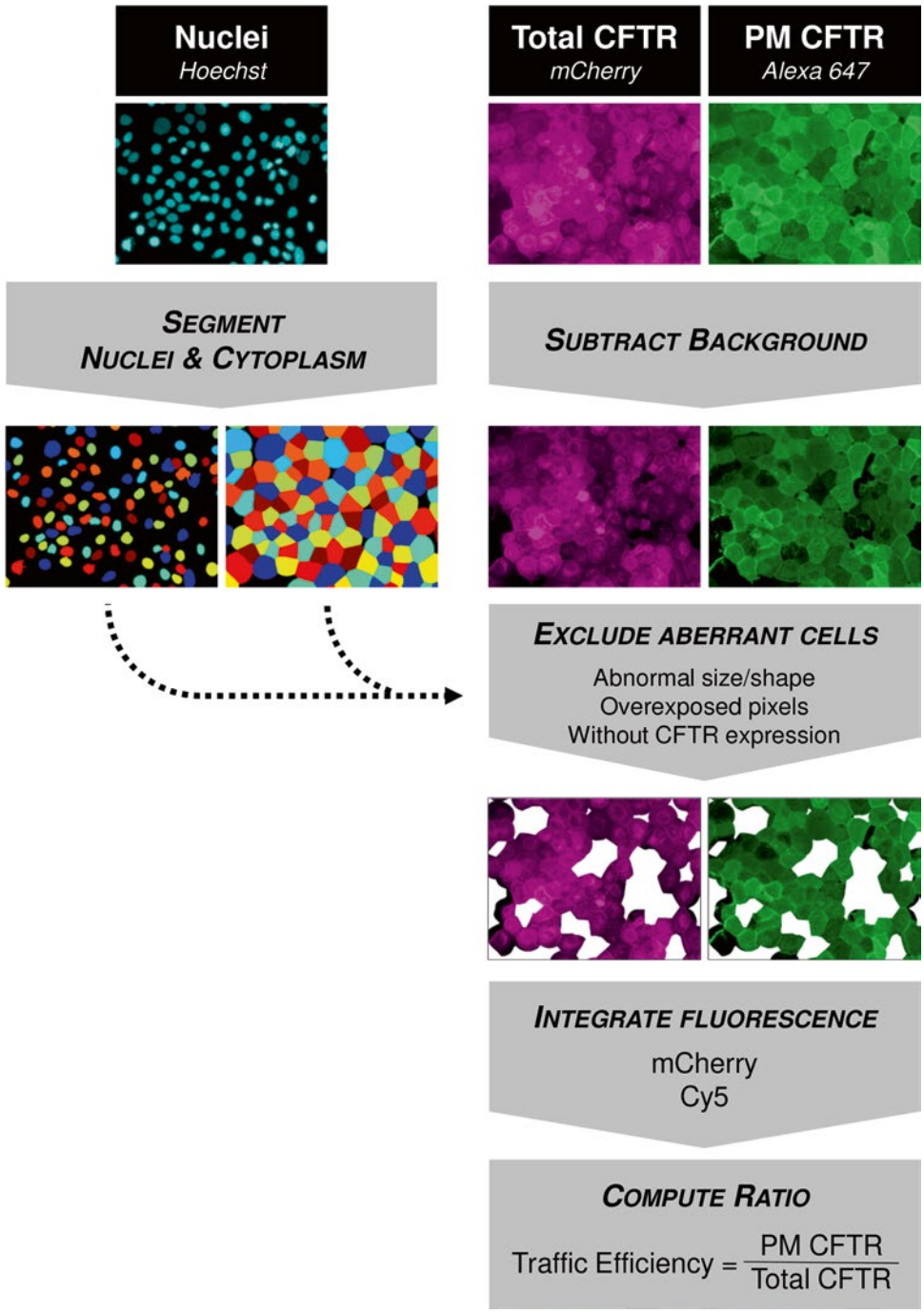

Fig. 6 Image analysis workflow for the quantification of CFTR traffic efficiency. The experimental outputs of the CFTR traffic assay are wide-field fluorescence microscopy images for three fluorophores: Hoechst 33342 (nuclei), mCherry (Total CFTR), Alexa 647 (CFTR molecules located at the PM). The Hoechst image is used for nuclei segmentation. The cytoplasm is segmented through dilation of the nuclei objects. Both CFTR channels are background corrected (via flat-field correction or subtracting illumination functions generated in silico). Using the segmentation data, cells presenting apoptotic- or mitotic-like morphology, without CFTR expression, or containing saturated pixels are excluded. Finally, a set of "approved cells" is established, all of them being characterized by the integrated mCherry and Alexa 647 fluorescence intensity as well as by the corresponding traffic efficiency (PM/Total CFTR). The analysis pipeline can be readily implemented in CellProfiler to enable the simultaneous analysis of large image datasets

7. In some cell types increasing acrylamide concentration up to 9% can decrease the smearing of band C allowing for better densitometric analysis of band intensity. The gel can be run until the 50 kDa marker is 0.5 cm above the bottom of the gel.
8. The pulse-chase procedure can be applied to different cell lines expressing CFTR. However, the duration of the pulse may need to be adjusted to obtain enough initial labeling.
9. In the pulse-chase experiment, each time point (durations of chase) corresponds to one P60 cell culture dish.
10. Addition of cycloheximide during the chase period is done to inhibit incorporation of the radioactive amino acids that were taken into the cell but not used for protein synthesis during the pulse period. As it is toxic, its use is optional and may depend on the cell line. So, perform first a test to assess if labeled CFTR levels go up in the first 30 min of chase. If so, use then cycloheximide.
11. Samples can be frozen (at –70 °C) either after clearing the lysates or before the immunoprecipitated proteins are loaded into the gel.
12. Other antibodies can be used in this procedure as long as they are efficient in CFTR immunoprecipitation.
13. To wash the beads, use a 2 or 5 ml syringe with a 21 G needle to aspirate RIPA. Do not vacuum aspiration as this tends to lead to sample loss.
14. In the first times you run a set of samples in a gel, you may consider using a radiolabeled protein molecular weight standard ([methyl-^{14}C] methylated, protein molecular weight markers, Perkin-Elmer NEC811001UC). The CFTR pattern is usually very clear, so in general there is no need for continued use of the molecular weight standard.
15. The initial washing steps should be carried out gently with easily detachable cells (such as BHK or HEK-293 cells) but vigorously with highly adherent cells (such as HeLa or specially CFBE cells) to ensure the removal of all unviable, biotin-permeable cells, so as to minimize contamination of the assays with biotin-labeled intercellular proteins.
16. CAUTION: Solubilizing the sulfo-NHS-SS-biotin in DMSO may increase its cell permeability leading to contamination of the assay with intracellular proteins. The water solubility of sulfo-NHS-esters eliminates the need to dissolve the reagent in an organic solvent.
17. Sulfo-NHS esters should be dissolved in water just before use because they hydrolyze easily.
18. The purity of the reagent is of the utmost importance to avoid the isolation of intracellular protein. Cheaper reagents may

contain high proportion of desulfonated NHS-biotin that is cell membrane permeable.

19. There is some flexibility in the conditions for conjugating NHS-esters to primary amines. Incubation temperatures can range from 4 to 37 °C, pH values in the reaction range from 7 to 9, and incubation times range from a few minutes to 12 h.
20. Buffers containing amines (such as Tris or glycine) must be avoided because they compete with the protein biotinylation reaction.
21. In some protocols, after the hydrolysis with the glycosidases, there is an additional protein precipitation step with either ethanol or trichloroacetic acid. The precipitate is then dissolved and loaded in the SDS-PAGE gel. This step is dispensable as the solubilization is usually difficult, and most of the times this reflects negatively on the band pattern obtained. The hydrolysis reaction can be loaded directly.
22. Alternatively to the use of extracts from metabolically labeled cells, the treatment with glycosidases can be done using total extracts (prepared as in Subheading 3.1). After cell lysis and protein quantification, the reactions can be prepared as in Subheading 3.4, **step 6**, using 50 μg of total protein. After overnight incubation at 37 °C, the samples can be analyzed by Western Blot with an anti-CFTR antibody (*see* above Subheading 3.1). Autofluorescence is minimized by choosing blue and red/infrared-emitting fluorophores.
23. Autofluorescence is minimized by choosing blue or red/infrared-emitting fluorophores.
24. Blasticidin and puromycin are the selection agents for the mCherry-Flag-CFTR construct.
25. Cell amounts are chosen such that a near-confluent cell monolayer is formed by the end of the assay.
26. From this point onwards, blasticidin and puromycin can be withdrawn from the culture media.
27. This ensures seeding homogeneity throughout all wells in a plate.
28. A 4 °C temperature is required to inhibit CFTR endocytosis.
29. These volumes minimize antibody usage and are provided as a suggestion
30. A low centrifugal field ensures that the cells are not disrupted.
31. Fixation masks the Flag epitope and therefore can only be performed after adding the primary antibody.
32. Paraformaldehyde solutions are irritating and carcinogenic. Such solutions must be handled in a chemical fume hood and disposed of in dedicated containers.
33. Overnight incubation is required for the Hoechst dye to fully diffuse into the nuclei, since cells are not permeabilized during this protocol.

34. Multiple plates can be stained in parallel. If stained plates are stored at 4 °C in the dark, imaging can occur within 1 week of staining without appreciable fluorescence loss.
35. A confocal microscope is not desirable because the assay readout is whole-cell fluorescence.
36. mCherry and Alexa 647 fluorescence cannot be spectrally separated with most conventional filter sets. To avoid mCherry fluorescence bleed-through, we use a custom filter cube for the Alexa 647 channel: excitation BP 645/30; emission LP 670.

Acknowledgements

Work supported by UID/MULTI/04046/2013 center grant (to BioISI) from FCT/MCTES/PIDDAC, Portugal. H.M.B. is recipient of SFRH/BPD/93017/2013 postdoctoral fellowship (FCT, Portugal) and P.M. is supported by grant IF/2012 (FCT, Portugal).

References

1. Farinha CM, Matos P, Amaral MD (2013) Control of cystic fibrosis transmembrane conductance regulator membrane trafficking: not just from the endoplasmic reticulum to the Golgi. FEBS J 280:4396–4406
2. Bannykh SI, Bannykh GI, Fish KN, Moyer BD, Riordan JR, Balch WE (2000) Traffic pattern of cystic fibrosis transmembrane regulator through the early exocytic pathway. Traffic 1:852–870
3. Farinha CM, Amaral MD (2005) Most F508del-CFTR is targeted to degradation at an early folding checkpoint and independently of calnexin. Mol Cell Biol 25:5242–5252
4. Chang XB, Cui L, Hou YX, Jensen TJ, Aleksandrov AA, Mengos A, Riordan JR (1999) Removal of multiple arginine-framed trafficking signals overcomes misprocessing of delta F508 CFTR present in most patients with cystic fibrosis. Mol Cell 4:137–142
5. Roxo-Rosa M, Xu Z, Schmidt A, Neto M, Cai Z, Soares CM, Sheppard DN, Amaral MD (2006) Revertant mutants G550E and 4RK rescue cystic fibrosis mutants in the first nucleotide-binding domain of CFTR by different mechanisms. Proc Natl Acad Sci U S A 103:17891–17896
6. Farinha CM, King-Underwood J, Sousa M, Correia AR, Henriques BJ, Roxo-Rosa M, Da Paula AC, Williams J, Hirst S, Gomes CM, Amaral MD (2013) Revertants, low temperature, and correctors reveal the mechanism of F508del-CFTR rescue by VX-809 and suggest multiple agents for full correction. Chem Biol 20:943–955
7. Nishimura N, Balch WE (1997) A di-acidic signal required for selective export from the endoplasmic reticulum. Science 277:556–558
8. Yoo JS, Moyer BD, Bannykh S, Yoo HM, Riordan JR, Balch WE (2002) Non-conventional trafficking of the cystic fibrosis transmembrane conductance regulator through the early secretory pathway. J Biol Chem 277:11401–11409
9. Gee HY, Noh SH, Tang BL, Kim KH, Lee MG (2011) Rescue of DeltaF508-CFTR trafficking via a GRASP-dependent unconventional secretion pathway. Cell 146:746–760
10. Wang X, Venable J, LaPointe P, Hutt DM, Koulov AV, Coppinger J, Gurkan C, Kellner W, Matteson J, Plutner H, Riordan JR, Kelly JW, Yates JR 3rd, Balch WE (2006) Hsp90 cochaperone Aha1 downregulation rescues misfolding of CFTR in cystic fibrosis. Cell 127:803–815
11. Simpson JC, Joggerst B, Laketa V, Verissimo F, Cetin C, Erfle H, Bexiga MG, Singan VR, Heriche JK, Neumann B, Mateos A, Blake J, Bechtel S, Benes V, Wiemann S, Ellenberg J, Pepperkok R (2012) Genome-wide RNAi screening identifies human proteins with a regulatory function in the early secretory pathway. Nat Cell Biol 14:764–774
12. Botelho HM, Uliyakina I, Awatade NT, Proença MC, Tischer C, Sirianant L,

Kunzelmann K, Pepperkok R, Amaral MD (2015) Protein traffic disorders: an effective high-throughput fluorescence microscopy pipeline for drug discovery. Sci Rep 5:9038

13. Almaça J, Faria D, Sousa M, Uliyakina I, Conrad C, Sirianant L, Clarke LA, Martins JP, Santos M, Heriché JK, Huber W, Schreiber R, Pepperkok R, Kunzelmann K, Amaral MD (2013) High-content siRNA screen reveals global ENaC regulators and potential cystic fibrosis therapy targets. Cell 154:1390–1400
14. Van Goor F, Hadida S, Grootenhuis PD, Burton B, Cao D, Neuberger T, Turnbull A, Singh A, Joubran J, Hazlewood A, Zhou J, McCartney J, Arumugam V, Decker C, Yang J, Young C, Olson ER, Wine JJ, Frizzell RA, Ashlock M, Negulescu P (2009) Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. Proc Natl Acad Sci U S A 106:18825–18830
15. Erfle H, Neumann B, Liebel U, Rogers P, Held M, Walter T, Ellenberg J, Pepperkok R (2007) Reverse transfection on cell arrays for high content screening microscopy. Nat Protoc 2:392–399
16. Kamentsky L, Jones TR, Fraser A, Bray MA, Logan DJ, Madden KL, Ljosa V, Rueden C, Eliceiri KW, Carpenter AE (2011) Improved structure, function and compatibility for cell profiler: modular high-throughput image analysis software. Bioinformatics 27:1179–1180
17. Amaral MD, Lukacs GL (2011) Introduction to section III: biochemical methods to study CFTR protein. Methods Mol Biol 741:213–218
18. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
19. Mendes AI, Matos P, Moniz S, Jordan P (2010) Protein kinase WNK1 promotes cell surface expression of glucose transporter GLUT1 by regulating a Tre-2/USP6-BUB2-Cdc16 domain family member 4 (TBC1D4)-Rab8A complex. J Biol Chem 285:39117–39126
20. Mendes AI, Matos P, Moniz S, Luz S, Amaral MD, Farinha CM, Jordan P (2011) Antagonistic regulation of cystic fibrosis transmembrane conductance regulator cell surface expression by protein kinases WNK4 and spleen tyrosine kinase. Mol Cell Biol 31:4076–4086
21. Moniz S, Sousa M, Moraes BJ, Mendes AI, Palma M, Barreto C, Fragata JI, Amaral MD, Matos P (2013) HGF stimulation of Rac1 signaling enhances pharmacological correction of the most prevalent cystic fibrosis mutant F508del-CFTR. ACS Chem Biol 8:432–442
22. Loureiro CA, Matos AM, Dias-Alves A, Pereira JF, Uliyakina I, Barros P, Amaral MD, Matos P (2015) A molecular switch in the scaffold NHERF1 enables misfolded CFTR to evade the peripheral quality control checkpoint. Sci Signal 8:48