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In Vitro Models for Studying Trophoblast Transcellular Transport

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Summary

In vitro models have proven to be effective in studying the placental transporters that play a role in the exchange of nutrients, waste products, and drugs between the maternal and fetal circulations. Although primary cultures of trophoblast cells can be used to perform uptake, efflux, and metabolism studies, only the rodent HRP-1 and the human BeWo cell lines have been shown to form confluent monolayers when grown on semi-permeable membranes. Protocols for the revival, maintenance, passage, and growth of BeWo cells for transporter expression and transcellular transport studies are provided.

Keywords

Trophoblast cells; BeWo cell; transcellular transport; efflux mechanisms

1. Introduction

As the fetus develops within the uterus, its sole link to the mother's blood circulation is through the placenta. Serving as a barrier between the mother and the fetus, the placenta mediates the transfer of nutrients and metabolic waste products while also secreting hormones that maintain pregnancy. The vectorial exchange of these compounds occurs because of the polarized nature of the placenta, and specifically the rate-limiting, single cell layer called the syncytiotrophoblast (1–3). In the process of forming the syncytiotrophoblast, blastocyst-derived cytotrophoblast stem cells invade the uterus to reach the maternal blood supply. Ultimately, the terminal differentiation of these cytotrophoblasts results in the complete fusion of lateral cell membranes, such that a polarized multinucleated syncytium forms (4). The maternal-facing plasma membrane of the syncytium consists of a microvillous brush border membrane that is in direct contact with maternal blood. On the opposite side, the basal membrane faces the fetal circulation and lacks microvillar projections. The two sides of the syncytium are not only structurally distinct, but also differ in the localization of transporters, enzymes, and hormone receptors.

In order to investigate the transport of nutrients, drugs, and pathogens across the placental syncytium, it is essential to utilize an effective in vitro model system in which cultured cells form a confluent monolayer on semi-permeable supports. Primary cultures of undifferentiated human cytotrophoblast cells can be isolated from term placentas (5). These cells syncytialize spontaneously when cultured and provide a good in vitro model system to successfully study uptake, efflux, metabolism, and hormone secretion. Unfortunately, instead of forming a tight-junctioned monolayer appropriate for transport studies, these nonproliferative, multinucleated cells form aggregates with large intercellular spaces when grown on semi-permeable membranes. In order to address this obstacle, Hemmings et al. have prepared confluent cell

layers of syncytiotrophoblasts on semi-permeable supports (6). This technique involves plating highly purified primary cultures of cytotrophoblasts in three successive cycles of seeding and differentiation. Although this method produces multiple overlapping layers of syncytialized cells, these cultures possess areas of microvillar projections on the apical surface and function as a barrier to low and high-molecular-weight molecules.

There are a variety of other experimental trophoblast systems available, including immortalized cell lines derived from normal and malignant tissues (reviewed in King et al. [7]). More recently, trophoblast cell lines have been developed from human embryonic stem cells (8) and the mouse trophectoderm and later extraembryonic ectoderm (9). Although trophoblast cells derived from human embryonic stem cells propagate poorly, likely because of as-yet unidentified growth factors, it is a potentially attractive biological resource because these cells form syncytia, express a range of trophoblast markers, and secrete placental hormones (8). In addition, stem-cell-derived cytotrophoblasts may provide a model for the early placenta, in contrast with primary cultures of cytotrophoblasts and choriocarcinoma-derived cell lines, which display characteristics of third-trimester placentas. As for in vitro model systems for studying trophoblast transcellular transport, currently there are only two trophoblast cell lines that have been shown to form confluent monolayers when grown on semi-permeable membranes, including the human BeWo cell line (10) and the rodent HRP-1 cell line (11).

The HRP-1 cell line is derived from normal midgestation rat chorioallantoic placenta. The HRP-1 cell line expresses cellular markers of and is morphologically similar to labyrinthine trophoblast cells (11). This cell line has been used to study the expression and/or function of fatty acid, glucose, glutamate, and organic anion transport systems (12–16). Because HRP-1 cells express the cytochrome P450 CYP1A1 isozyme, these cells also serve as a useful in vitro model for placental metabolic studies (11).

The human, choriocarcinoma-derived, BeWo cell line (17) forms a confluent, polarized monolayer that provides a good in vitro model system to study the transcellular distribution of nutrients and drugs across the placental trophoblast (10). The BeWo cell line is easy to maintain by passage and grows in a relatively short period of time. BeWo cells also demonstrate hormonal secretion properties common to typical trophoblasts and display many of the characteristics of third-trimester trophoblasts (18). In contrast with primary cultures of cytotrophoblasts, BeWo cells are unable to spontaneously differentiate and consist predominantly of undifferentiated cytotrophoblasts with a few syncytialized cells (19). Although treatment with forskolin or cyclic-adenosine monophosphate will stimulate BeWo cells to syncytialize (19), these treatments cause cellular aggregation and destroy monolayer confluency. Fortunately, undifferentiated BeWo cells are morphologically similar to primary cultures of trophoblasts, exhibiting close cell apposition and microvillar projections on the apical side of the monolayer (10). Furthermore, the monolayer is polarized in the expression of apical and basolateral marker enzymes and transporters in a manner consistent with primary trophoblasts (20). Overall, the BeWo cell line has been successfully utilized to investigate the asymmetric transcellular transport of multiple nutrients and compounds, including amino acids (21–23), glucose (24,25), cholesterol (26), folic acid (27), fatty acids (10), transferrin (28,29), serotonin (30), choline (31), and immunoglobulin G (32).

Transport studies can be performed in Transwell® inserts, Side-bi-Side™ diffusion chambers, or a similar setup (Fig. 1). Detailed instructions for carrying out transplacental transport experiments with BeWo cells in Transwell inserts appear in the protocols that follow. Outlined procedures include cell revival, maintenance of the BeWo cells in culture, freezing cells, plate preparation, seeding the cells, and transport protocols.

2. Materials

1. The BeWo cell line was originally derived from a human choriocarcinoma. The BeWo clone (b30) in use in our laboratory was obtained from Dr. Alan Schwartz (Washington University, St. Louis, MO). The original BeWo cell line is also available through American Type Culture Collection (ATCC; Manassas, VA, cat. no. CCL-98); however, it does not have the same monolayer-forming behavior in culture as the Schwartz clone.
2. Cell culture medium. This is prepared using Dulbecco's modified Eagle's medium (DMEM) (with L-glutamine and 4500 mg glucose/L, without sodium bicarbonate; Sigma Chemical Co., St. Louis, MO, cat. no. D-5648), heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA), penicillin/streptomycin (10,000 U/mL; Invitrogen, Carlsbad, CA), and MEM nonessential amino acids (Invitrogen).
3. HBSS-Glc. Hanks' balanced salt solution (HBSS) with sodium bicarbonate and without phenol red (Sigma, cat. no. H-8264) containing 25 mM D-glucose.
4. Tissue culture flasks (25, 75, 150 cm²). Available from Corning Costar (Corning, NY).
5. Trypsinization solution. Trypsin–ethylenediamine tetraacetic acid (EDTA) (10X) (Sigma, cat. no. T-4174) diluted as stated in Methods in 1X PBS. PBS is prepared from 10X phosphate buffered saline (PBS), pH 7.4, without calcium chloride, without magnesium chloride (Invitrogen, cat. no. 70011-044).
6. Freezing canister. Nalgene Cryo 1° Freezing Container (Nalgene Nunc International, Rochester, NY, cat. no. 5100-0001).
7. Freezing medium. Cell culture medium containing 10% dimethylsulfoxide (DMSO).
8. Lysis buffer. The formulation is listed in Methods. Use Triton X-100 (Sigma, cat. no. T-9284). Ensure that lysis conditions are compatible with detection methods for the substrate used.
9. Human placental collagen (Fluka Chemical, Milwaukee, WI, cat. no. WA13270).
10. Protease inhibitor cocktail. Complete Mini Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Indianapolis, IN, cat. no. 1 836 153).
11. 12-well Transwell polycarbonate plates (Corning Costar, cat. no. 3460).
12. Trypan Blue (0.4% solution, Sigma, cat. no. T-8154).
13. BCA Protein assay (Pierce, Rockford, IL, cat. no. 23227).
14. Hot box/incubator. Several options are available; we use the Boekel Jitterbug (Boekel Scientific, Feasterville, PA).
15. Fibronectin. Dissolve 5 mg of lyophilized fibronectin (Sigma) in 20 mL of sterile 1X PBS. The solution may be warmed to 37°C for brief periods to ensure dissolution. The stock (250 µg/mL) should be stored at 4°C and is stable for up to 2 mo. Immediately before use, a working solution of fibronectin (50 µg/mL) is prepared by diluting the stock solution 1:4 in sterile PBS.
16. Poly-D-lysine (PDL) hydrobromide (MW 70,000–150,000; Sigma, cat. no. P-6407).

3. Methods

3.1. BeWo Cell Culture Medium

1. Dissolve the contents of one bottle (enough for 1 L which is 13.4 g) DMEM powdered media in 870 mL of double-distilled water (ddH₂O).
2. Supplement with 3.5 g NaHCO₃ and stir for 20 min.
3. Adjust the pH to 7.4 using 1 N HCl.
4. Filter media through a 0.22- μ m disposable filter in a laminar flow hood (*see Note 1*).
5. Add 10 mL of 200 mM L-glutamine, 10 mL of 10,000 U/mL penicillin with 10 mg/mL streptomycin and 10 mL of 10 M nonessential amino acids to media.
6. Add FBS for 10% (v/v) (*see Note 2*).

3.2. BeWo Cell Revival From Liquid Nitrogen

1. Warm all the solutions needed for cell culture, e.g., DMEM and PBS, to 37°C.
2. Remove a vial of frozen BeWo cells from a liquid nitrogen storage tank and thaw the cells in a 37°C water bath.
3. Immediately after the cells are thawed, transfer the cell suspension to a 15-mL falcon tube containing 10 mL DMEM, and mix the cell suspension gently.
4. Centrifuge to pellet the cells at 335g for 8 min at room temperature.
5. Aspirate the supernatant and resuspend the cell pellet in 10 mL of media.
6. Centrifuge again, remove the supernatant and gently resuspend the cell pellet in 8 mL of media using a sterile Pasteur pipet.
7. Place the entire cell suspension into a 25-cm² tissue culture flask and label the flask with the cell line, passage number, and date.
8. Grow cells in a 37°C incubator supplied with 5% CO₂ and 95% relative humidity (*see Note 1*).
9. When the cells reach 70–80% confluence, the monolayer can be treated with trypsin to detach and disperse the cells.
10. Aspirate media from 25-cm² tissue culture flask using a sterile Pasteur pipet.
11. Wash flask with 5 mL of PBS twice in order to remove serum.
12. Detach the cells from a 25-cm² flask by incubating the cells with 5 mL of 1X trypsin-EDTA solution in PBS for 30 s and then aspirate off all but few drops of the liquid (*see Note 3*).
13. Place the flask in the 37°C incubator for approx 2 min.
14. Take the 25-cm² flask out of the incubator and examine the cell layer under the microscope to ensure that cells have detached from the substratum and “ball up.”
15. Suspend the cells uniformly in 12 mL of media and transfer the entire cell suspension to seed cells in a 75-cm² tissue culture flask. Label the flask with the cell line, passage number, and date.
16. When the cells become 70–80% confluent, passage cells to a 150-cm² tissue culture flask.

17. Detach and disperse cells as described in **Subheading 3.2., steps 10–14**, except use about 10 mL of the trypsin–EDTA solution.
18. Suspend the cells uniformly in 10 mL of media and seed 5 mL of such cell suspension into one 150-cm² tissue culture flask, which has 20 mL of media in it. Label the flask with the cell line, passage number and date.
19. Subsequent passages can be performed and are described as follows.

3.3. BeWo Cell Passage

1. Aspirate media from flask of cells to be passaged and wash flask with 10 mL of PBS twice.
2. Detach and disperse cells with trypsin-EDTA solution (2 mL of 10X trypsin-EDTA stock solution in 9 mL of PBS). Place in a 37°C incubator for approx 3–5 min and visually confirm cell detachment (*see Note 4*).
3. Add 10 mL of DMEM in the flask to stop trypsinization. Suspend cells and transfer entire cell suspension into a 50-mL Falcon tube.
4. Centrifuge to pellet the cells at 300g for 8 min at room temperature.
5. Aspirate the supernatant media and resuspend the cell pellet in 10 mL of DMEM.
6. Seed 1 mL of this cell suspension to each 150-cm² tissue culture flask, which has 25 mL of media in it (*see Note 5*).
7. Add one to the passage number shown on the initial frozen vial from liquid nitrogen. Label the flask with the cell line, passage number, and date.
8. Feed the cells the next day and every other day subsequently.
9. Visualize cells under a microscope. The morphology of cells is shown in Fig. 2.

3.4. BeWo Cell Storage

1. Obtain the cell pellet from trypsinization of a 150-cm² flask as described in **Subheading 3.3., steps 1–4**.
2. Suspend cells in 10 mL of DMEM media, determine the cell density, and create a cell suspension with 1×10^6 cells/mL in DMEM containing 10% DMSO.
3. Locate and label 2 mL tissue culture vials appropriate for deep-freezing.
4. Transfer 1.5 mL of cell suspension to each vial.
5. Freeze cells by placing in a freezing canister and placing in a –80°C freezer for 12 h. Cell vials should then be transferred to a liquid nitrogen storage container for long-term storage (*see Note 6*).

3.5. Efflux and Uptake Transporter Expression in BeWo Cells

Efflux transporters can be characterized by examining their expression at the level of protein in cultured cells, identifying substrates of the transporter by measuring transmonolayer permeability in a Transwell system (Fig. 1) and determining inhibitors of the transporter using uptake assays. All these techniques can also be applied to study uptake transporters.

1. Plate cells in either 75-cm² or 150-cm² tissue culture flasks as described under **Subheading 3.3.**
2. Harvest cells when they are approx 80% confluent.

3. Rinse cells with 5 mL of prewarmed (37°C) PBS three times.
4. Scrape cells from flask in 5 mL of PBS using a cell scraper and transfer the cells to a 15-mL Falcon tube.
5. Rinse the flask with another 5 mL of PBS and combine with the scraped cells.
6. Resuspend by pipetting up and down with a 5-mL pipet to break up cell clumps.
7. Count the cells using the trypan blue exclusion method.
8. Pellet cells by centrifugation at 335g for 15 min in a refrigerated centrifuge.
9. Resuspend cell pellet in fresh lysis buffer (1% Triton X-100, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride [PMSF]) to give a cell density at 1×10^8 cells/mL lysis buffer.
10. Incubate the cell lysate on ice for 30 min. Vortex briefly midway through incubation.
11. Centrifuge at 18,300g for 10 min at 4°C.
12. Collect supernatant, which contains solubilized protein and store aliquots at -80°C.
13. Determine protein concentrations using standard assays (e.g., BCA assay).
14. Transporter protein expression can be detected using standard procedures for Western blot analysis.

3.6. Growth of BeWo Cells in Transwell Plates

1. Prepare a stock solution of the coating material by dissolving human placental collagen in 0.1% acetic acid solution (1 mg in 0.345 mL) and store at 4°C. Prepare a working solution of the coating material immediately before use by diluting the stock solution 1:3 in 70% ethanol (one part human placental collagen in 0.1 % acetic acid and three parts 70% ethanol).
2. Pipet 70 µL of the coating material on the membrane of each well for a 12-well Transwell plate (adjust the volume for plates with different size wells), making sure that the membrane is coated evenly.
3. Dry for 2–3 h in a laminar flow hood with lid open (no ultraviolet [UV] light).
4. Sterilize the plates for 1 h under UV light with the lid open. Longer exposure times can cause the membranes to split.
5. If the plate is to be used immediately, skip to **step 7** below. Otherwise, wrap the plates in aluminum foil and store at 4°C. The plates may be used up to one week after coating with collagen.
6. If coated plates were stored in the refrigerator, allow the plates to warm to room temperature for approx 30 min before use.
7. Prewet the membranes with the addition 1 mL of prewarmed PBS (37°C) to the apical chamber and 2 mL to the basal chambers for 30–45 min.
8. Meanwhile, prepare cells for plating as described under **Subheading 3.3.** and determine the density of viable cells using trypan blue exclusion.
9. Calculate the dilution of the cell suspension needed to get a seeding density of 50,000 to 100,000 cells per mL.
10. Dilute the cells with DMEM to get the proper seeding density and mix well to ensure uniformity.

11. Aspirate PBS from both apical and basolateral chambers.
12. Seed the apical chamber with 0.5 mL per well of cell suspension using a repeat pipettor. Shake to evenly distribute the cells.
13. Add 1.5 mL of media in the basolateral chamber.
14. Feed cells the next day and every other day subsequently. Media on both the apical and basolateral sides should be replaced at each feeding.
15. Transport assays can be performed when the cells become confluent, which usually takes 5–6 d.

3.7. Transporter Assays

1. Warm up a bench-top incubator and all cell culture solutions to be used in the transport assay to 37°C for 30–45 min before preparing cells.
2. Solutions for pre-incubation and incubation steps should be prepared and warmed to 37°C. Generally, HBSS containing 25 mM L-glucose (HBSS-Glc) is the medium used for transport when using fluorescent substrates. The pre-incubation solution usually consists of HBSS-Glc along with any inhibitors that may be used in the experiment; the incubation solution should be identical to the pre-incubation solution except for the inclusion of the transporter substrate (*see Note 7*).
3. Remove media and wash the cells twice with prewarmed (37°C) HBSS-Glc (0.5 mL for apical chambers and 1.5 for basolateral chambers for a 12-well Transwell plate).
4. Aspirate HBSS-Glc from all the chambers.
5. Incubate cells in the appropriate pre-incubation solution for 30–60 min at 37°C on a rotating platform (*see Note 8*).
6. Aspirate the pre-incubation solution.
7. Add dosing solution to either the apical or basolateral chamber. For apical (A) to basolateral (B) transport, load 0.5 mL of drug/substrate to the top and 1.5 mL buffer to the bottom. Load the drug/substrate to the bottom for B to A transport.
8. At designated time points, sample 100 µL from the receiver chamber (to measure A to B transport, sample from the basolateral side; to measure B to A transport, sample from the apical side). Place the samples in a 96-well plate for fluorescent assay or scintillation vials, as appropriate.
9. Replace the sample withdrawn with 100 µL of fresh buffer identical to that originally added to that compartment.
10. Continue taking time points until designated time has elapsed.
11. To determine the rate of paracellular transport, [¹⁴C]-sucrose flux can be measured. This is usually done after a fluorescence experiment, but can be performed concurrently with the primary experiment when using a [³H]-labeled compound.
12. Fluorescence analysis or scintillation counting can then be performed for each time point.

3.8. Calculation of Apparent Permeability Coefficients

Apparent permeability coefficient for the monolayers of cells, P_{app} (in cm/s), can be calculated according to the following equation: $P_{app} = \Delta Q / \Delta t / (A * C_0)$, where $\Delta Q / \Delta t$ is the linear appearance rate of mass in the receiver solution, A is the membrane/cell surface area, and C_0

is the initial concentration of the test compound. The net efflux of a test compound can be assessed by calculating the ratio of P_{app} in the basolateral to apical direction vs P_{app} in the apical to basolateral direction (33).

3.9. Growth of BeWo Cells on Standard Cell Culture Plates

1. Prepare a PDL coating solution in 28% ethanol (5 μ g/mL).
2. Pipet coating solution to each well of culture plates, for example 100 μ L per well for a 12-well plate.
3. Shake the plate to coat the bottom of the well evenly and dry for 3 h in a laminar flow hood with the lid open (no UV light).
4. Sterilize the plates for 1 h under UV light. The plates could be used right away by skipping to **step 6** or kept in the refrigerator up to 2 wk if wrapped in aluminum foil.
5. If PDL coated plates were stored in the refrigerator, warm up the plate to room temperature for about 30 min before use.
6. Add 1 mL of PBS to each well of 12 well plates and leave in a laminar flow hood for 30 min.
7. Aspirate PBS and add three drops fibronectin (50 μ g/mL in PBS).
8. Shake the plates to spread the fibronectin and coat the wells evenly.
9. Dry the plates in hood for about 45 min.
10. Meanwhile, prepare cell suspension as described under **Subheading 3.3.** and determine viable cell density by using the trypan blue exclusion method.
11. Calculate the dilution of the cell suspension needed to get a density of 50,000 to 100,000 cells per mL and prepare a cell suspension of the proper seeding density by dilution with DMEM.
12. Remove excess fibronectin from the well by washing with 1 mL of DMEM.
13. Seed with 0.5 mL of cell suspension per well for 12 well plates using a repeat pipettor.
14. Add another 1 mL of DMEM to each well of a 12-well plate and shake to evenly distribute the cells.
15. Feed with 1.5 mL of DMEM every 2 d.
16. Use cells for uptake assay when they are at least 70–80% confluent; this usually occurs 5–6 d after plating.

3.10. Uptake Assays on Standard Cell Culture Plates

Uptake assays can be performed as described (34) with some modifications.

1. Warm up the bench top incubator and HBSS-Glc to 37°C for 30–45 min before preparing cells.
2. Place some HBSS-Glc on ice (approx 36–40 mL for a 12-well plate or 72–80 mL if using a 12-well Transwell plate).
3. Prepare solutions for pre-incubation and uptake as described previously for transport assays (*see Subheading 3.7.*).

4. Remove media and wash the cells twice with prewarmed (37°C) HBSS-Glc (1 mL per well for a 12-well plate or if using a 12-well Transwell plate, 0.5 mL for apical chamber and 1.5 mL for basolateral chamber).
5. Aspirate HBSS-Glc from all the wells.
6. Incubate cells in pre-incubation solutions for 30–45 min at 37°C in the bench top incubator on a rotating platform (add pre-incubation solution into both apical and basolateral chambers for a 12-well Transwell plate).
7. Aspirate the pre-incubation solution and initiate the uptake with the addition of 1 mL of the uptake incubation solution $\pm$ inhibitor to each well for a 12-well plate. For a 12 Transwell plate, either add 0.5 mL of uptake incubation solution $\pm$ inhibitor into apical chamber and 1.5 mL of HBSS-Glc $\pm$ inhibitor into the basolateral chamber or vice versa.
8. Incubate the cells in the incubation solution for the desired time period; this will depend on the substrate transmembrane permeability.
9. Remove the culture plate from the incubator and aspirate the uptake incubation solution.
10. Wash cells in each well three times with ice cold HBSS-Glc to stop uptake and remove excess substrate.
11. Add 1 mL of lysis buffer (0.5% Triton X-100 in 0.2 N NaOH; *see Note 9*) to each well for a 12-well plate. For a 12 Transwell plate, add 0.5 mL of lysis buffer into the apical chamber.
12. Cover and incubate the plate in the bench top incubator at 37°C for 2 h, or at 4°C overnight.
13. Mix the contents of each well by pipetting.
14. Assay the lysate from each well to determine the amount of substrate present and the protein concentration.
15. Uptake can be normalized to the number of cells in each well by expressing the data as: concentration of substrate/protein concentration. It is important that each data point be determined by substrate and protein determinations from the same well.

4. Notes

1. Unless otherwise noted, procedures should be performed under a laminar flow hood for sterile conditions. Cell lysis (**Subheading 3.5.**) and transporter (**Subheading 3.7.**) and uptake (**Subheading 3.10.**) assays may be performed in nonsterile surroundings. Culture conditions are 37°C, 5% CO₂, and 95% relative humidity.
2. The medium can be divided into two aliquots and filtered into 500-mL bottles. FBS (50 mL for 10% v/v) should be added to one bottle and the other stored without FBS until ready for use. Once serum is added, the medium may be used for up to 2 wk.
3. During the revival period and for several subsequent passages, the BeWo cells are treated with greater care. The cells are detached from the flasks by rapid exposure to 1X trypsin-EDTA and immediately resuspended. Centrifugation is avoided during this time to improve cell survival and attachment rates.
4. Once normal culture conditions are reached, the cells are passaged at a higher trypsin concentration. The final concentration used to passage BeWo is roughly 1.8X trypsin-EDTA (2 mL 10X trypsin-EDTA and 9 mL PBS).

5. Cell density is not precisely determined for seeding BeWo flasks. Adding 1 mL of suspension is a good place to start, but the amount may need to be adjusted accordingly. If cells reach confluency before 4–5 d, decrease the amount. Likewise, increase the amount of suspension added if it is more than 6 d before the cells are ready for passage.
6. Using a freezing canister filled with isopropyl alcohol (*see* Materials) will help to freeze the cell suspension more gradually and improve cell viability upon revival.
7. Radiolabeled compounds are common substrates for transport and uptake assays. These experiments may be performed under nonsterile conditions, but personal protective equipment and shielding appropriate for the selected isotope must be used.
8. Pre-incubation with an inhibitor may be necessary depending on its particular effect on the chosen transporter. Initial assays with a positive control should be performed to determine whether or not pre-incubation is needed.
9. Lysis buffer including NaOH may quench some fluorescent compounds, such as calcein. An alternative lysing solution is 2% Triton X-100.

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Fig. 1. Diagrams of Transwell® inserts (**A**) and Side-bi-Side™ diffusion chambers (**B**) used in transcellular transport experiments. In the 12-well Transwell plates described in the text, the apical chamber holds 0.5 mL of transport buffer and the basolateral chamber holds 1.5 mL. For both the Transwell inserts and Side-bi-Side apparatus, the membrane separating the two chambers is coated with a matrix, such as human placental collagen, and cells are seeded at the appropriate cell density. Both chambers can be sampled for analysis. To achieve uniform mixing, Transwell plates should be placed on a rotating platform; stir bars are placed within the Side-bi-Side diffusion chambers.

Fig. 2. Morphology of BeWo cells in culture. The BeWo cells form a monolayer when they are approx 100% confluent. Magnification 200 $\times$.