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Assessment of Drug Transporter Function Using Fluorescent Cell Imaging

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Abstract

ATP-binding cassette (ABC) proteins, including the breast cancer resistance protein (BCRP) and the multidrug resistance proteins (MDRs), actively transport structurally diverse chemicals from a number of tissues. Moreover, transporters are being increasingly cited as mediators of clinically relevant drug-drug interactions. The potential outcomes of concomitantly administering two drugs that interact at the same transporter include altered disposition and toxicity and/or efficacy of one or both of the drugs. Research demonstrating the role of transporters in clinical pharmacokinetics has shed light on the need for *in vitro* screening methods that detect drug-transporter interactions during preclinical development. This paper describes a cell-based model for the detection of functional inhibitors of BCRP and MDR1 by measuring fluorescent substrate accumulation in suspended cells that overexpress or endogenously express these proteins using an automated cell counter. An alternate protocol is provided describing the use of a spectrophotometer with fluorescence detection capabilities to identify functional inhibitors of BCRP and MDR1 in transporter overexpressing cells. While a spectrophotometer is available in most laboratories, an automatic cell counter offers convenience, sensitivity, and speed in measuring the cellular accumulation of fluorescent substrates and identification of novel inhibitors.

Keywords

ABC transporter; MDR1; BCRP; ABCB1; ABCG2

INTRODUCTION

The role of ATP-binding cassette (ABC) efflux transporters as mediators of drug-drug interactions is currently a topic of great interest due to the potential for altered drug disposition, therapeutic efficacy, and toxicity. ABC transporters, such as the multidrug resistance protein 1 (MDR1/*ABCB1*) and the breast cancer resistance protein (BCRP/*ABCG2*), are transmembrane proteins that transport chemicals out of the cell with energy derived from the hydrolysis of ATP. Expressed in a number of tissues, transporters contribute to the excretion of chemicals (such as in the intestine, liver, kidneys) as well as to

the protection of sensitive organs (such as in the brain and placenta) (reviewed in Klaassen and Aleksunes, 2010). The chemicals that interact with ABC transporters as substrates and/or inhibitors include a wide variety of xenobiotics such as pharmaceuticals and environmental chemicals (Gedeon et al., 2006; Tanigawara et al., 1992; van Herwaarden et al., 2006). Chemotherapeutic agents such as doxorubicin (Shen et al., 2008) and vinblastine (Hammond et al., 1989), as well as the cardiac glycoside, digoxin (Tanigawara et al., 1992), interact with MDR1. Examples of BCRP specific substrates and/or inhibitors include the antibiotic drug nitrofurantoin (Wright et al., 2011), hypoglycemic drug glyburide (Gedeon et al., 2008) and dietary carcinogens, such as heterocyclic amines and aflatoxin B₁ (van Herwaarden et al., 2006). Many drugs exhibit substrate specificity for only MDR1 or BCRP, however there are some chemicals that interact with both transporters such as the blood pressure medication prazosin and the chemotherapy drug topotecan (de Vries et al., 2007; Zhou et al., 2009). The significant effect of transporters on the pharmacokinetics of new chemical entities *in vivo* has encouraged the publication of a report by the International Transporter Consortium that describes the importance of screening for drug-transporter interactions and provides initial guidelines for evaluating transporter function during drug development testing (Giacomini et al., 2010).

Chemicals that are functional inhibitors of ABC transporters can interfere with the transport of substrates by competitive or non-competitive inhibition (Giacomini et al., 2010). The functional inhibition of transporters can be determined by measuring the accumulation of a fluorescent substrate in cells that overexpress the ABC transporter of interest in the presence and absence of the test chemical. Detection of fluorescent substrates presents advantages over radioactive and analytical (i.e., mass spectrometry) methods including the sensitive detection of fluorescent substrates, relatively low cost, and convenience. Visualization of fluorescent substrate retention may be performed using a fluorescence microscope which does not provide a quantitative measure. A spectrophotometer with fluorescence detection capabilities has been used as a quantitative measure of fluorescent substrate accumulation (Barthomeuf et al., 2005; Ozveg-Laczka et al., 2004), however the procedure utilizes cell lysates rather than whole cells and the overall sensitivity of detection is lower. A more sensitive method, flow cytometry, has been used previously to detect and quantify the intracellular cellular accumulation of fluorescent substrates in the presence of ABC transporter inhibitors (García-Escarp et al., 2004; Ivnitski-Steele et al., 2008; Kim et al., 2012). While flow cytometry is able to measure the fluorescence intensity of individual cells with optimal sensitivity, the high cost, and required access to a Core Facility emphasize the need for additional simple and user-friendly methods for the identification of functional inhibitors of ABC transporters.

This unit describes methods for detecting the effect of test chemicals on the function of ABC transporters using fluorescent dyes in MDR1- and BCRP-overexpressing cell lines as well as cell lines endogenously expressing both transporters. A fluorescence detection method that utilizes an automated cell counter, the Cellometer® Vision (Nexcelom Bioscience, Lawrence, MA), was demonstrated to be similarly effective at identifying ABC transporter inhibitors as flow cytometry (Robey et al., 2011). The Cellometer® Vision offers sensitivity, rapid detection of intracellular fluorescence intensity, convenience of use, and is cost effective. The first protocol includes a step-by-step procedure of the method introduced by Robey et al. for quantifying transporter function by measurement of intracellular fluorescent substrate retention with an automated cell counter (Cellometer® Vision). For laboratories without access to the Cellometer® Vision, alternate instructions for fluorescence detection in cell lysates using a 96-well plate format and a microplate spectrophotometer are also provided.

NOTE: All protocols using human-derived cells must follow appropriate blood-borne pathogen procedures approved by an Institution.

BASIC PROTOCOL. MEASUREMENT OF TRANSPORTER FUNCTION IN ABC TRANSPORTER-OVEREXPRESSING CELLS USING AN AUTOMATED FLUORESCENT CELL COUNTER

This protocol provides a detailed account of the steps involved in the quantification of ABC transporter function in suspended cells using an automated cell counter, the Cellometer® Vision. The Cellometer® Vision is able to detect the effect of specific ABC transporter inhibitors on the accumulation of a fluorescent substrate quickly and with great sensitivity. Because the Vision has interchangeable fluorescence optic modules, a wide variety of chemicals that fluoresce (excitation/emission) at 375/450 nm, 475/535 nm, 525/595 nm, and other wavelengths can be used. Fluorescent substrates and positive control inhibitors including recommended concentrations for this procedure are listed in Table 1 for the MDR1 and BCRP transporters. This basic protocol focuses on analysis of chemical transport by a single transporter over-expressed in a cell line that exhibits low basal expression and activity of other transporters. A cell line transfected with an empty vector plasmid can be used as a control. This approach allows an investigator to focus on a single transporter protein and investigate novel chemicals as potential inhibitors. Because this method uses overexpressing cells, one can expect significant differences in substrate fluorescence intensity between the active and the inhibited transporter.

Materials

Appropriate transporter-overexpressing cell lines:

- *Laboratory-Generated Cell Lines:* Cells can be transfected with plasmids containing various ABC efflux transporters using Lipofectamine™ and other transfection reagents. Plasmids for the various ABC efflux transporters as well as empty vector controls can be purchased at Origene Technologies (Rockville, MD) as well as other companies.
- *Commercially-Available Cell Lines:* Companies have already prepared cells that overexpress various ABC transporters which are licensed for use. Examples include the SB MDCKII BCRP transfected cell line, SB MDCKII MDR1 transfected cell line, SB HL60 MRP1 selected cell line, and SB K562 MRP1 selected cell line from Solvo Biotechnology (Boston, MA).

Complete cell culture medium

Cell dissociation medium (i.e., 0.25% Trypsin)

Dimethylsulfoxide (DMSO)

Fluorescent substrates:

- Rhodamine 123 (Sigma-Aldrich, St. Louis, MO) dissolved in DMSO, 10 mM stock
- Hoechst 33342 (Sigma-Aldrich, St. Louis, MO), dissolved in deionized water, 10 mM stock

Positive control inhibitors:

- PSC833 (Xenotech, Lenexa, KS), dissolved in DMSO, 1mM stock
- Ko143 (Sigma-Aldrich, St. Louis, MO), dissolved in DMSO, 1 mM stock

Test inhibitors, dissolved in DMSO, 1 mM and 10 mM stock solutions for each chemical

Two clear 96-well round bottom microtiter plates with lids (one plate to balance the centrifuge rotor)

Microtiter plate centrifuge set to 5°C, $500 \times g$ for 5 min.

Tubes (amber-colored if available)

- One 15 ml tube
- Eleven 1.5 ml tubes
- Ten 2 ml tubes

Cell culture incubator (37°C, 5% CO₂)

Automatic serological pipettor

Multichannel (8) automatic pipettor (100–1000 µl)

Multichannel (8) manual pipettor (5–50 µl)

Chilled phosphate-buffered saline (PBS)

Ice

Aluminum foil to cover plate

Paper towels

Cellometer® Vision and computer software

Cellometer® counting chamber slides

Cellometer® Vision fluorescence optics modules

- VB-450-302 (Ex/Em: 375/450 nm for Hoechst 33342) and/or
- VB-595-502 (Ex/Em: 525/595 nm for Rhodamine 123)

Prepare chemicals

- 1 Prepare stock solutions of a fluorescent substrate (FS) and positive control inhibitor (I) (Table 1). Selection of the appropriate FS and I depends on the transporter of interest.
- 2 Choose two test inhibitors and prepare two stock solutions for each inhibitor in DMSO at concentrations of 1 mM and 10 mM.
- 3 Dilute FS in 7 ml complete cell culture medium to the desired final concentration in a 15 ml tube (Table 1).

Remember to limit the light exposure of the fluorescent substrates until the end of the experiment by keeping the lights dim, using amber-colored tubes, or covering the tubes and/or plate with aluminum foil.

- a. Aliquot the volumes of FS solution into 1.5 ml tubes labeled 1–10 (Figure 1, Table 2). **NOTICE** that tube 11 is a negative control, which should receive the indicated volume of complete cell culture medium without FS.

- 4 Add test inhibitors (or I) and DMSO to the tubes (1–11) from step 3a (Table 2). The final concentration of DMSO will be 1%. When complete, place solutions to the side of the cell culture hood and out of the light.

Prepare cells

- 5 Add 2 ml of cell dissociation medium to confluent cells overexpressing empty vector or the transporter of interest in T75 cell culture flasks for 3–4 min or until cells begin to detach from the flask.
- 6 Add 6 ml of complete cell culture medium to each flask, pipette cells and medium into 15 ml tubes and centrifuge at $400 \times g$ for 4 min at room temperature.
- 7 Remove supernatant (containing cell dissociation medium) from the tube without disturbing the cell pellet. Then resuspend cells in 10 ml of fresh medium.
- 8 Determine the concentration of each cell type (transporter overexpressing and empty vector control) using the cell count function on the Cellometer® Vision and dilute to 500,000 cells/ml.

Ideally, you will need at least 6 ml of cell suspension (500,000 cells/ml) for each cell type in order to plate the appropriate number of cells. It is possible to use lower concentrations of cells ($>100,000$ cells/ml), however, this will result in fewer cells for imaging. Keep in mind that cells will be lost during the experiment. A pilot experiment varying cell concentration may target the optimal concentration of cells for a specific cell line.

- 9 Add 200 μ l of diluted cells to the appropriate wells of a clear 96-well round bottom plate according to Figure 2.
- 10 Centrifuge the plate at $500 \times g$ for 5 min at 5°C . *Do not forget to balance the centrifuge rotor with a second plate. 5°C was chosen to ensure the transporters are inactive in between incubations.*
- 11 Throw the medium into a biohazard waste container with a forceful flick. Blot the 96-well plate with a paper towel to remove excess medium from the surface of the plate (Video 1).

Loading phase

- 12 Add 100 μ l of previously prepared solutions (Table 2) to the appropriate wells (Figure 2) (4 wells/treatment).
- 13 Cover plate with lid and incubate in cell culture incubator for 30 min at 37°C and 5% CO_2 (keep dark).

Most cell types will remain in suspension during incubation. If the cells attach to the walls of the 96-well plate during incubation this will not alter the course of the experiment, however care should be taken at the end of the experiment to adequately resuspend the cells in PBS (see Step 28). During the incubation, chill the medium in a 4°C refrigerator or on ice.

Prepare for efflux phase

- 14 Label ten 2 ml tubes A–J (Table 3).

- a. Add 500 μ l chilled medium to tubes A–H.
- b. Add 1000 μ l chilled medium to tube I.
- c. Add 2000 μ l chilled medium to tube J.

- 15 Prepare tubes (A–J) with and without inhibitors and DMSO (Table 3). The final concentration of DMSO will be 1%.
- 16 After the 30 min incubation has ended, centrifuge the plate at $500 \times g$ for 5 min at 5°C.
 If the centrifuge is in a different room than the cell culture hood, keep plate on ice and covered with aluminum foil when walking between laboratories. The low temperature and foil will assure that the transporters remain inactive during this time and that the fluorescent substrates are not quenched by light.
- 17 Throw the medium into a biohazard waste container with a forceful flick. Blot the 96-well plate with a paper towel to remove medium from the surface of the plate (Video 1).
- 18 Add 100 μ l chilled medium to each well using a multichannel (8) automatic pipettor. This will wash the cells of residual substrate and/or inhibitor.
 The chilled medium ensures that the transporters will not begin to efflux substrates prior to the designated efflux phase. If a multichannel automatic pipettor is unavailable, keep the plate on ice while adding chilled medium.
- 19 Centrifuge the plate at $500 \times g$ for 5 min at 5°C.
- 20 Throw the medium into a biohazard waste container with a forceful flick. Blot the 96-well plate with a paper towel to remove medium from the surface of the plate (Video 1). *Keep the plate on ice until 60 min incubation at 37°C (Step 22).*

Efflux phase

- 21 Add 100 μ l of previously prepared inhibitor solutions (Table 3) to appropriate wells of the 96-well plate (Figure 2). *Note: FS (row A) and No FS (row G) receive Tube J, medium with no inhibitor.*
- 22 Cover plate with lid and incubate in cell culture incubator for 60 min at 37°C and 5% CO₂ (keep dark). *During incubation, chill PBS in 4°C refrigerator or on ice.*

Prepare to measure intracellular fluorescence

- 23 After the 60 min efflux incubation, centrifuge the plate at $500 \times g$ for 5 min at 5°C.
- 24 Throw the medium into a biohazard waste container with a forceful flick. Blot the 96-well plate with a paper towel to remove medium from the surface of the plate (Video 1).
- 25 Place the plate on ice and rinse cells by adding 100 μ l chilled PBS to each well using a multichannel (8) automatic pipettor.
- 26 Centrifuge the plate at $500 \times g$ for 5 min at 5°C.

- 27 Throw the PBS into a biohazard waste container with a forceful flick. Blot the 96-well plate with a paper towel to remove PBS from the surface of the plate (Video 1).
- 28 Place the plate on ice for the remainder of the experiment and resuspend the cells in up to 50 μ l of PBS by pipetting up and down with a multichannel manual pipettor to mix cells and lift from the bottom of the well. If cells attach to the plate, use extra force to remove the cells from the bottom of the wells by individually pipetting each well to ensure the cells are in suspension. After resuspended in PBS, the cells should no longer attach to the plate because the plate will be on ice for the remainder of the experiment.

Measure intracellular fluorescence using the Cellometer® Vision

- 29 Turn on the Cellometer® Vision and open the Cellometer® Vision computer software.
- 30 From the dropdown menu choose the appropriate ABC transporter assay (Hoechst 33342 or Rhodamine 123) and make sure the appropriate fluorescence optics module is installed in the instrument (Hoechst 33342: VB-450-302; Rhodamine 123: VB-595-502).
- 31 The parameters for both assays should be:
 - a. Cell type: Choose cell type (will not distinguish between overexpressing and empty vector cell types) from dropdown menu. Common cell types available with the Cellometer® Vision software include: Human embryonic kidney 293 (HEK) cells, Madin-Darby canine kidney epithelial (MDCK) cells, Human promyelocytic leukemia cells (HL60), and Human erythromyeloblastoid leukemia cells (K562). If the desired cell type is not listed use 'Initial cell type' which uses a wide range of parameters that fit many different types of cells. However, if greater specificity is desired, it is possible to add the new cell type with the appropriate parameters by taking an image of your cells with the Cellometer® Vision and electronically sending it to specialists at Nexcelom Bioscience. After characterization, the cell type (and parameters) can be remotely added to the cell type dropdown menu on your computer. Be sure to send the image a few days before performing the experiment as it may take some time for Nexcelom to generate the parameters and add the cell type to the computer program.
 - b. Imaging Mode: Brightfield and Fluorescence
 - c. Dilution: 1.0
 - d. Brightfield exposure: 10.9 ms.
 - e. Fluorescence exposure: 2.0 s. Alter fluorescence exposure time based on signal observed when previewing the F1 image (see Step 35). The range should not be less than 90%.
- 32 Load 20 μ l of cell suspension from one well of the 96-well plate (on ice) into one chamber of a Cellometer® counting chamber slide (Figure 3).
- 33 Insert loaded chamber slide into the Cellometer® Vision and preview brightfield image. Focus the brightfield image with the knob located on the right side of the Cellometer® Vision.
- 34 Stop preview brightfield image.

- 35 Preview F1 image and if range is greater than 90%, click count.
- 36 After counting, an excel file is generated containing size and fluorescence intensity data for every individually recognized cell that fits the parameters of the chosen cell type. The excel file along with brightfield and fluorescent images of the cells can be exported to save.
- 37 Repeat steps 32–36 for the remaining wells taking care to cover the plate from the light in between sample retrieval.

ALTERNATE PROTOCOL 1. MEASUREMENT OF TRANSPORTER FUNCTION IN CELLS THAT ENDOGENOUSLY EXPRESS ABC TRANSPORTERS USING AN AUTOMATED FLUORESCENT CELL COUNTER

This alternate protocol describes the use of the Cellometer® Vision to analyze fluorescent chemical transport in cells that endogenously express ABC transporters. Use of cells that normally express ABC transporters, rather than cells that are genetically manipulated to express the proteins at high levels may bring one a step closer to understanding how a specific ABC transporter functions in the presence of other normal cellular activities occurring in that cell type (i.e., other transporters and drug metabolizing enzymes). In order to delineate the specific role of one ABC transporter on the transport of a substrate, it is important to first characterize the expression and function of all of the transporters that are present. When choosing an appropriate fluorescent substrate for functional assays, co-incubation of the cells with a specific inhibitor for each ABC transporter that is present is necessary to understand the contribution of individual transporters to the transport of the fluorescent substrate. Once a substrate is identified, a dose-response experiment should be performed to determine the concentration of substrate that elicits the greatest difference in fluorescence intensity in the presence and absence of the specific transporter inhibitor. After the substrate concentration is optimized, steps from the Basic Protocol can be followed to measure the effect of two test inhibitors on the transport of a fluorescent substrate using the Cellometer® Vision automated cell counter. Keep in mind that you will not need wells for empty vector cells, eliminating half of the wells on the 96-well plate and half of the solutions that are required. If your chemical requires a different fluorescence optics module (Ex/Em: 475/535 nm; i.e., Calcein AM), be sure to install the correct one prior to using the Cellometer® Vision.

ALTERNATE PROTOCOL 2. MEASUREMENT OF TRANSPORTER FUNCTION IN ABC TRANSPORTER-OVEREXPRESSING CELLS USING A 96-WELL PLATE FLUORESCENCE READER

Without access to an automated cell counter, measuring intracellular fluorescence intensity of suspended cells that overexpress an ABC transporter using a 96-well plate fluorescence reader may similarly identify potential inhibitors of ABC transporter function. Disadvantages of this method include lower sensitivity as well as a lack of data that reports cell size and the frequency distribution of cell fluorescence intensity that is provided by the Cellometer® Vision. However, since most laboratories have access to a 96-well plate reader with fluorescence detection, this method may provide sufficient results. Additionally, previous work has identified tyrosine kinase inhibitors and the coumarin cniadin, as inhibitors of BCRP and MDR1 function, respectively, using a multiplate reader to measure the intracellular accumulation of fluorescent substrates in cell overexpression systems (Ozvegy-Laczka et al., 2004, Barthomeuf et al., 2005). This alternate protocol provides modifications to the Basic Protocol to measure intracellular fluorescent substrate accumulation using a 96-well plate fluorescence reader.

The concentration of fluorescent substrate used should be similar to the recommendations in Basic Protocol 1 (Table 1), however an initial dose-response experiment should be performed to determine the concentration of fluorescent substrate that can be altered by a prototypical inhibitor (MDR1: PSC-833, BCRP: Ko143) with the greatest degree of transporter inhibition. The materials and steps described in Basic Protocol 1 may otherwise be followed with a few alterations: (1) two 96-well flat bottom plates (one for each test inhibitor) must be used instead of round bottom plates; (2) a different plate setup providing a greater sample size for each cell type and treatment due to the decreased sensitivity of the instrument and variability within groups. An additional row of cells should be plated to determine the final concentration of cells within each well in order to properly report the data (i.e., fluorescence intensity/ 10^5 cells); (3) after step 27, lyse the cells in 50 μ l of 50% methanol rather than resuspending in PBS; (4) read the plate at the appropriate excitation and emission wavelengths for each substrate (Hoechst 33342: 355/465 nm; Rhodamine 123: 511/534 nm) using a 96-well plate reader with fluorescence detection such as a SpectraMax Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA). Of note, the cells should be washed very well before lysis.

COMMENTARY

Background Information

ABC efflux transporters influence the pharmacokinetics of many structurally diverse chemicals. As their name implies, these transporters require ATP to remove chemicals from the tissues in which they are located including the intestines, liver, kidneys, brain, and placenta (reviewed in Klaassen and Aleksunes, 2010). There is increasing evidence for the role of efflux transporters in the disposition and subsequently efficacy and/or toxicity of xenobiotics *in vivo*. In particular, there is a need for thorough investigations of substrate-transporter interactions particularly with new chemical entities (Giacomini et al., 2010). Currently, clinical transporter-drug interactions are beginning to be listed on the package inserts for drugs such as the immunosuppressant Sandimmune® (cyclosporine). The package insert indicates that up to 35 drugs, each when taken concomitantly with cyclosporine, may alter the plasma concentration of cyclosporine as a result of interacting with MDR1 and/or the drug metabolizing enzyme, cytochrome P450 3A4 (Sandimmune (cyclosporine) [package insert], 2012). Additionally listed are the effects of cyclosporine on the toxicity of other drugs that are transported by MDR1 such as digoxin. Digoxin is a cardiac glycoside with a narrow therapeutic index. Impaired clearance of digoxin because of MDR1 inhibition may increase the risk for cardiac toxicities including ventricular tachycardia and atrioventricular block (Dorian et al., 1988). Cyclosporine has also been observed to increase the exposure of patients taking the chemotherapy drug Hycamtin® (topotecan) by 2- to 3-fold when the two drugs are taken together (Hycamtin (topotecan) [package insert], 2011). The BCRP transporter has similarly been recognized as a potential mediator of clinically relevant drug-drug interactions considering the overlap in the chemicals that interact with the transporter. The growing list of drugs that interact with MDR1 and BCRP emphasizes the particular relevance of screening for drug-transporter interactions during preclinical development.

This paper describes cell-based methods for determining the effect of test inhibitors on ABC transporter (BCRP and MDR1) function. This can be accomplished by measuring fluorescent substrate accumulation within the cells that have been incubated with and without the test inhibitors. Table 4 lists additional fluorescent substrates and positive control inhibitors that are often used in measuring ABC transporter function by flow cytometry including chemicals that interact with another class of transporters, the multidrug resistance-associated proteins (MRPs). If a particular substrate of interest is not available as a fluorescent molecule, radiolabeled substrates may be used in a similar cell-based model to

measure the intracellular accumulation of the radioactive chemical in the presence of a test inhibitor. Drawbacks of the model include the high cost of working with radioactivity, the need for custom synthesis of a substrate radioisotope if the substrate is not commercially available, and the potential for isotope binding to the filter plate and/or cellular constituents which may confound the results. An alternate *in vitro* model for studying chemical-transporter interactions includes the inverted membrane vesicles that are isolated plasma membranes from cells that overexpress a particular ABC transporter. The vesicle model system may not be ideal for measuring certain chemical interactions, as it is not a cell-based model and therefore cannot indicate if a metabolite may interact with the transporter. Flow cytometry utilizes cells in suspension and fluorescent substrates to detect chemical-transporter interactions, however this method can often require access to a Core Facility. The automated cell counter, the Cellometer® Vision (Nexcelom Bioscience, Lawrence, MA) is able to rapidly measure the intracellular substrate fluorescence within cells that overexpress or endogenously express ABC transporters. Unlike a 96-well plate fluorescence reader, the Cellometer® Vision is a more sensitive measure of fluorescence intensity within cells, which ensures that the measure is a reflection of intracellular substrate accumulation. Experiments performed using the Cellometer® Vision exhibit between 15.5–28.5% standard deviation for fluorescent substrates commonly used in our laboratory using human embryonic kidney 293 (HEK) cells overexpressing BCRP, demonstrating the repeatability of the procedure (Table 5). For a modest cost, laboratories can purchase their own Cellometer® Vision which may be an affordable and convenient alternative to flow cytometry. The Cellometer® Vision has also been used to examine how transcriptional regulation may increase or decrease the functional activity of ABC transporters in brain microglia cells (Gibson et al., 2012). With the ability of the Cellometer® Vision to measure fluorescence intensity of individual cells, another potential application of the automated cell counter may include the detection of cell surface expression of ABC transporters to determine transcription efficiency using fluorescently-labeled antibodies. Similarly, if transfection plasmids include the gene that codes for green fluorescent protein, transfection efficiency may be monitored with the Cellometer® Vision using the appropriate fluorescence optics module (VB 535-402).

Critical Parameters and Troubleshooting

Recommended substrate concentrations are provided for the Basic Protocol using the ABC transporter-overexpressing cells. Keep in mind that it may be necessary to adjust the concentration of the fluorescent substrate based on the cell type used to achieve the greatest difference in fluorescence intensity in the presence and absence of inhibitors. If you choose to use a different cell type or fluorescent substrate, an initial concentration-response experiment with and without the positive control inhibitor will aid in optimizing the fluorescent substrate concentration. The speed of the centrifugation is also critical to troubleshoot to ensure that cells are not lost when the medium is thrown from the 96-well plate. The speed recommended in this protocol ($500 \times g$) is based upon experiments using a microcentrifuge with a radius of 32 cm. Be sure to consider centrifuge speed if your centrifuge has a radius larger than 32 cm.

Because some cell types may exhibit a range of cell sizes, you should consider using the “normalize fluorescence to cell size” option (Gibson et al., 2012). It should also be noted that data can be re-analyzed with different parameters by using the original images acquired rather than repeating the experiment.

The purpose of the loading phase is to equally load all of the cells with the fluorescent substrate before the efflux phase. To ensure that there is equal loading of cell types and that

the test inhibitors do not interfere with the uptake of a substrate, baseline fluorescence should also be quantified in each cell type and treatment following the uptake phase.

This assay may generate false-negative results as it is possible that your cell line may not express the appropriate uptake transporters needed for the test inhibitor to gain entry to the cell for access to the intracellular portion of the transporter.

Anticipated Results

Results generated by the Cellometer® Vision allow you to present the data as a frequency distribution, as is commonly seen with flow cytometry, as well as mean fluorescence intensity. The range of fluorescence intensity will vary based upon the substrate and cell type. No matter what the substrate or cell type is, the cells co-incubated with positive control inhibitor (and the empty vector cells) should generate a frequency distribution that is shifted to the right as compared to the ABC transporter overexpressing or endogenously expressing cells incubated without inhibitor (Robey et al., 2011; Figure 4A). This reflects greater fluorescence retention in cells where MDR1 or BCRP has been inhibited (i.e., no efflux of substrate). Moreover, the mean fluorescence intensity of the cells plus positive control inhibitor and empty vector cells should be much greater than the transporter overexpressing cells without the inhibitor (Figure 4B). If the test chemicals inhibit the transport of a specific ABC transporter substrate, then one would expect both a graded shift of the frequency distribution to the right and increasing average fluorescence intensity, with increasing concentration of test inhibitor. After finding a chemical that inhibits transporter efflux with the initial screen, it may be desirable to increase the number of wells per treatment to obtain a higher sample size in order to perform statistics. Furthermore, an extended concentration-response experiment with various substrate and the test inhibitor concentrations can help to characterize the nature of the chemical-transporter interaction.

Time Considerations

Approximately six to seven hours should be allotted to conduct the cell uptake and efflux steps and to individually pipette and analyze each well from the 96-well plate using the Cellometer® Vision.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Sample preparation

Distribution of fluorescent substrate (FS) solution and medium in preparation for the addition of positive control inhibitor and various concentrations of test inhibitors in subsequent steps.

			Test Inhibitor 1				Test Inhibitor 2						
		1	2	3	4	5	6	7	8	9	10	11	12
FS	A												
FS + 1 μ M test inhibitor	B												
FS + 10 μ M test inhibitor	C												
FS + 50 μ M test inhibitor	D												
FS + 100 μ M test inhibitor	E												
FS + I	F												
No FS	G												
	H												

Overexpressing cells

Empty vector cells

Figure 2. Plate layout

The layout for a 96-well round bottom plate demonstrates the placement of cell types and prepared solutions (100 μ l). The following abbreviations are used: fluorescent substrate (FS), fluorescent substrate plus various concentrations of test inhibitors (FS + test inhibitor), fluorescent substrate plus positive control inhibitor (FS + I), and no fluorescent substrate (No FS).

Figure 3. Cell loading procedure for imaging

This image demonstrates the addition of suspended cells (20 μ l) into a counting chamber slide. It should be noted that a blue dye was used to visualize loading. During a typical experiment, the solution will be colorless.

Figure 4. Example results with fluorescent substrates

Human embryonic kidney 293 cells stably-transfected with human BCRP or MDR1 genes or empty vector plasmids were incubated with fluorescent substrates (BCRP: Hoechst 33342 and MDR1: Rhodamine 123). Positive inhibitors (BCRP: Ko143 and MDR1: PSC833) were used to block efflux of fluorescent substrates. (A) Line graphs represent the distribution of individual cell fluorescence. Each point represents the mean percent of cells $\pm$ SE exhibiting a quantity of fluorescence. (B) Data (bar graphs) are presented as mean relative fluorescence $\pm$ SE normalized to cell size.

Table 1
Recommended substrates and inhibitors

Recommended stock and final concentrations of fluorescent substrate (FS) and positive control inhibitor (I) for each efflux transporter.

	Fluorescent Substrate (FS)	Positive Control Inhibitor (I)
BCRP	Hoechst 33342	Ko143
Diluent	Deionized water	DMSO
Stock	10 mM	1 mM
Final	7 μ M	1 μ M
MDR1	Rhodamine 123	PSC833
Diluent	DMSO	DMSO
Stock	10 mM	1 mM
Final	5 μ M	1 μ M

Preparation of control [fluorescent substrate (FS), fluorescent substrate plus positive control inhibitor (FS + I), no fluorescent substrate (No FS)] and test inhibitor (FS + test inhibitor) solutions.

Table 2
Preparation of inhibitor solutions for the loading phase

Tube	Solution	FS (μl) ¹	Stock test inhibitor 1 (μl)	Stock test inhibitor 2 (μl)	Stock I (μl)	DMSO (μl)	Total Volume (μl)
1	FS	1000	-	-	-	10	1010
2	FS + 1 μM test inhibitor 1	500	0.5 (1 mM stock)	-	-	4.5	505
3	FS + 10 μM test inhibitor 1	500	0.5 (10 mM stock)	-	-	4.5	505
4	FS + 50 μM test inhibitor 1	500	2.5 (10 mM stock)	-	-	2.5	505
5	FS + 100 μM test inhibitor 1	500	5 (10 mM stock)	-	-	0	505
6	FS + 1 μM test inhibitor 2	500	-	0.5 (1 mM stock)	-	4.5	505
7	FS + 10 μM test inhibitor 2	500	-	0.5 (10 mM stock)	-	4.5	505
8	FS + 50 μM test inhibitor 2	500	-	2.5 (10 mM stock)	-	2.5	505
9	FS + 100 μM test Inhibitor 2	500	-	5 (10 mM stock)	-	0	505
10	FS+I	1000	-	-	1	9	1010
11	No FS	1000 ²	-	-	-	10	1010

¹The FS volumes are from Figure 1.

²Tube 11 has medium without FS.

Table 3

Preparation of inhibitor solutions for the efflux phase.

Tube	Solution	Medium (μl) ^I	Stock test inhibitor 1 (μl)	Stock test inhibitor 2 (μl)	Stock I (μl)	DMSO (μl)	Total Volume (μl)
A	1 μM test inhibitor 1	500	0.5 (1 mM stock)	-	-	4.5	505
B	10 μM test inhibitor 1	500	0.5 (10 mM stock)	-	-	4.5	505
C	50 μM test inhibitor 1	500	2.5 (10 mM stock)	-	-	2.5	505
D	100 μM test inhibitor 1	500	5 (10 mM stock)	-	-	0	505
E	1 μM test inhibitor 2	500	-	0.5 (1 mM stock)	-	4.5	505
F	10 μM test inhibitor 2	500	-	0.5 (10 mM stock)	-	4.5	505
G	50 μM test inhibitor 2	500	-	2.5 (10 mM stock)	-	2.5	505
H	100 μM test inhibitor 2	500	-	5 (10 mM stock)	-	0	505
I	I	1000	-	-	1	9	1010
J	No inhibitor	2000	-	-	-	20	2020

^I Medium chilled in 4°C refrigerator or on ice.

Table 4

Fluorescent substrates and inhibitors frequently used for the quantification of ABC transporter function.

	ABC Transporter	References
Fluorescent Substrates		
Bodipy-prazosin	BCRP, MDR1	Kimchi-Sarfaty et al., 2002; Robey et al., 2011
Pheophorbide A	BCRP	Dohse et al., 2010; Robey et al., 2004
Bodipy-paclitaxel	MDR1	Gow et al., 2008; Kimchi-Sarfaty et al., 2002
Hoechst 33342	BCRP, MDR1	Kim et al., 2002; Shapiro and Ling, 1997
Calcein AM	MDR1, MRPs	Homolya et al., 1996; Olson et al., 2001; Roy et al., 2009
Inhibitors		
Elacridar	BCRP, MDR1	de Bruin et al., 1999 Woodahl et al., 2004
Verapamil	MDR1, MRPs	Konya et al., 2006 Vellonen et al., 2004
Probenecid	MRP1	Dogan et al., 2004
MK571	MRPs, BCRP, MDR1	Matsson et al., 2009

Table 5
Repeatability of transporter function using fluorescent cell imaging

Human embryonic kidney 293 cells stably transfected with the BCRP gene or empty vector plasmid were incubated with fluorescent substrate (Hoechst 33342 or BODIPY-glyburide). Fluorescence intensity was measured by the CellometerR Vision. Data represent percent (%) standard deviation of absolute fluorescence intensity values measured in five independent experiments.

	Percent (%) Standard Deviation	
	Hoechst 33342	BODIPY-glyburide
BCRP Overexpressing	15.5	18.5
Empty Vector	19.3	28.5