Chapter 2

High-Throughput Screening of Libraries of Compounds to Identify CFTR Modulators

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Abstract

Small molecules acting as selective activators (potentiators), inhibitors, or "correctors" of the CFTR chloride channel represent candidate drugs for various pathological conditions including cystic fibrosis and secretory diarrhea. The identification of CFTR pharmacological modulators may be achieved by screening highly diverse synthetic or natural compound libraries using high-throughput methods. A convenient assay for CFTR function is based on the halide sensitivity of the yellow fluorescent protein (YFP). CFTR activity can be simply assessed by measuring the rate of YFP signal decrease caused by iodide influx. This assay can be automated to test thousands of compounds per day.

Key words: Cystic fibrosis, CFTR, fluorescent protein, high-throughput screening, drug discovery.

1. Introduction

The CFTR chloride channel is an important pharmacological target to treat various genetic and nongenetic diseases (1). In cystic fibrosis (CF), mutations affecting the *CFTR* gene cause a large variety of defects including altered CFTR channel gating (class III mutations such as G551D and G1349D) or impaired CFTR protein maturation (class II mutations such as F508del). Therefore, compounds increasing CFTR-dependent chloride transport are potentially useful as drugs to treat CF patients. In particular, pharmacological activators of CFTR, called potentiators, are useful to overcome the gating defect caused by class III CF mutations. Conversely, other compounds, called correctors, may help the F508del-CFTR protein to escape the endoplasmic reticulum and reach the plasma membrane. Potentiators are also useful

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for F508del. Indeed, this mutation causes also a gating defect, although less severe than that of classical class III mutations. On the other hand, CFTR inhibitors are potentially useful to treat secretory diarrhea and polycystic kidney disease, two pathological conditions characterized by increased CFTR activity (1).

The identification of novel pharmacological modulators (potentiators, correctors, and inhibitors) of the CFTR chloride channel may be achieved by performing high-throughput screenings of large chemical libraries using a functional assay. This assay has to measure the main function of the CFTR protein, i.e., the transmembrane transport of chloride and other small anions. CFTR function may be determined directly with electrophysiological techniques. However, these methods are time consuming and expensive. Usually, high-throughput screenings are better performed using fluorescence-based techniques. For CFTR, a convenient fluorescent probe is the halide-sensitive yellow fluorescent protein (HS-YFP).

YFP is a derivative of the green fluorescent protein (GFP). Its fluorescence is quenched in the presence of chloride at high concentrations. Its sensitivity to anions has been further improved by mutagenesis. The replacement of histidine by glutamine at position 148 (H148Q) was initially found to enhance the affinity of the YFP protein to iodide and chloride, the corresponding K_i values being 20 and 100 mM, respectively (2). Subsequently, a second isoleucine to leucine mutation at position 152 (I152L) further increased the affinity for halides (K_i ~ 2 mM for iodide and 20 mM for chloride) (3).

The different sensitivity of the HS-YFP toward iodide and chloride allows to perform assays measuring the transport of anions through the plasma membrane as changes in cell fluorescence. For this assay, the cells expressing HS-YFP are equilibrated in a physiological chloride-rich saline solution (e.g., Dulbecco's PBS). During fluorescence reading, cells are exposed to a high concentration of iodide. Iodide influx quenches the cell fluorescence with a rate that depends on the halide permeability of cell membrane, and therefore, on the activity of anion channels or transporters (**Fig. 2.1**).

The YFP assay has been applied to CFTR (4–7), pendrin (8), and the Ca^{2+}-activated Cl^- channel (9). Because of its simplicity and sensitivity, it can be automated. Therefore, it has been applied to perform high-throughput screenings of large chemical libraries in order to identify pharmacological modulators of CFTR.

Here we present a schematic description of procedures used to evaluate CFTR function in high-throughput format with HS-YFP. We also describe an adaptation of the HS-YFP assay for transiently transfected cells (HEK-293). Although this method has a significantly lower throughput, it is useful for initial evaluation of novel CFTR mutants or activity of a limited number of compounds.

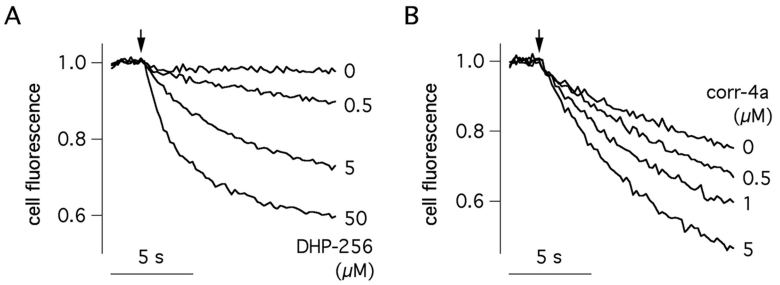

Fig. 2.1. Evaluation of CFTR correctors and potentiators with the HS-YFP assay. The figure shows representative cell fluorescence recordings acquired with a microplate reader. (**a**) Potentiator assay: FRT cells expressing G551D-CFTR. Cells were acutely stimulated with forskolin (20 μM) plus the potentiator DHP-256 at the indicated concentrations. (**b**) Corrector assay: FRT cells expressing F508del-CFTR. Cells were treated for 24 h with corr-4a at the indicated concentrations. After treatment, cells were acutely stimulated with forskolin (20 μM) and genistein (50 μM). In both panels, the *arrow* indicates iodide addition.

2. Materials

2.1. Cell Culture Media and Transfection Reagents

1. Cell culture medium for FRT cells: Coon's modification of F-12 (Sigma-Aldrich). This is a powder medium that requires addition of sodium bicarbonate as indicated by the supplier. After solubilization, the medium is supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum.
2. Cell culture medium for A549 and HEK-293 cells: Dulbecco's modified Eagle's medium–Ham's F-12 (DMEM/F-12 1:1) (Euroclone) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum.
3. The selection agents zeocin, hygromycin B (Invitrogen), and geneticin (Calbiochem) are dissolved in tissue culture-grade water at 100 mg/ml, stored in aliquots at −20°C (zeocin) or at 4°C (hygromycin B and geneticin), and then added to cells as required.
4. PBS solution without Ca^{2+} and Mg^{2+} (Euroclone).
5. Trypsin solution (0.05%) and ethylenediaminetetraacetic acid (EDTA) (0.02%) (Euroclone).
6. Transfection reagent: Lipofectamine 2000 (Invitrogen).
7. Synthetic medium to use for DNA–Lipofectamine 2000 complex formation: Opti-MEM (Invitrogen).

2.2. CFTR and YFP Plasmids

Common plasmids or other vectors carrying the CFTR coding sequence and suitable for stable transfections can be used. Plasmids for the YFP-H148Q or YFP-H148Q/I152L can be

obtained from our laboratory or from Dr. A.S. Verkman (verkman@ucsf.edu).

2.3. Saline Solutions

1. Standard Dulbecco's PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$ (pH 7.4).
2. Iodide-rich Dulbecco's PBS: 137 mM NaI, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$ (pH 7.4).

2.4. Equipment

1. For automated testing on stable transfected cells: a microplate reader equipped with one or two syringe pumps, temperature control, and high-quality excitation (HQ500/20X: 500 ± 10 nm) and emission (HQ535/30 M: 535 ± 15 nm) filters for EYFP (Chroma Technology Corp., Brattleboro, VT).
2. For manual testing on transiently transfected cells: an inverted fluorescence microscope equipped with optical filters for EYFP (excitation: 505 nm; emission: 535 nm; dichroic: 515 nm). Optical filters for EGFP or similar probes (485 for excitation and 520 for emission) are also acceptable. The microscope needs one of the following acquisition systems: (1) photomultiplier tube (PMT, Hamamatsu) connected to an analog-to-digital converter (e.g., PowerLab 2/25; ADInstruments) or a camera (e.g., CoolSNAP cf; Photometrics) with relatively fast acquisition rate (at least four images per second).

2.5. Compounds

Compounds are dissolved as 10 mM stock solutions in dimethyl sulfoxide (DMSO) and arrayed in 96-well master plates. Daughter plates are made at 1–2 mM concentration in DMSO. Plates are stored at -80°C. Compounds are added to cells dissolved in saline solution (potentiators and inhibitors) or in the culture medium (correctors). Final DMSO concentration should not exceed 0.5–1.0%. Appropriate controls to evaluate effect of DMSO alone should be included throughout the screening.

Screenings are usually performed by testing a single compound per well. However, screenings of small compound pools (four per well) have also been carried out.

3. Methods

3.1. Generation and Use of Stable Transfectants for CFTR Functional Assays

Clones of Fischer rat thyroid (FRT) or A549 cells stably coexpressing human CFTR and the halide-sensing YFP (9) are generated with the following scheme:

1. Cells are first transfected with the plasmid carrying the coding sequence of wild-type or mutant CFTR using Lipofectamine 2000 (Invitrogen).
2. Twenty-four hours after transfection, cells are treated with the appropriate selection agent (e.g., 0.6 mg/ml zeocin or 0.75 mg/ml geneticin).
3. After 3–6 days, surviving cells are plated in 96-well microplates at clonal density (0.5–1 cell per well) for further selection.
4. Positive clones are identified by immunofluorescence or with functional assays (e.g., short-circuit current measurements for FRT cells).
5. Cells with stable CFTR expression are retransfected with the plasmid carrying the coding sequence for the halide-sensitive YFP and treated with a second selection agent to isolate pure fluorescent clones (*see* **Note 1**).
6. Stable clones co-expressing CFTR and the fluorescent protein are evaluated with functional assays. If needed, the cells can be re-cloned again to isolate cells with homogeneous expression of both proteins. After expansion of the best clone(s), the cells are frozen in several aliquots to keep a large stock for future screenings.

FRT and A549 cells expressing CFTR and YFP are cultured in the continuous presence of the corresponding selection agents. When needed for screenings, the cells are plated (50,000 cells/well) on clear-bottomed 96-well black microplates (Corning Life Sciences, Acton, MA) (*see* **Note 2**).

3.2. Generation of Transiently Transfected Cells for CFTR Functional Assays

This method is suitable for transient transfection in cells like HEK-293:

1. Cells are plated in 96-well microplates (25,000 cells/well) in 100 μl of DMEM/F-12 medium supplemented with 10% serum *without antibiotics.*
2. After 6 h, the cells are co-transfected with the plasmids coding for CFTR and the halide-sensitive YFP.
3. The transfection complex solution contains 0.2 μg total plasmidic DNA and 0.5 μl of Lipofectamine 2000 (Invitrogen) in 50 μl of Opti-MEM (Invitrogen) per well.
4. The mixture is incubated for 1 h at room temperature to allow formation of DNA/Lipofectamine 2000 complexes before addition to the cells (final volume in the well is therefore 150 μl).
5. After 24 h, the complexes are removed by replacement with fresh medium (*see* **Note 3**).

3.3. CFTR Assay by Microplate Reader

This method has been successfully used for high-throughput screening of large compound libraries to find CFTR inhibitors (4), potentiators (5, 6), and correctors (7):

1. At the time of the assay (24–48 h after cell plating in 96-well microplates), the cell culture medium is removed and the cells are washed and incubated for 30–45 min with 60 μl of Dulbecco's PBS containing compounds for CFTR stimulation/inhibition (e.g., forskolin, CPT-cAMP, and potentiators).
2. Microplates are transferred to a microplate reader for CFTR activity determination.
3. The assay in each well consists of a continuous 14 s fluorescence reading with 2 s before and 12 s after injection of 165 μl of the iodide-rich Dulbecco's PBS. This step ensures that the cells are exposed to a final iodide concentration of 100 mM. Injection flow rate is set at 100–160 μl/s. Cell incubation and assay are carried out at 37°C.

3.4. CFTR Assay by Fluorescence Microscope

This method has been successfully used to study the effect of CFTR inhibitors (10) and potentiators (11) after transient transfection in cells like HEK-293:

1. At the time of assay (48 h after transfection in 96-well microplates), cells are washed twice with standard Dulbecco's PBS.
2. After washing, the cells are incubated for 30–45 min with 60 μl PBS with and without compounds for CFTR stimulation/inhibition (e.g., forskolin, CPT-cAMP, and potentiators).
3. After this step, the microplates carrying the cells are transferred to the microscope to perform the assay, one well at a time.
4. Cell fluorescence is continuously recorded (with a PMT or a camera) before and after addition of 165 μl of iodide-containing Dulbecco's PBS.

3.5. Conditions for Screening (Potentiators, Inhibitors, and Correctors)

3.5.1. Conditions for the Screening of Correctors on F508del-CFTR Cells

1. Eighteen to twenty-four hours before the assay, the cells are treated with test compounds at the desired concentration in the culture medium. Each microplate is used to test 80 compounds with the rest of wells available for positive controls (known correctors) and negative controls (DMSO vehicle alone). Additional microplates can be incubated at 27°C (in a 5% CO_2–95% air atmosphere) as another positive control.
2. At the time of assay, cells are washed with standard Dulbecco's PBS and stimulated for 30–45 min with forskolin (20 μM) plus genistein (50 μM) in a final volume of 60 μl of the same saline solution.

3.5.2. Conditions for the Screening of Potentiators on F508del-CFTR Cells

1. Eighteen to twenty-four hours before the assay, the cells are incubated at 27°C (in a 5% CO_2–95% air atmosphere) to allow rescue of the mutant protein to the plasma membrane.
2. At the time of assay, cells are washed with standard Dulbecco's PBS and stimulated for 30–45 min with forskolin (20 μM) and test compounds (at the desired concentration) in a final volume of 60 μl of the same saline solution. Positive controls include genistein and/or other known potentiators at maximally effective concentrations.

3.5.3. Conditions for the Screening of Potentiators on G551D- and G1349D-CFTR Cells

The conditions are identical to those used to test potentiators on F508del-CFTR cells except that the step of incubation at low temperature is omitted. Furthermore, higher concentrations of potentiators are required for G551D cells since this mutant is more refractory to pharmacological stimulation (e.g., genistein needs to be tested at 100–200 μM instead of 50 μM).

3.5.4. Conditions for the Screening of Inhibitors on Wild-Type CFTR Cells

1. Twenty-four to forty-eight hours after plating in 96-well microplates, cells with stable expression of wild-type CFTR and the halide-sensitive YFP are washed with Dulbecco's PBS.
2. Cells are treated for 30–45 min with a stimulating cocktail containing forskolin (20 μM) and IBMX (100 μM) plus test compounds (at the desired concentration) in a final volume of 60 μl Dulbecco's PBS.

3.6. Data Analysis

Data analysis is usually performed as follows:

1. Subtracting the background fluorescence (i.e., well with saline solution but no cells);
2. Normalizing each trace by the initial fluorescence;
3. Fitting of the fluorescence decay (skipping the first few points to avoid the injection artifact) with a single exponential function (**Fig. 2.2a**);
4. Determination of maximal slope by differentiation of the fit (*see* **Note 4**).

Fluorescence subtraction and normalization can be done with Excel. Fitting needs a program such as Igor (Wavemetrics). The entire calculation, or part of it, can be done using automated procedures.

An alternative method to quantify fluorescence quenching is based on the determination of the decrease in signal at a fixed time point (**Fig. 2.2a**). This approach is simpler and may be more adequate for cells with a complex shape of the cell fluorescence decrease that does not follow a single exponential decay.

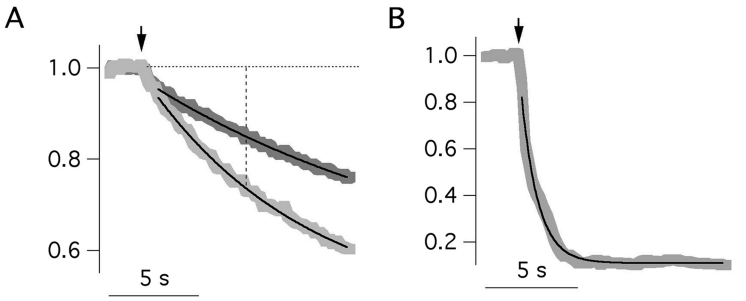

Fig. 2.2. Analysis of HS-YFP assay data. (**a**) Representative traces from FRT cells showing two different levels of F508del-CFTR activity (*light gray* and *dark gray lines*). The fluorescence decay was fitted with exponential functions (*smooth black curves*) to calculate initial maximal slope. As an alternative method, decay was also measured as the fraction of initial fluorescence at a fixed time point (*dashed vertical line*). (**b**) Example of artifact generated by incubation with a toxic compound. Presence of toxic compounds during corrector screenings causes cell detachment at the time of iodide solution injection during the HS-YFP assay. This results in a sharp fluorescence drop that may appear as a "high CFTR activity."

A software to analyze cell fluorescence intensity (e.g., MetaMorph; Molecular Devices) needs to be utilized if fluorescence changes are acquired as a series of images (microscope equipped with a fast acquisition camera). This method is time consuming compared to acquisition of an integrated signal from a cell population, as done with a microplate reader or with a microscope equipped with a PMT. However, it gives the advantage to determine CFTR activity in single cells.

4. Notes

1. In theory, transfection of cells with the HS-YFP in first place and then with the CFTR plasmid should be more practical. Indeed, after the second transfection, the HS-YFP assay can be used to rapidly screen hundreds of clones to identify the ones that express CFTR. However, for unknown reasons, this procedure is associated in our hands with a very low probability of positive clones, at least in FRT cells.
2. Besides FRT and A549 cells, other cell lines can also be used to perform the HS-YFP assay. One of the requirements is that the cell line has no endogenous activity of other anion channels and transporters that could interfere with the CFTR activity. Another important requirement is that the chosen cell line must be strongly attached to the plastic of the microplate to resist washings and iodide solution

injection. Cell plating density needs to be determined for each cell line.

3. Percentage of HEK-293 cells expressing HS-YFP is in the 30–50% range using the indicated conditions.
4. The initial points after iodide injection are an important source of artifacts. Solution addition causes a fast drop in fluorescence that is usually smaller than 5% of total signal. If not skipped during the fitting step, it could erroneously generate a large value of fluorescence decay rate. The extent of the artifact caused by solution injection should be monitored each time by running wells in which the cells are not stimulated. During screenings and automatic analysis of data, the presence of toxic compounds may generate false positives (**Fig. 2.2b**) because of large decreases in cell fluorescence upon iodide injection (due to cell detachment). These artifacts can be easily detected by visual inspection of traces (they appear as sharp drops in cell fluorescence) and cells (there will be a visible detachment at the center of the well).

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