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Lucifer Yellow is a Robust Paracellular Permeability Marker in a Cell Model of the Human BBB

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January 11, 2019

Nandita Singh, Ph.D.
Senior Science Editor
JoVE

Dear Dr. Singh,

Thank you for arranging the reviews for JoVE58900R1. The reviewers' comments considerably improved the manuscript and I have appended a separate file titled 'Response to Reviewers' where we have addressed all comments. We have also added new data in the manuscript to address the reviewer's concerns.

I respectfully submit a revised mansucript titled "Lucifer Yellow is a Robust Paracellular Permeability Marker in a Cell Model of the Human BBB." We present a simple and robust assay to determine the apparent permeability coefficient of Lucifer Yellow in a cell model of the human blood-brain barrier (BBB). Drug delivery to the BBB is an exciting area of research where advances can lead to great impacts in the treatment of CNS diseases. *In vitro* BBB models are useful tools for research but must be carefully validated for accurate interpretation of experimental data. We present a simple and robust assay to determine the time required for the formation of functional tight junctions, a key characteristic of the BBB. Going further, we demonstrated an additional utility of this assay to determine changes in tight junction barrier characteristics in DNA nanoparticle-transfected cells.

Thank you for your consideration.

Yours sincerely,

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TITLE:

Lucifer Yellow - A Robust Paracellular Permeability Marker in a Cell Model of the Human Blood-brain Barrier

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KEYWORDS:

lucifer yellow, paracellular permeability, blood-brain barrier, hCMEC/D3, DNA nanoparticles, transfection.

SHORT ABSTRACT:

We present a fluorescence assay to demonstrate that Lucifer Yellow (LY) is a robust marker to determine the apparent paracellular permeability of hCMEC/D3 cell monolayers, an in vitro model of the human blood-brain barrier. We used this assay to determine the kinetics of a confluent monolayer formation in cultured hCMEC/D3 cells.

LONG ABSTRACT:

The blood-brain barrier BBB consists of endothelial cells that form a barrier between the systemic circulation and the brain to prevent the exchange of non-essential ions and toxic substances. Tight junctions (TJ) effectively seal the paracellular space in the monolayers resulting in an intact barrier. This study describes a LY-based fluorescence assay that can be used to determine its apparent permeability coefficient (P_{app}) and in turn can be used to determine the kinetics of the formation of confluent monolayers and the resulting tight junction barrier integrity in hCMEC/D3 monolayers. We further demonstrate an additional utility of this assay to determine TJ functional integrity in transfected cells. Our data from the LY P_{app} assay shows that the hCMEC/D3 cells seeded in a transwell setup effectively limit LY paracellular transport 7 days-post culture. As an additional utility of the presented assay, we also demonstrate that the DNA nanoparticle transfection does not alter LY paracellular transport in hCMEC/D3 monolayers.

INTRODUCTION:

Blood-brain barrier (BBB) is the protective barrier limiting the influx of plasma components into

the brain tissue and consists of brain endothelial cells along with supporting cells such as pericytes. The major role of BBB is to serve as a barrier that seals the space between peripheral blood and central nervous system (CNS) to maintain hemostasis of the neural microenvironment[1,2]. The brain capillary endothelial cells effectively seal the paracellular pathway via formation of intercellular tight junctions (TJs)[1]. This protective barrier allows glucose and selected nutrients to enter the brain while it prevents the majority of ions, toxic substances and drugs from passing through this tight barrier. Apart from its protective role, the natural barrier function of the BBB poses a severe challenge in the development of drug delivery systems targeting the CNS.

In vitro cell culture models of the BBB are helpful tools to study its biology and to understand the effects of drug treatment on TJ barrier integrity. We used the human cerebral microvascular endothelial cell line (hCMEC/D3) as an in vitro model since it is an accepted model of human brain endothelium[3] and recapitulates many functions of the human BBB. hCMEC/D3 is one of the most commonly used cell lines for modeling the BBB in vitro[4-9]. Despite its comparatively low values of transendothelial electrical resistance (TEER), a measure of barrier tightness, this cell line retains most of the morphological and functional properties of brain endothelial cells, even as a monoculture in the absence of cocultured glial cells[6,7]. The hCMEC/D3 cell line expresses multiple BBB markers including active transporters and receptors until approximately passage 35 without undergoing dedifferentiation to unstable phenotypes[6,7,9-11]. The most striking characteristic of hCMEC/D3 cell line as an in vitro BBB model is its ability to form TJs[5,9,11,12]. It should be noted that although stem cell-derived BBB models showed higher permeability in many studies compared with hCMEC/D3 cell line and they do express some BBB markers, they are yet to evolve as the most common BBB cell model[13]. Importantly, stem-cell derived BBB models remain to be characterized with respect to maximum passage numbers that allow the cells to maintain stable BBB phenotypes[14].

Three primary methods are commonly used to determine the TJ barrier integrity, including the measurement of TEER, measurement of apparent permeability coefficient (P_{app}) of small hydrophilic tracer molecules such as sucrose, inulin, Lucifer Yellow, etc. and immunostaining of known molecular markers of TJs such as claudin-5, ZO-1, occludin, etc.[5]. TEER is a relatively simple and quantitative method that measures the electrical resistance across the cell monolayers cultured on a porous membrane substrate[5]. However, TEER values can be influenced by experimental variables such as composition of the culture medium and the type of measurement instrument. A likely combination of these factors leads to a broad distribution of TEER values ranging from 2 to 1150 Ω cm^2 in the hCMEC/D3 cell line cultured for 2-21 days[13]. Immunostaining is a visual method to determine the presence of TJ proteins by labelling the targeted protein using antibodies. However, immunostaining involves a series of experimental steps, including the need to fix/permeabilize cells that may result in experimental artifacts and the fluorescent signals may fade over time. The above factors may lead to subjective errors affecting data quality.

The primary focus of this work is to present a LY-based apparent permeability assay determine the kinetics of a confluent monolayer formation in cultured hCMEC/D3 cells. Although other advanced in vitro BBB systems, such as co-culture systems, microfluidic systems, are

physiologically more relevant mimics with significantly improved barrier function[15-17], the hCMEC/D3 transwell setup is a simple and reliable model to estimate the kinetics of TJ formation and rapidly screen the effect of different drug formulations on barrier function. In general, P_{app} values are consistent for various hydrophilic solutes in hCMEC/D3 monolayers. For example, the reported P_{app} values for various low molecular mass solutes (such as sucrose, mannitol, LY, etc.) in different in vitro BBB models are in the order of 10^{-4} cm/min[5,18-20]. In our experimental setup, the brain endothelial cells are seeded on a collagen-coated microporous membrane for cell attachment and monolayer formation to mimic the in vivo barrier. The LY added in the apical side is expected to traverse the intercellular tight junctions and accumulate in the basolateral side. Greater concentrations of LY in the basolateral side indicate an immature, not-fully functional barrier while lower concentrations reflect restricted transport due to the presence of functional TJs resulting in a mature barrier.

LY is a hydrophilic dye with distinct excitation/emission peaks and avoids the need to radiolabel tracer molecules such as sucrose, mannitol or inulin. Thus, the fluorescence values of LY can be used to directly calculate its paracellular permeability across the BBB monolayers. Also, compared to many commercially available dyes used in biomedical fields that suffer from small Stokes shifts such as fluorescein[21], the Stokes shift of LY is about 108 nm with sufficient spectral separation, thus allowing LY fluorescence data as a robust readout to determine paracellular permeability. We used Western blotting as an orthogonal technique to demonstrate changes in expression of the tight junction marker protein, ZO-1, over culture time. ZO-1 expression detected via Western blotting is used to supplement the LY P_{app} data and in combination, these data suggest that the observed changes in LY P_{app} values is reflective of the formation of a monolayer with gradual increase in expression of the tight junction marker, ZO-1.

As pointed out earlier, the central focus of this work is to demonstrate a LY assay as a simple technique to monitor the formation of a confluent monolayer with functional tight junctions. However, to demonstrate an additional utility of the developed assay, we measured the LY P_{app} in DNA nanoparticle-transfected hCMEC/D3 monolayers. Nucleic acids can be condensed into polyelectrolyte nanoparticles with a diameter of 100-200 nm via electrostatic interaction between the positively charged groups of polymers and the negatively charged phosphate groups of nucleic acids[22,23]. We refer to these complexes as DNA nanoparticles (DNA NPs) in our work. While our intention is to transfect cells and express the desired protein, we must ensure that the barrier properties of the hCMEC/D3 monolayers are not compromised. Our data suggests that a standard 4 h luciferase gene transfection regime does not measurably change the LY permeability demonstrating the utility of the LY P_{app} assay to determine changes in TJ barrier integrity.

PROTOCOL:

1. General hCMEC/D3 cell culture

1.1. Resuscitation of frozen cells

NOTE: All cell culture maintenance and experiments were performed inside a sterile biosafety

hood. Culture media, supplements and reagents were either purchased as sterile products or sterilized via filtration using a 0.22 µm membrane filter to prevent microbial contamination.

1.1.1. Add 8.5 mL of collagen solution (0.15mg/mL) in a tissue culture flask (75 cm^2 growth area; referred henceforth as T75) and place it in an incubator (37 °C, 5% CO_2) for 1 h.

1.1.2. Remove the collagen solution and gently wash the flask with sterilized phosphate-buffered saline (PBS). Add 15 mL of complete growth medium to the flask and leave in the CO_2 incubator for 15 min.

NOTE: The complete medium (final concentration) contained Endothelial Cell Growth Basal Medium-2 (500 mL) supplemented with fetal bovine serum (5%), penicillin-streptomycin (1%), hydrocortisone (1.4 µM), acid ascorbic (5 µg/mL), chemically defined lipid concentrate (1/100), HEPES (10 mM) and basic fibroblast growth factor (1ng/mL).

1.1.3. Move a cryovial of frozen hCMEC/D3 cells from the liquid nitrogen tank and rapidly thaw the vial in a 37 °C water bath (< 1 min).

1.1.4. Once only a tiny flake of ice is visible, quickly aspirate and transfer the cells to the flask containing pre-warmed medium. Gently shake the flask to allow mixing of the cells with the growth medium.

1.1.5. Place the flask in the incubator (37 °C, 5% CO_2) and observe the cells under a light microscope after 2 h to make sure that the cells are attached.

1.1.6. Once the cells attach to the bottom of flask, remove the old growth medium and add 10 mL of fresh pre-warmed growth medium to replace dimethylsulfoxide in the old growth medium[24].

1.1.7. After 24 h, check under a light microscope to observe spindle-shaped cells and replace the old growth medium with pre-warmed fresh growth medium.

1.2. Cell culture maintenance

1.2.1. Replenish the growth medium every other day until 100% confluence. Check the cells under the microscope before removing the old growth medium and also after adding fresh growth medium. Take out the flask from the incubator and examine hCMEC/D3 cells under phase contrast microscope to ensure they appear healthy.

NOTE: The majority of cells should be attached to the bottom of the flask, have a spindle-shaped morphology and often times, light refracting around their membranes is also seen. Growth medium should be transparent (non-cloudy) and pinkish-orange in color.

1.2.2. Remove old growth medium from the flask and transfer 10 mL of pre-warmed fresh

medium into the flask.

NOTE: The medium should be added to the top side of the flask and not directly on the surface of the cells to avoid affecting cell attachment.

1.2.3. Turn the flask back into horizontal position and gently rock it several times and check hCMEC/D3 cells under the microscope before returning the flask to the incubator (37 °C, 5% CO_2).

1.2.4. Observe the cells under an inverted light microscope each time before and after handling the cells, both during regular culture work and during experiments. Record any noticeable changes in cell number or morphology in the laboratory notebook.

1.3. Cell passaging

1.3.1. Incubate a new T75 flask with 8.5 mL of collagen solution for 1 h in the incubator (37 °C, 5% CO_2).

1.3.2. Remove the collagen solution and gently wash the flask with sterilized PBS. Add 10 mL of pre-warmed hCMEC/D3 medium to the new flask and place the flask in the incubator (37 °C, 5% CO_2).

1.3.3. Take out the flask from the incubator and examine hCMEC/D3 cells under a phase contrast microscope to check if the cells are 100% confluent.

1.3.4. Remove hCMEC/D3 cell medium from the flask containing cells and wash hCMEC/D3 cells with 10 mL of PBS.

NOTE: FBS added to growth medium contains protease inhibitors such as α1-antitrypsin and α2-macroglobulin. These inhibit the trypsinization process. Thus, it is essential to wash the cells with PBS to remove traces of FBS to prevent the inhibition of the trypsinization process.

1.3.5. Add 1 mL of 0.25% trypsin solution containing 0.02% EDTA and trypsinize for 2-5 min in the incubator (37 °C, 5% CO_2) (tap the flask gently on the sides to help detachment).

NOTE: Never leave the cells on trypsin/EDTA for more than 6 min.

1.3.6. Add 10 mL of pre-warmed hCMEC/D3 medium to stop the trypsinization process and resuspend hCMEC/D3 cell by pipetting up and down several times. Then, remove the entire cell suspension from the flask into a 15 mL tube.

1.3.7. Transfer 1 mL of the cell suspension from the 15 mL tube to the new flask with pre-warmed fresh medium (splitting cells 1:10) and return the new flask back to the incubator.

NOTE: Before transferring to the new flask, pipette the cell suspension up and down several times

to minimize cell concentration gradients.

2. Cell plating

2.1. Place tissue culture inserts with microporous membranes (pore size: 0.4 µm, material: polyethylene terephthalate (PET)) into a 24-well culture plate.

2.2. Add 400 µL of collagen type I (0.15 mg/mL) in each tissue culture insert and incubate for 1 h in the CO_2 incubator (37 °C, 5% CO_2). Rock the 24-well plate gently to allow even spreading of the collagen solution over the microporous membrane in the tissue culture inserts.

2.3. Remove the collagen solution and gently wash the microporous membrane with 0.4 mL of 1x PBS buffer.

2.4. Plate hCMEC/D3 cells with the density of 50,000 cells/cm^2 in the cell inserts (15,000 cells in 500 µL of medium).

NOTE: In order to minimize the differences in cell number in each tissue culture insert, the cell suspension was resuspended with a 10 mL pipette before adding cells to the inserts.

2.5. Place the 24-well plate with tissue culture setup in an incubator (37 °C, 5% CO_2) to allow cell attachment and proliferation.

2.6. Incubate the plate for 7 days to allow the cells to reach 100% confluency. Remove the growth medium every other day and transfer 0.5 mL of pre-warmed fresh media into tissue culture inserts.

2.7. Repeat the plating procedure (steps 2.2-2.6) on a 12-well plate, 48-well plate and 96-well plate. Use the 12-well plate for Western blotting to determine changes in ZO-1 expression. Use the 48-well plate for DNA NP transfection. Use the 96-well plate for the ATP assay to determine cell viability in transfected cells.

3. Kinetics of cell growth.

3.1. Seed the cells at a density of 50,000 cell/cm^2 in a collagen-coated 24-well tissue culture plate.

3.2. On each day of the experiment, remove the growth medium and gently wash the cells twice with 500 µL of 1x PBS. Then, add 30 µL of 0.25% trypsin solution containing 0.02% EDTA and leave the plate for about 2-5 min in an incubator (37 °C, 5% CO_2).

NOTE: Gradual formation of confluent monolayer may affect the extent of cell detachment and it is necessary to increase the volumes of trypsin/EDTA as indicated here: 30 µL for 1-5 days post-seeding, 60 µL for 6-7 days post-seeding and 100 µL for 8-10 days post-seeding.

3.3. Add either 470 µL, 440 µL or 400 µL of growth medium based on the volume of trypsin/EDTA solution added in the step 3.2 to prepare 500 µL of cell suspension in each well.

3.4. Suspend the cells by pipetting up and down in each well several times and observe the cells under a microscope to make sure all cells are suspended in the growth medium. If some cells are still attached to the plate bottom after pipetting several times, gently scrape the cells using a plastic cell scraper to facilitate cell detachment.

3.5. Remove 0.1 mL of cell suspension from 500 µL cell suspension in step 3.3 and add to a 1.5 mL tube. Then, add 0.1 mL of 0.4% Trypan blue solution to the cell suspension and mix well.

3.6. Clean a hemacytometer with 70% isopropyl alcohol. Add 20 µL of mixture from step 3.5 on each side in the V-groove and locate the 16 squares under the microscope. The 16 squares are considered as one grid. Locate two random grids on each side of the hemacytometer and count all the living, non-blue cells.

NOTE: Cells that appeared blue in color from excluded from counting, <1% of the cells stained blue at all time points.

3.7. Calculate the cell density (cells/cm^2) based on following formulas.

$$\text{Average \# of cells/grid} = \frac{Viable\ cells}{number\ of\ grids} \qquad \text{(Eq.1)}$$

$$\text{Dilution Factor} = \frac{Volume\ of\ mixture\ of\ cell\ suspension\ and\ 0.4\%\ trypan\ blue}{Volume\ of\ cell\ suspension\ removed} \qquad \text{(Eq.2)}$$

$$\text{Cell density (viable cells/cm}^2\text{)} = \frac{Average\ \#\ of\ cells/grid \times Dilution\ factor \times 10^4 \times volume\ of\ cell\ suspension\ in\ each\ well}{Growth\ area\ of\ tissue\ culture\ plate} \qquad \text{(Eq.3)}$$

Equations 1-3. Viable cells are the number of cells counted in each grid, number of grids correspond to the number of grids located under the microscope, Volume of mixture of cell suspension and 0.4% Trypan blue is the volume prepared in step 3.5, volume of cell suspension removed is the volume removed from 500 µL cell suspension in step 3.5, the volume of cell suspension in each well is the 500 µL cell suspension from step 3.3, growth area of tissue culture plate is the growth area of single well in 24-well plate.

4. Lucifer yellow apparent permeability (LY P_{app}) assay

4.1. For determining LY P_{app} on each day post-seeding, follow steps starting 4.3. For determining the P_{app} in transfected hCMEC/D3 cells, add 8.3 µL of the transfection formulation (**Figure 1**) mixed with 50 µL of complete growth medium and incubate for 4 h. Transfection formulations are described in section 5.

4.2. After the 4 h transfection, gently wash the apical side twice using sterile 1x PBS buffer to remove any residual transfection mixture. Varying volumes of PBS buffer left behind after removal can affect the concentration of LY in the apical side. Take care to ensure that residual PBS buffer in tissue culture inserts is completely removed. Carefully aspirate residual reagents and medium to minimize cell detachment.

NOTE: This step is skipped when measuring every-day apparent permeability (P_{app}) of hCMEC/D3 cells.

4.3. Remove the growth medium and add 1.5 mL of pre-warmed (37 °C) transport buffer (25 mM HEPES, 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mM NaH_2PO_4, 5 mM glucose, pH 7.4) to the basolateral side.

NOTE: The volume of transport buffer in all basolateral compartments should be equal to ensure accuracy of permeability coefficient calculation.

4.4. Add 58.3 µL of 20 µM LY solution to the apical side of each transwell insert. Save 50 µL of the 20 µM LY solution for fluorescence measurements. After removing residual PBS buffer from apical side completely, add LY solution as quick as possible to avoid drying hCMEC/D3 cells. Ensure accurate volumes of LY solution in the apical side.

NOTE: To minimize the decay of LY fluorescence intensity, light exposure should be limited. Once the LY powder is reconstituted, the solution should be stored at 4 °C, protected from light.

4.5. Incubate in a rotary plate shaker (37 °C, 100 rpm) for 60 min. Then, remove 30 µL of the LY sample from each apical compartment. Then transfer the 20 µM LY solution and the apical side samples to pre-labeled tubes and dilute the sample 10-fold using transport buffer.

NOTE: It is required to dilute the 20 µM LY stock solution and the apical side samples because the high fluorescence intensity of these samples may potentially overload and damage the fluorescence detector of the fluorescence microplate reader.

4.6. Remove 500 µL from each basolateral compartment and transfer the sample to pre-labeled tubes.

NOTE: Samples are removed from separate transwell at indicated time points. The time points are each day post-seeding starting on day 1 until day 10.

4.7. Prepare a series of LY standards for the standard curve (39.00 nM, 78.13 nM, 156.25 nM, 312 nM, 625 nM, 1250 nM, 2500 nM).

4.8. Add 100 µL of each standard (in duplicate), apical and basolateral sample to each well in a black 96-well plate (**Figure 1**).

NOTE: Black plates absorb light and reduce background and fluorescence crossover among wells.

4.9. Use a fluorescence microplate reader (set points: excitation 428 nm, emission 536 nm) to measure the LY fluorescence intensity to calculate the P_{app}. A fluorescence plate reader is used for this study.

4.10. Calculate the P_{app} and %LY recovery values as described in the manuscript text.

Equation 4*. Formulae for calculating P_{app} values.*

Calculate the apparent permeability (P_{app}) coefficient and the %LY recovery using following equations. It should be noted that the P_{app} values can be calculated either based on mass[25] or concentration of LY.

$$P_{app}\left(\frac{cm}{min}\right) = V_A/(AM_{A0}) \times (\Delta M_B/\Delta t) \textbf{ or } P_{app}\left(\frac{cm}{min}\right) = V_B/(AC_{A0}) \times (\Delta C_B/\Delta t)$$

V_A-volume in the apical compartment
V_B- volume in the basolateral compartment
A- the surface area of the transwell insert membrane (0.3 cm^2)
M_{A0} - the initial mass in the apical compartment
$\Delta M_B/\Delta t$ - the change of mass over time in the basolateral compartment
C_{A0} - the initial concentration in the apical compartment
$\Delta C_B/\Delta t$- the change in concentration over time in the basolateral compartment.

Equation 5. *Formulae for calculating % LY recovery.*

Recovery (%) = $\frac{M_{Af}+M_{Bf}}{M_{A0}} \times 100\%$

M_{Af} is the mass in the apical compartment at the end time point, M_{Bf} is the mass in the basolateral compartment at the end time point, M_{A0} is the initial mass in the apical compartment.[26]Note: The initial mass is calculated based on the volume of the 20 µM LY solution in the step 4.5. This experiment was always done using cells under passage number 35 and was conducted four independent times.

5. Calcium depletion

5.1. Remove the growth medium from the transwell inserts and 12-well plate and gently wash the apical side and 12-well plate using pre-warmed calcium-free medium (Minimum Essential Medium Eagle Spinner (S-MEM) medium) to remove calcium ion from the transwell inserts and 12-well plate.

5.2. Add 500 µL or 2 mL of pre-warmed S-MEM 1xmedium to the inserts or 12-well plate and incubate for 24 h in the incubator (37 °C, 5% CO_2). After incubation, remove the S-MEM 1x medium and wash the cells once using pre-warmed 1x PBS buffer.

5.3. Follow steps 4.4-4.10 described in section 4 for LY treatment and subsequent steps. Follow steps 8.1.2-8.2.4 described in section 8 for Western blotting and subsequent steps.

6. Transfection

6.1. Preparation of DNA nanoparticles (DNA NPs).

6.1.1. Dilute the stock solution of gWIZ-Luc plasmid in 10 mM sodium acetate (NaAc) buffer (pH 5.0) and allow the DNA solution to stand for 10 min at room temperature (RT).

NOTE: The DNA NP containing predominantly single DNA molecules can be prepared at DNA concentrations 20–40 μg/mL[23]. Thus, gWIZ-Luc plasmid stock solution needs to be diluted. The frozen gWIZ-Luc plasmids stock needs to be thawed completely on ice to minimize temperature stress. Gently vortex the diluted DNA stock solution for 30 s on a standard benchtop vortexer set at knob position 3-5.

6.1.2. Calculate the desired N/P ratios, used here as a numerical parameter to reflect NP composition.

$$N/P\ ratio = \frac{\frac{Mass\ of\ cationic\ polymer}{(Mass/Charge)_{cationic\ polymer}}}{\frac{Mass\ of\ plasmid\ DNA}{(Mass/Charge)_{plasmid\ DNA}}}$$

Equation 6. N/P ratio calculation: the ratio of moles of the amine groups of cationic polymers to those of the phosphate groups of DNA. Mass of cationic polymers means total weighed amount of cationic polymer; $(Mass/Charge)_{cationic\ polymers}$ refers to the molecular weight of cationic polymer (poly (ethyleneglycol)5k-block-polyaspartamide with 48 diethylenetriamine side chains (PEG-DET)) normalized to the number of charged primary amines (48) per cationic polymer (mol/mol), this value for our polymer is 306 Da; Mass of DNA means total amount of DNA used in the formulation obtained by multiplying the volume and concentration in mg/mL; $(Mass/Charge)_{DNA}$refers to the molecular weight of DNA normalized to number of phosphate group per double-stranded DNA (325 Da per nucleobase).

NOTE: The DNA NP preparation table (**Table 1**) contains the formulation recipe for the different samples tested in our experiments. DNA NP were prepared using a rapid titration technique. The PEG-DET polymer solution was added along the walls of the tube while holding the tube in a horizontal position. Then the tube was switched to a vertical position, followed by quickly vortexing at maximum speed for 10s. The DNA NP were allowed to stand for 30 min at RT prior to use. The rule-of-thumb for DNA NP dosing is 0.5 μg DNA for ca. 1 cm^2 growth area. So, for each transwell insert/each well in a 48-well plate/each well in a 96-well plate, prepare DNA NP at N/P 10 containing 0.157/0.5/0.195 μg/well of gWIZ-Luc DNA.

6.1.3 For samples containing indicated concentration of Poloxamer P84 (P84), add P84 to DNA NP and vortex for 5 s. The final concentration of P84 in each sample is either 0.01% or 0.03% wt.

6.2. DNA NP transfection in a 48-well plate setup

6.2.1. Seed the cells with the density of 50,000 cell/cm^2 in a 48 well plate and grow until confluence in the incubator (37 °C, 5% CO_2).

6.2.2. For each treatment group, mix 25 µL of the indicated sample (transfection formulation) and 150 µL of complete growth medium and 175 µL of this mixture to each well.

6.2.3. Observe the hCMEC/D3 cells under a microscope to ensure the cells appear healthy and are 100% confluent at the time of the experiment.

6.2.4. Remove the growth medium from wells and 175 µL transfection mixture to each well. Then, incubate the plate for 4 h in the incubator (37 °C, 5% CO_2).

6.2.5. After 4 h, remove the transfection mixture and wash the hCMEC/D3 cells with pre-warmed sterile 1x PBS buffer.

NOTE: To minimize the accidental detachment of hCMEC/D3 cells from the plate/insert surface during the washing steps, carefully pipette enough sterile PBS along the walls of the wells and remove any nanoparticles in the residual culture media.

6.2.6. Gently rock the plate a few times and carefully aspirate and discard the PBS wash and add 500 µL of hCMEC/D3 pre-warmed culture medium.

6.2.7. Microscopically examine the cells and record observations on cell morphology and any possible effects of transfection.

6.2.8. Incubate for 24 h in the 37 °C cell culture incubator to allow luciferase production. After 24 h, remove growth medium completely and wash the cells once with pre-warmed 1x PBS.

6.2.9. Lyse the transfected cells by adding 100 µL of ice-cold luciferase cell culture lysis 1x reagent per well.

6.2.10. For measurement of the luciferase protein content, add 20 µL of cell lysate and 100 µL of luciferase assay buffer (20 mM glycylglycine (pH 8), 1 mM $MgCl_2$, 0.1 mM EDTA, 3.5 mM DTT, 0.5 mM ATP, 0.27 mM coenzyme A) into a 1.5 mL tube.

6.2.11. Read the luminescence of the sample described in the step 5.2.10 on a Luminometer with a Single Auto-injector.

NOTE: The luminescence should be integrated over 10 s before reading.

6.2.12. Measure the total amount of cellular protein in the lysate using a bicinchoninic acid assay (BCA assay) kit by following the manufacturer's protocol.

6.2.13. Calculate and express luciferase gene expression as Relative Light Units (RLU) per total cellular protein.

7. Luminescent ATP assay

7.1. Seed the cells with the density of 50,000 cell/cm^2 in a 96 well plate and grow until confluence in the incubator (37 °C, 5% CO_2).

7.2. Transfect the cells with 9.7 µL of the transfection formulation (preparation details are in section 6) and 58.4 µL of complete growth medium for 4 h.

7.3. Remove the transfection mixture and gently wash the cells with pre-warmed PBS 1x buffer twice to remove the treatment reagents completely.

NOTE: Different volumes of residual buffer could dilute the ATP assay reagents to different extent and can potentially affect the data.

7.4. Mix 75 µL of fresh pre-warmed medium and ATP assay reagent in a 1:1 dilution using a multichannel pipette. Make sure that the liquid level in all the multichannel pipette tips is the same.

7.5. Place the plate on a nutating shaker for 15 min at room temperature. After 15 min of adding the reagents, transfer 60 µL of each sample into a white 96-well plate.

NOTE: White plates are better to reflect output light than clear or black plates.

7.6. Pop any air bubbles using a needle prior to reading the plate. Read the plate on a luminometer with a 1 s integration time. Read the plate within 20 min after adding of ATP assay reagents. Timing is critical for comparison across different plates, because the luminescence signal is transient with a fast decay rate.

7.7. Calculate the percent (%) cell viability using this formula: (luminescence of transfected cells/luminescence of control, untreated cells) x 100.

8. Western blotting for measurement of tight junction protein ZO-1

8.1. Cell lysis and protein extraction

NOTE: All the steps for protein extraction from cells must be carried out at 2-8 °C.

8.1.1. Seed the cells at a density of 50,000 cell/cm^2 in a collagen-coated 12-well tissue culture plate.

8.1.2. On day 3, day 5, day 7, day 10 post-seeding and on day 7 (cells pre-incubated with calcium free medium), remove the growth medium and gently wash the cells twice with 2 mL of ice-cold 1x PBS. Then, add 300 µL mixture of ice-cold 1x RIPA lysis buffer containing 3 µg/mL aprotinin in each well.

NOTE: The mixture should be freshly made and kept on ice. Aprotinin is used to inhibit proteases present in lysates from degrading the protein of interest.

8.1.3. After two freeze-thaw cycles (-80 °C), scrape the cells using a cold plastic cell scraper. Collect the cell lysates in microfuge tubes. Then, centrifuge the tubes at 200 x *g* for 30 min at 4 °C.

8.1.4. Collect the supernatant into clean tubes and place them on ice. Measure the total amount of cellular protein in the lysates using the BCA assay kit by following the manufacturer's protocol.

8.2. Western blotting for detecting of tight junction protein ZO-1

8.2.1. Denature aliquots of total homogenates containing 40 µg of total protein with 1x Laemmli buffer at 95 °C, 5 min and subject to electrophoresis in a reducing 6–7.5% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (90 V, 10 min through stacking gel, 120 V through resolving gels).

NOTE: When loading the samples or standards, remember to load slowly and carefully into each lane, being careful not to break the well in the process.

8.2.2. Transfer the separated proteins onto a nitrocellulose membrane with pH 8.5 transfer buffer which contains 192 mM Glycine, 25 mM Tris Base, 10% methanol and 0.1% SDS (75 V, 110 min at room temperature).

NOTE: Do not touch the membrane. Use 70% isopropanol-washed plastic forceps to handle the membrane. In order to successfully transfer the ZO-1 protein (MW 200 kDa) to the membrane, pH should be around 8.3-8.5. If the transfer buffer is more acidic than that, transfer would not happen. If the ladder bands are visible still on the gel, it would be helpful to increase transfer time and SDS concentration.

8.2.3. After washing the membrane by using Tris-buffered saline containing 0.1%Tween 20 (T-TBS), use blocking solution (1:1 LiCOR-Odyssey Block: 1x Tris buffered saline) to block the membranes for 60 min.

8.2.4. Carefully cut the membrane into two strips. Incubate with two primary antibodies (ZO-1 monoclonal antibody, dilution, 1: 900, and GAPDH antibody, dilution, 1: 10,000) overnight at 4 °C.

Then, incubate the ZO-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) membranes with donkey anti-mouse IgG (dilution, 1:50,000). After washing the membranes using T-TBS, image the membranes in the 700 channel on a 16-bit imager.

REPRESENTATIVE RESULTS:

First, we determined the effect of culturing time on LY permeability to determine the apparent kinetics of TJ formation. The mean LY P_{app} values from day 1 to 10-post seeding are shown in **Figure 2a**. On day 1, the mean P_{app} was 4.25×10^{-4} cm/min and slightly dropped to 3.32×10^{-4} cm/min on day 2. The mean P_{app} value slightly increased to 3.93×10^{-4} cm/min on day 3 and fluctuated with no significant changes until day 6. The P_{app} values significantly decreased to 2.36×10^{-4} cm/min on day 7 compared to day 1 ($P < 0.05$) probably suggesting that the barrier became tighter. The P_{app} values stabilized in the range between 2.14×10^{-4} and 2.36×10^{-4} cm/min from day 7 to day 10, which implied the barrier formation was complete and functional resulting in decreased LY paracellular transport. We calculated the percentage (%) LY recovered on each day to be ca. 80%, a value that is considered optimal to reliably calculate the P_{app} value[27] (**Figure 2b)**. Recovery% is an important index in LY assay. For example, if cells metabolize most LY, LY is immobilized in cells or sticks to the cell membrane or LY degrades during incubation, it would be inaccurate to interpret that observed low LY signal in basolateral compartment indicates a tight barrier. Thus, recovery% gives more confidence that we did not lose significant amount of LY owing to one or more of the above possibilities and allowes to confidently estimate LY P_{app} values. As noted earlier in the introduction section, greater concentrations of LY in the basolateral side indicates an incomplete barrier while lower concentrations reflect restricted transport, suggesting a mature, complete barrier due to the presence of functional TJs. We also present additional evidence using an orthogonal technique, Western blotting detection of ZO-1 protein (**Figure 4**), to confirm that the observed changes in LY P_{app} correlates with the formation of tight junctions.

Since the transwell insert setup does not allow to directly track changes in cell density, we determined changes in cell density using a standard Trypan blue exclusion assay. We therefore determined the changes in cell density on a transparent tissue culture plate that readily allowed us to monitor the cell growth kinetics. The increase in cell density from $5.5\times10^4 \pm 1.0$ cells/cm^2 to $1.9\pm0.2\times10^5$ cells/cm^2 from day 1 to 10-post seeding (**Figure 3**) was linear with a regression coefficient of 0.94. These data also suggest that the observed changes in LY P_{app} (**Figure 2a**) are a result of the formation of a confluent monolayer over the 10-day period. We observed the cells under an inverted light microscope on each day and visaully documented a gradual increase in cell number and monolayer formation.

We used Western blotting to detect changes in the expression of the tight junction protein ZO-1 over time (**Figure 4**). The changes in ZO-1 expression is used to orthogonally supplement the LY P_{app} data and to ensure that the observed changes in LY P_{app} indicates the formation of a tight barrier. ZO-1 band intensities were analyzed by densitometry and normalized relative to the expression of a housekeeping gene, GAPDH. The two bands in **Figure 3a** represent the two ZO-1 isoforms (ZO-1α^+ and ZO-1α^-)[28]. Densitometry analysis revealed that the pixel value of ZO-1 increased from day 3-7 post-seeding, suggesting that the TJ protein ZO-1 formed continually from

day 3-7. After calcium depletion treatment on day 7 post-seeding, the band of ZO-1 was almost undetectable, which indicates that ZO-1 was unable to form in the absence of calcium ions. Moreover, the pixel value of ZO-1 markedly decreased at day 10 post-seeding. The signal intensities of GAPDH from day 3-10 appeared were comparable, except on day 7 when the cells were treated with calcium-free medium. A possible reason for the lower GAPDH expression in calcium-treated cells may be due to the lesser total protein (28.9 µg total protein), again, likely due to calcium depletion. Overall, the band densitometry analysis revealed a gradual increase in ZO-1 expression (relative to GAPDH) until day 7 and a decrease in expression on day 10 when cells cultured in complete growth medium. The analysis also revealed a lower expression of ZO-1 (relative to GAPDH) on day 7 in cells treated with calcium-free medium.

While the focus of this work is to present the LY P_{app} assay as a method to determine the kinetics of a monolayer formation, to demonstrate an additional utility of the developed assay, we determined whether DNA NP transfection influenced the TJ barrier integrity by measuring LY P_{app} through hCMEC/D3 cells 4 h post-transfection (**Figure 5**). Our DNA NPs containing Poloxamer P84 mediate high levels of gene expression in the hard-to-transfect hCMEC/D3 cell line (**Figure 6a**). Specifically, we wanted to determine if the hydrophobic domains of Poloxamer P84 in our DNA NPs may perturb TJ integrity in transfected cells. The %LY recovered in each treatment group was ca. 92%, suggesting that the calculated P_{app} values are reliable (**Figure 5b**). We noted that the transfection procedure using various formulations did not affect the LY P_{app}, relative to non-transfected cells exposed to LY alone. Extracellular calcium is a critical component for the maintenance of cell-cell junctions in various cell types[29-31], including the brain microvessel endothelial cells. Thus, maintaining cells in calcium-free medium (CFM) leads to the disruption of TJs[32,33]. Therefore, we used cells treated with CFM for 24 h as a positive control.

Our data shows that the LY P_{app} for cells incubated with CFM was 2-fold higher compared to control cells incubated with the regular growth medium. This 100% increase in P_{app} suggests that the cells had lost their TJs because of the loss of calcium ions needed for its formation. Notably, the actual value (5.14×10^{-4} cm/min) was slightly higher than the average P_{app} value from day 1-6 post seeding (**Figure 2**) when the TJs were not yet fully formed. Albeit not significant, cells transfected with DNA NP containing 0.01-0.03% Poloxamer P84 showed a tiny increase in LY P_{app} values compared to the untreated cells maintained in regular culture medium. This observation suggested that DNA NP + P84 transfection had no significant effect on TJ barrier integrity. Overall, the LY P_{app} values in the transfected cells approximately averaged to $2.5x10^{-4}$ cm/min and this value corresponded to the average value noted during day 7-10 post-seeding (**Figure 2**) when the LY P_{app} was the least, suggesting the presence of functional TJs that effectively restricted LY paracellular transport.

We present additional transfection data to show that the lack of changes in LY P_{app} (**Figure 5**) is not an inconsequential observation. Despite the high levels of transfection observed in the DNA NPs+P84 group, we noted no changes in LY P_{app} suggesting that our formulations do not perturb the TJ barrier. The relatively low transfection efficiency in the naked DNA-treated cells is typical because the anionic nature of plasmid DNA (due to phosphate groups in its backbone) and its hydrophilic nature limit cellular uptake. DNA condensed in DNA NP mediated a 50-fold increase

in transfection compared to naked pDNA **(Figure 6)**. Addition of 0.01% P84 to the DNA NP resulted in an 18-fold increase compared to DNA NP-alone ($P < 0.01$). Increasing the P84 concentration to 0.03 wt.% resulted in a 30-fold increase compared to DNA NP-alone ($P < 0.001$). These results are noteworthy given that brain endothelial cells are a hard-to-transfect cell type.

We measured the cell viability of cells transfected under different conditions to confirm that the transfection procedure does not cause cell stress. Adenosine triphosphate (ATP) is the energy currency of life and reflects cellular metabolic function. We used a luciferase-based ATP assay where the luminescence values are directly proportional to ATP levels. Possimo et al. reported that the luminescence ATP assay was a robust measure of metabolic cell viability[34]. The cell viability of hCMEC/D3 cells transfected by the various formulations was comparable to untreated cells **(Figure 5b)**, suggesting that the ATP levels were similar as well. Therefore, DNA NPs containing Pluronic P84 (0.01% to 0.03% w/w) are safe gene delivery formulations.

FIGURE AND TABLE LEGENDS:

Figure 1. **Experimental setup for LY P_{app} study**. 24-well plate setup containing transwell inserts (adapted to show DNA nanoparticle transfection as an example, data in **Figure 5**). Each column was treated with the indicated sample for 4 h. Control indicates hCMEC/D3 cells treated with complete growth medium while calcium depletion 24 h indicates that the cells were incubated with calcium free medium prior to LY exposure. The right template depicts LY fluorescence intensity measurement in a black 96-well plate.

Figure 2. **Apparent kinetics of TJ barrier formation determined using the LY P_{app} assay**. (**a**) LY P_{app} through hCMEC/D3 monolayers cultured on transwell inserts were measured everyday post-seeding cells. (**b**) %LY recovered in each treatment group. Data represents average ± SD of two independent experiments (n=3/experiment). Statistical comparisons were made using unpaired t-test (*$P < 0.05$, N.S. not significant).

Figure 3. Kinetics of cell growth determined using a Trypan blue exclusion assay. hCMEC/D3 cells were seeded in a 24-well plate at a cell density of 50,000 cells/cm^2. On each day of the experiment, cells were dissociated and mixed with an equal volume of 0.4% Trypan blue before counting viable cells on a hemacytometer. Data represents average $\pm$ SD of three independent measurements.

Figure 4. Apparent kinetics of ZO-1 expression detected using western blotting. (**a**) The hCMEC/D3 cells were seeded in a 12-well plate at a cell density of 50,000 cells/cm^2. On each day of the experiment (day 3, day 5, day 7 and day 10-post seeding), cells were lysed by 400 µL of 1x RIPA buffer containing 3 µg/mL aprotinin. Cell lysates containing 40 µg of total protein were loaded on a 4-7.5% SDS-polyacrylamide gel. (**b**) Band densitometry analysis allowed normalizing expression of ZO-1 protein to GAPDH.

Figure 5. **Effects of DNA NP transfection on TJ barrier tightness measured using the LY P_{app} assay**. (**a**) hCMEC/D3 cells were cultured on transwell inserts for 7 days, transfected with PEG-

DET containing gWIZLuc plasmid DNA with/without Pluronic P84 for 4 h and then replaced with pre-warmed transport buffer containing 50 µM LY for 1 h. Control represents the hCMEC/D3 cells incubated with growth medium for 4 h followed by LY exposure (n=4, *P<0.05, N.S. not significant). (**b**) %LY recovered in each treatment group, values presented are average ± SD (n = 4).

Figure 6. **DNA NPs mediate high levels of transgene expression in hCMEC/D3 monolayers**. (**a**) hCMEC/D3 cells were cultured 7 days and transfected with PEG-DET containing gWIZLuc plasmid DNA with/without Pluronic P84 (N/P 10, DNA dose per well: 0.5 µg) for 4 h, transfection mixture was removed and cells were cultured for 24 h in complete growth medium prior to measuring gene expression. Levels of luciferase gene expression was expressed as relative light units (RLU) nornalized to total cellular protein content. Data presents average ± SD of three independent experiments. Statistical comparisons were made using unpaired t-test (** P< 0.01, *** P< 0.001). (**b**) DNA NPs are safe transfection formulations in hCMEC/D3 monolayers. Effects of DNA DNA NP transfection on cell viability was evaluated using a luminescent ATP assay. hCMEC/D3 cells were transfected with the indicated samples for 4 h following which the ATP assay was conducted by following manufacturer's protocol. Percent (%) cell viability was calculated as follows: (luminescence of transfected cells/luminescence of control, untreated cells)x100. Data represents average ± SD of two independent experiments (n=3/experiment).

Table 1. The NP formulations used for obtaining the data reported.

DISCUSSION:

A key role of the BBB is to prevent the exchange of non-essential ions and toxic substances between the systemic circulation and the brain to maintain hemostasis of neural microenvironment. One of the characteristic features of the BBB is the ability of the capillary endothelial cells to form tight junctions (TJs) that effectively seal the paracellular route of transport. We demonstrated a LY P_{app} assay as a quantitative method to determine the apparent kinetics of TJ barrier formation in cultured hCMEC/D3 monolayers. ZO-1 expression detected via Western blotting orthogonally validated the data from the LY P_{app} studies as discussed in detail in the following paragraph. As an additional utility of the developed assay, we further demonstrated that DNA NP transfection did not measurably change the LY P_{app} indicating the suitability of this assay to determine changes in TJ barrier characteristics in an experimental setup.

Western blot data revealed a clear increase in ZO-1 expression on days 3, 5, 7-post seeding and a slight reduction on day 10-post seeding **(Figure 4a)**. From **Figure 1**, it can be seen that the LY P_{app} decreased from day 1-7 post-seeding suggesting the formation of TJs. After calcium depletion treatment, the LY P_{app} showed a marked increase (**Figure 2a**) while the ZO-1 expression showed marked reduction **(Figure 4a)**. Extracellular calcium is a critical component for the maintenance of cell-cell junctions in various cell types[29-31], including the brain microvessel endothelial cells. Lack of calcium in the growth medium disrupted and dissociated the TJs[33]. As expected, the P_{app} value of calcium–depleted cells was significantly higher than the untreated cells and close to the P_{app} value of the blank inserts containing no cells **(Figure 2a)**. The LY revealed a steady plateau in

P_{app} values from day 7-10 post-seeding (**Figure 2a**) suggesting that the barrier had fully formed by day 7 that resulted in restricted LY transport to the basolateral side. The Western blot data showed a slight decrease in ZO-1 expression on day 10 compared to day 7-post seeding. This difference in observation is likely due to the contribution of other extracellular proteins, apart from ZO-1, in maintaining barrier tightness. In summary, we have successfully used data from two orthogonal techniques to determine the kinetics of tight junction barrier formation in a human cell model of the BBB. While the LY P_{app} assay measured the functionality of the TJ barrier, the Western blot data traced the formation of the TJs using ZO-1 as a marker protein.

As an additional utility of the developed assay, we confirmed that DNA NP transfection in hCMEC/D3 monolayers does not affect the integrity of TJs (**Figure 5**). Untreated cells incubated with growth medium was used as the negative control and cells pre-incubated with calcium free medium for 24 h was used as a positive control. Cells in which the TJs were disrupted as a result of the calcium depletion showed a 210% increase in LY P_{app} compared to the untreated cells. Our data suggests that DNA NP transfection either in the presence or absence of P84 does not affect the TJ integrity. It should be noted that the lack of LY P_{app} changes in transfected cells is not an inconsequential observation. In fact, our DNA NPs mediate significant levels of gene expression in the hard-to-transfect hCMEC/D3 monolayers[9,35-37]. DNA NPs containing 0.01 or 0.03 wt. % P84 increased luciferase gene expression by ca. 18-and 30-fold, respectively, compared to DNA NPs alone (**Figure 6a**). We also demonstrated that our NP treatments do not adversely affect the cellular ATP levels, used here as an indicator of functional cell viability (**Figure 6b**). Our results underscore the expanded utility of this LY P_{app} assay to determine the TJ barrier integrity in an experimental transfection setup.

One critical step in the execution of the LY assay is to keep the same amount of LY in apical side and equal volume of transport buffer in the basolateral side across the various time points in the entire experiment. If different amounts of LY or unequal volumes of transport buffer were used in wells, the calculation of P_{app} would be unreliable, resulting in artificially large standard deviations. Another critical aspect is to limit the light exposure to minimize the decay of LY fluorescence intensity. Also, the liquid needs to be removed completely before adding exact same of LY solution and transport buffer into apical side and basolateral side, respectively. This ensures that the fluorescence readout can be reliably used for P_{app} calculation. Another critical step in this experiment is to immediately measure the LY fluorescence of the basolateral samples and avoid the need to freeze the samples after collection. Subjecting the LY-containing samples to freeze-thaw cycles results in a larger intra-group variation of the fluorescence signal. One limitation of using the transwell setup is that the live cells are difficult to be visualized by conventional microscopy or imaged by confocal microscopy. Moreover, it does not easy translate into a setup that allows co-culturing different cell types unless mixed culturing of say, glial and endothelial cells is a possibility. Nevertheless, the LY assay has the following advantages: LY is a dye with distinct excitation/emission peaks and a relatively large Stokes shift compared to dyes like sodium fluorescein, which allows for a robust measurement of the probe crossing the BBB and avoids the need for additional radiolabel as in the case of tracers such as sucrose or mannitol. High recovery %s of LY provides reliable data to confidently calculate P_{app} values.

Based on our findings, we conclude that the LY P_{app} method is a simple and robust assay to quantify the paracellular permeability across hCMEC/D3 cell monolayers. By using LY P_{app} method, we optimized the culture time for the formation of intact barrier, and we demonstrated an additional utility of the developed assay by determining TJ integrity in DNA NP-transfected cell monolayers.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

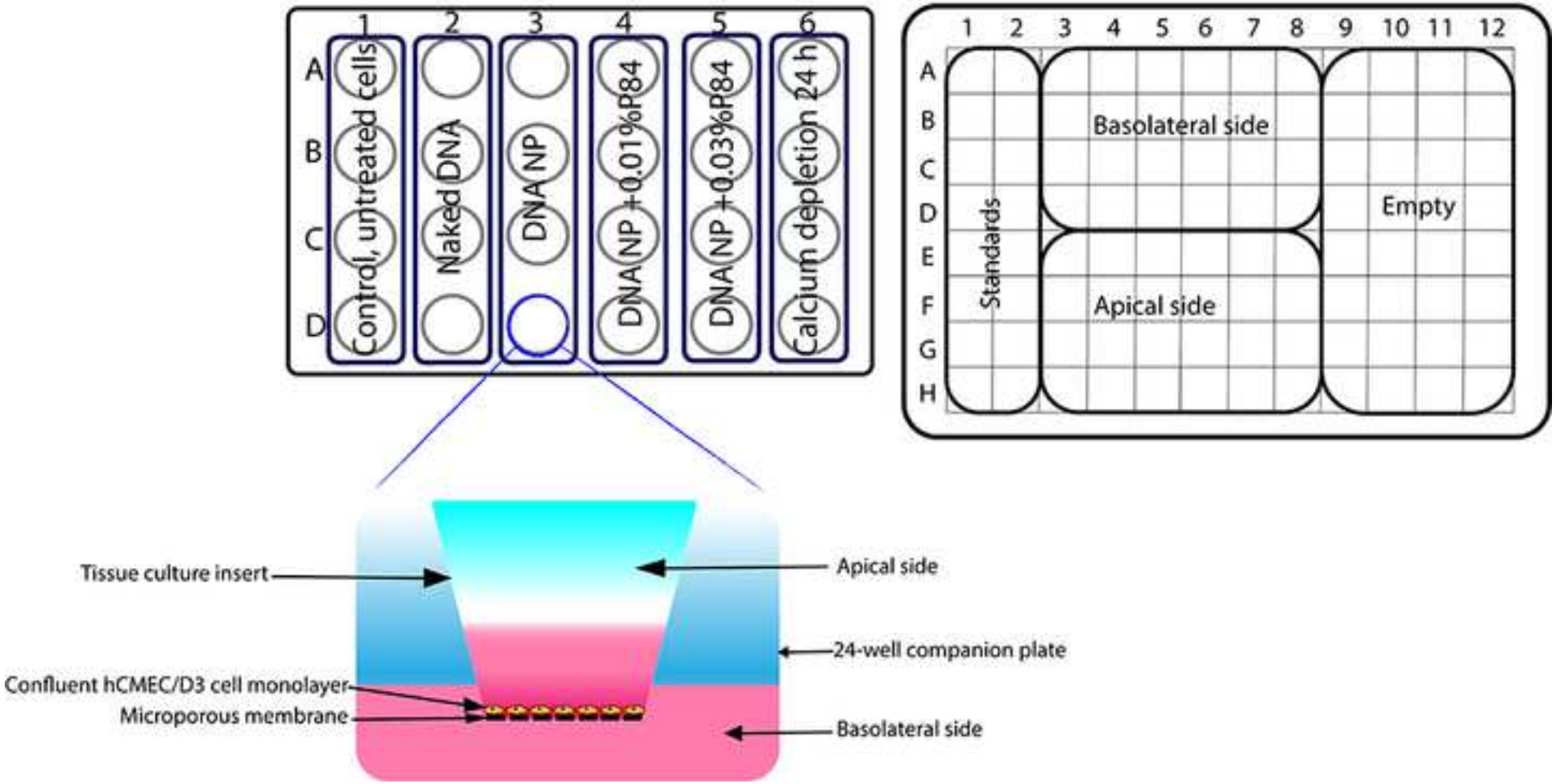


Figure 2

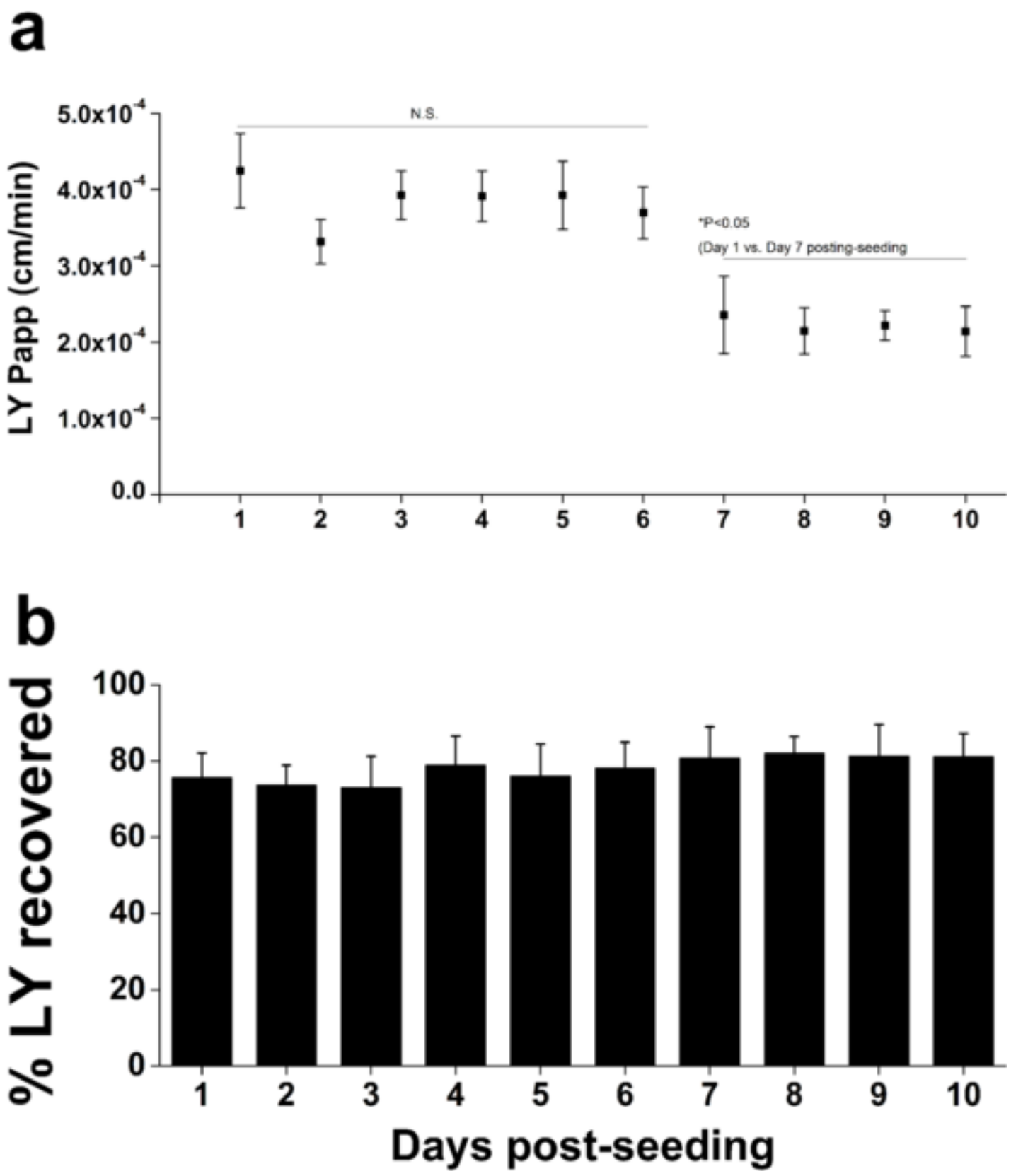


Figure 3

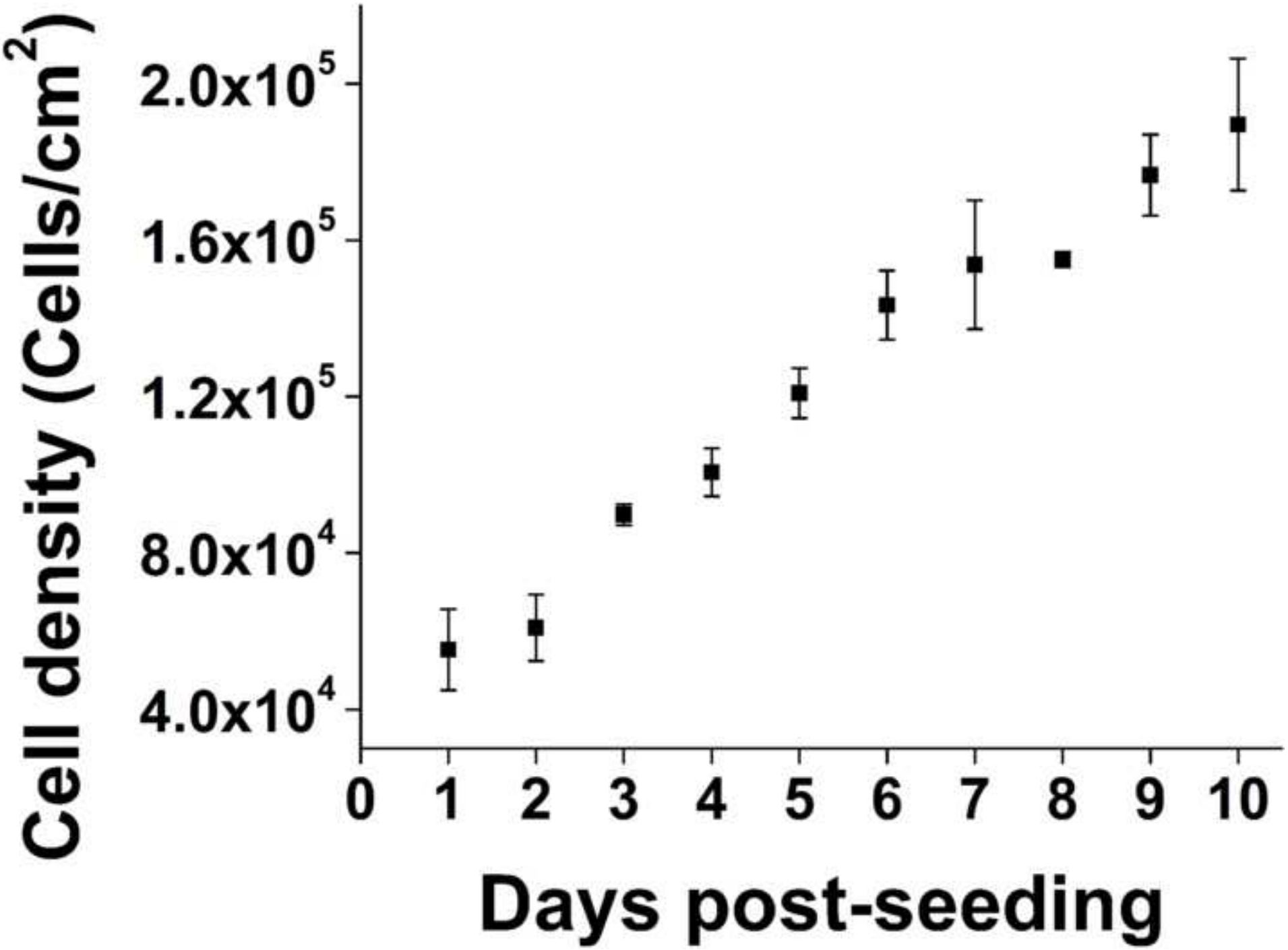
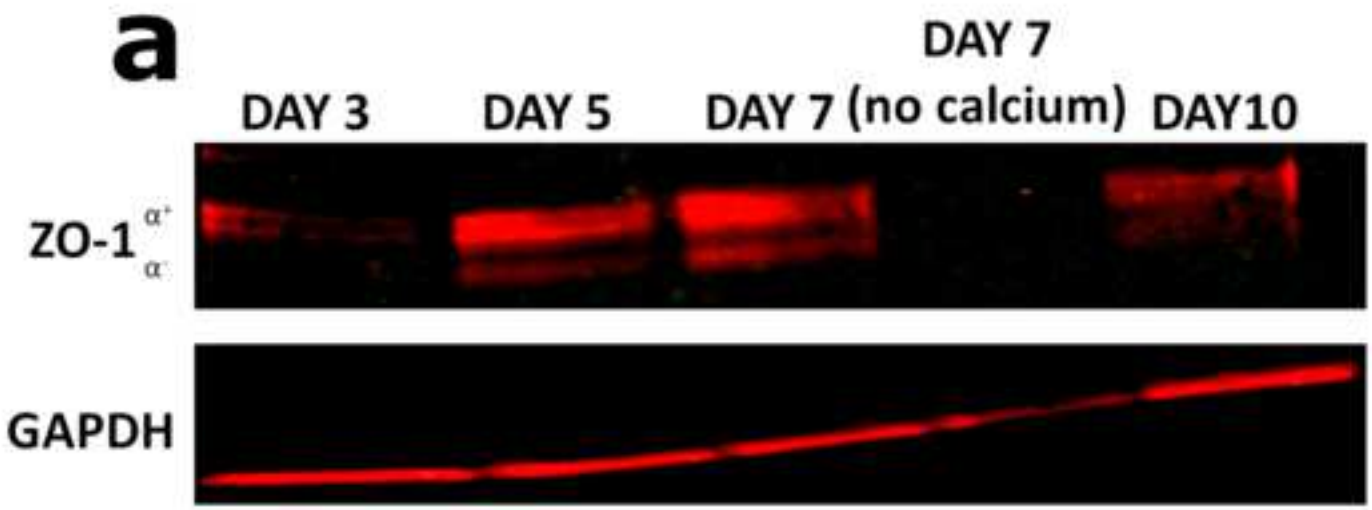


Figure 4



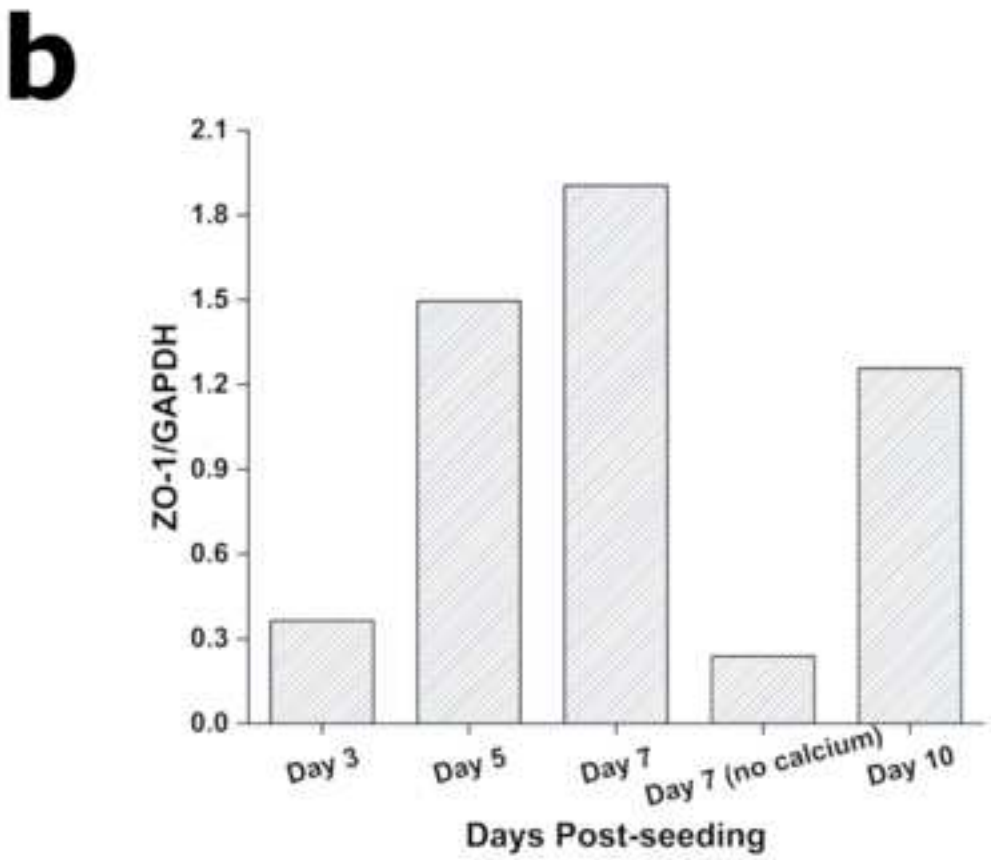


Figure 5

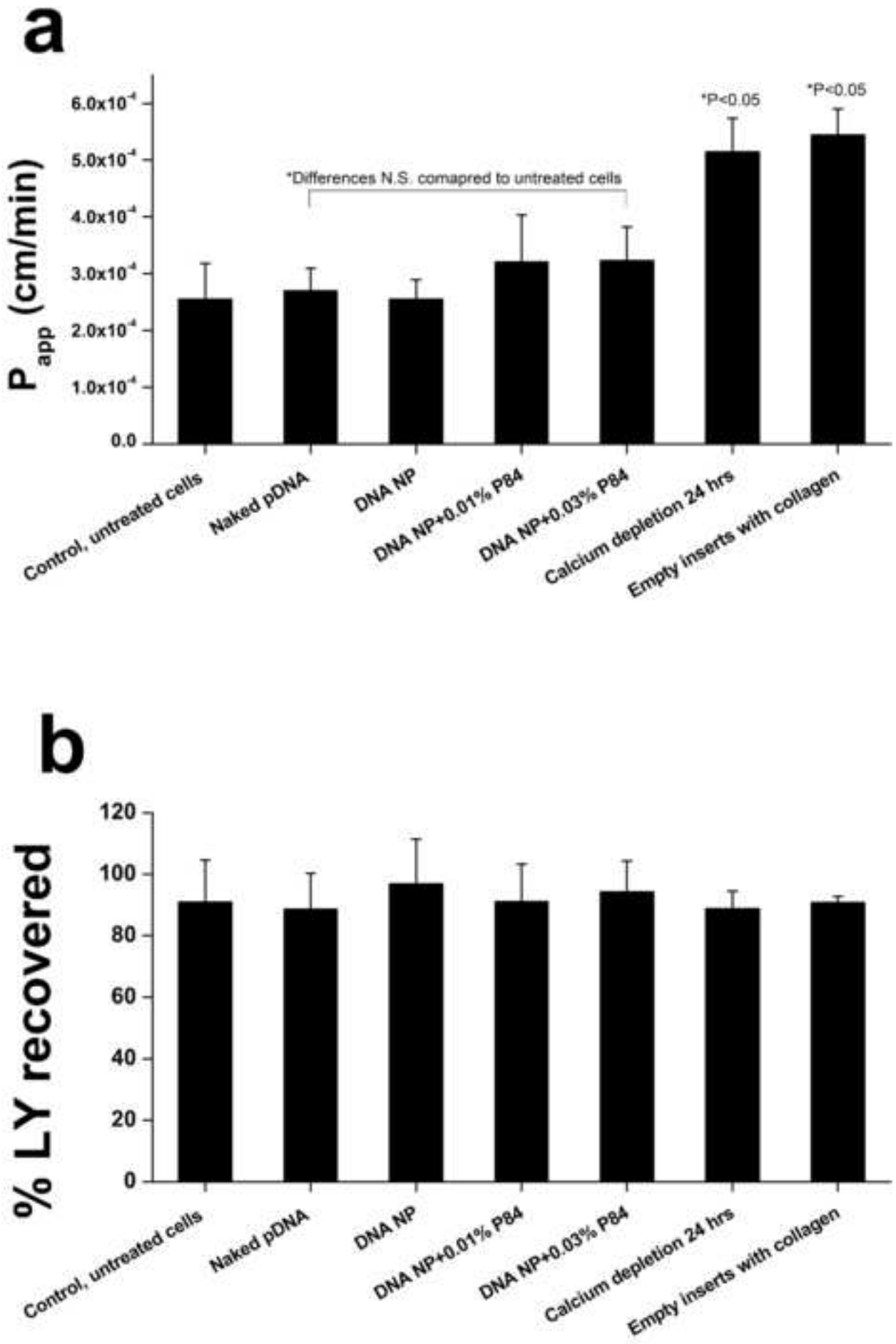
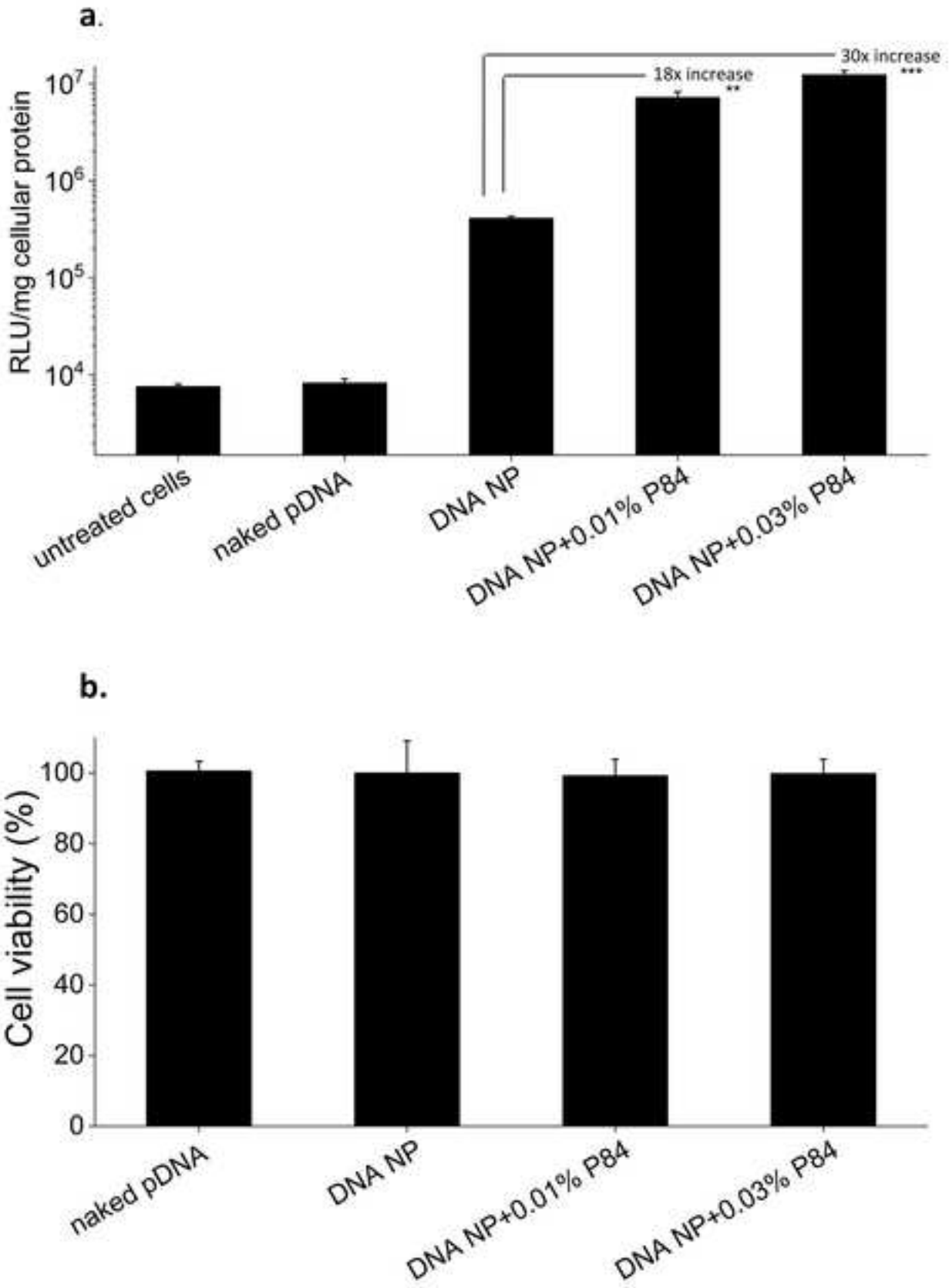


Figure 6

Click here to access/download;Figure;Figure 6.tif



DNA NP preparation for tissue culture plates

Description: This table shows the NP formulations we used for obtaining the data reported in figure

Experimental setup	Sample name	Volume of 1mg/mL plasmid DNA (µL)
Tissue culture inserts[a]	Control, untreated cells	0
	Naked DNA	0.157
	DNA NP	
	DNA NP + 0.01%P84	
	DNA NP + 0.03%P84	
48-well plate	Control, untreated cells	0
	Naked DNA	0.5
	DNA NP	
	DNA NP + 0.01%P84	
	DNA NP + 0.03%P84	
96-well plate	Control, untreated cells	0
	Naked DNA	0.195
	DNA NP	
	DNA NP + 0.01%P84	
	DNA NP + 0.03%P84	

[a]Tissue culture insert characteristics: Mfr. No. 353095; Filter diameter, 6.4 mm; Culture Are

s 4 and 5.

Volume of 10 mM NaAc buffer, pH 5(µL)	Volume of 5 mg/mL polymer (µL)
0	0
8.143	
7.843	0.3
7.6681	
7.26	
0	0
24.5	
23.56	0.94
23.385	
23.035	
0	0
9.35	0.37
8.9307	
9.0669	

a, 0.3 cm^2; material, polyethylene terephthalate (PET); Pore size, 0.4µm.

Volume of 10% w/w. P84 (µL)	Volume of growth medium (µL)
0	58.3
0.1749	50
0.0583	
0	175
0.175	150
0.525	
0	68.1
0.0681	58.4
0.2043	

Name of Material/ Equipment	Company	Catalog Number
hCMEC/D3 cell line	Cedarlane Laboratories	102114.3C-P25
gWizLuc	Aldevron	5000-5001
lucifer yellow CH dilithium salt	Invitrogen	155267
Transwell inserts with polyethylene terephthalate (PET) track-etched membranes	Falcon	353095
Tissue culture flask	Olympus Plastics	25-207
24-well Flat Bottom	Olympus Plastics	25-107
Black 96-Well Immuno Plates	Thermo Scientific	437111
S-MEM 1X	Gibco	1951695
EBM-2	Clonetics	CC-3156
phosphate-buffered saline 1X	HyClone	SH3025601
Collagen Type I	Discovery Labware, Inc.	354236
Pierce BCA Protein Assay Kit	Thermo Scientific	23227
Cell Culture Lysis 5X Reagent	Promega	E1531
Beetle Luciferin, Potassium Salt	Promega	E1601
SpectraMax i3	Molecular Devices	
Trypan Blue Solution, 0.4%	Gibco	15250061
ZO-1 Polyclonal Antibody	ThermoFisher	61-7300
anti-GAPDH antibody	abcam	ab8245
Alexa Fluor680-conjugated AffiniPure Donkey Anti-Mouse LgG(H+L)	Jackson ImmunoResearch Inc	128817
12-well, Flat Bottom	Olympus Plastics	25-106
RIPA buffer (5X)	Alfa Aesar	J62524
Aprotinin	Fisher BioReagents	BP2503-10
Odyssey CLx imager	LI-COR Biosciences	

Comments/Description
human cerebral microvascular endothelial cell line
Plasmid DNA encoding luciferase gene
Spinner-minimum essential medium (S-MEM)
Endothelial cell basal medium-2(EBM-2)
Fluorescence Plate Reader
for scanning western blot membranes

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Author(s): Wanzhu Zhao, Younsoo Bae and Devika S Manickam

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Reviewer 3

Major Concerns:

1. While I will not argue about the LY assay itself as it is established protocol and validation already described previously, my concerns are related to the method validation following the transfection that is not strong enough. The authors didn't validate their data following the transfection with another assay or permeability marker such as TEER measurement and/or inulin.

We thank the reviewer for this suggestion. We have included new western blot data reporting expression of ZO-1, a tight junction protein (Figure 3) to orthogonally confirm the changes in LY apparent permeability. We noted a clear increase in ZO-1 expression on days 3, 5, 7-post culture. In addition, the ZO-1 expression was lower on day-10 post-culture and cells incubated in calcium-free growth medium showed a marked reduction in ZO-1 expression.

We would like to reiterate that the central focus of this manuscript is to present a LY assay to determine the apparent permeability (P_{app}) of LY. We reported data from the transfection experiment only as an additional utility of the assay to determine LY P_{app} in transfected cells. So, the orthogonal western blotting technique was used to complement data from the LY P_{app} assay in cultured cell monolayers. It should be noted that TEER values can be influenced by experimental variables such as the type of proteins used for extracellular matrix coating, culture medium and the type of measurement instrument. A likely combination of these factors leads to a broad distribution of TEER values ranging from 2 to 1150 Ω cm^2 in the hCMEC/D3 cell line cultured for 2-21 days [1].

2. I am not sure why the authors are relating the transfection results to the validity of LY assay. The issues associated with transfection are not related the type of permeability marker (LY, inulin, sucrose, TEER…etc) as much as the nature of transfection vehicles that necessitate the use of polymers. Cells exposure to PEG or Pluronic or others can decrease cells intactness and thus increase the permeability marker transport, any permeability marker.

We thank the reviewer for this comment and apologize that this was not made clearer in the manuscript. The reviewer is right that relating the transfection results to the validity of the LY assay is not appropriate. However, as noted under ***(1)***, the central focus of this manuscript is to use the LY assay to determine the apparent permeability of LY. We reported data from the transfection experiment as an additional utility of the reported assay to determine LY P_{app} in transfected cells. We further would like to emphasize that LY P_{app} data from the transfected cells are only an extended application of the developed assay but does not independently validate the assay itself.

3. The robustness validation of the LY assay following transfection is not strong enough. The authors performed the LY permeability only on 4 wells (lines 546-552). A onetime experiment with 4 wells is not enough to conclude robustness and validity. In addition, another permeability marker and/or TEER measurements should be assessed.

The reviewer is right that this is a onetime experiment with four wells, but we conducted the experiment for a total of four independent runs to ensure the results were stable and accurate. The

data presented in the manuscript is representative of our observations. We have included new data reporting ZO-1 expression detected via western blotting (Figure 3) to orthogonally confirm the changes in LY apparent permeability. We noted a clear increase in ZO-1 expression on days 3, 5, 7-post culture. In addition, the ZO-1 expression was lower on day-10 post-culture and cells incubated in calcium-free growth medium showed a marked reduction in ZO-1 expression. We would also like to emphasize that the central focus of this manuscript is to use the LY assay to determine the apparent permeability of LY. We reported data from the transfection experiment as an additional utility of the reported assay to determine LY P_{app} in transfected cells. So, the orthogonal western blotting technique was used to complement data from the LY P_{app} assay. It should be noted that TEER values can be influenced by experimental variables such as the type of proteins used for extracellular matrix coating, culture medium and the type of measurement instrument. A likely combination of these factors leads to a broad distribution of TEER values ranging from 2 to 1150 Ω cm^2 in the hCMEC/D3 cell line cultured for 2-21 days [1].

4. Also, for validation that monolayer was not affected by the transfection, a dose dependent as well as time dependent effect of amount of polymer on LY permeability is necessary. Showing such profiles are important. Transfection of some proteins may require more than 4 hours that was used by the authors, thus time dependent studies are necessary.

We thank the reviewer for this comment and apologize that this was not clear in the manuscript. As described in the earlier responses, we reported data from the transfection experiment as an additional utility of the reported assay to determine LY P_{app} in transfected cells. We certainly agree that a dose as well as time-dependent effects of polymer can be useful information, and we would like to point out that those data are the subject of a future manuscript. The reviewer is also right that transfection of some genes may take longer than 4 hours and in this particular case, we have used luciferase as a model reporter gene to demonstrate that the presented assay is a useful tool to determine changes in LY P_{app} in transfected BBB monolayers. It should be pointed out that luciferase gene transfection is typically done for 4 h [2-5].

5. I am not sure what is the importance of calculating recovery% post seeding. The added LY will distribute between apical and basolateral sides and the recovery won't change regardless how much in the apical or basolateral. How recovery is relevant to permeability? Further clarification is required.

Recovery% is an important and useful parameter in the LY assay. For example, if cells metabolized most of the added LY, LY is immobilized in cells or sticks to the cell membrane or if LY degrades during incubation, it would be inaccurate to interpret that the observed low LY signal in basolateral compartment indicates a tight barrier. Thus, LY recovery% gives more confidence that we did not lose significant amount of LY owing to one or more of the above possibilities and allows to confidently estimate LY P_{app} values. This has been now clarified in the manuscript on lines 533-539.

6. The number of cells continue to increase up to day 10, shouldn't this be associated with stricter LY permeability? Please explain.

Thank you for making this comment. The hCMEC/D3 cells were seeded on a collagen-coated tissue culture plate and not on Transwell inserts for the cell growth kinetics experiment. This was done to ensure regular growth kinetics similar to what we observe in a collagen-coated tissue culture flask and also allow us to microscopically track the changes in cell number. It should be noted that the LY permeability studies were conducted in collagen-coated Transwell inserts. The reasons for the observed discrepancy between growth kinetics in the tissue culture plate and LY permeability in the Transwell insert can be explained as follows. Despite the collagen-coating, tissue culture plates and Transwell inserts have different basal (initial) surfaces and we posit that the inserts may show a combination of faster growth kinetics and earlier maturation of cell junctions compared to a tissue culture plate/well. As an orthogonal piece of evidence, published literature have reported a decrease in TEER value to about 30-1500 Ω*cm^2 after 2-3 culture days with increase in cell numbers [6, 7].

In figure 1a, on day 1 post-seeding, the mean P_{app} was the highest (4.25×10^{-4} cm/min), indicating the cells, which attached on the microporous membrane did not form a tight barrier to prevent LY paracellular transport. The mean P_{app} values decreased on day 2 and stayed constant until day 6. This likely suggests that the actin cytoskeleton accumulated in the areas of cell-cell contacts while continually assembling nascent TJ proteins [8-10]. The LY P_{app} dropped on day 7 and remained stable until day 10. This likely suggests that the organized TJ proteins formed mature apical junctional complex and sealed the paracellular diffusional pathway between the cells.

Minor Concerns:

1. Transfection is section 6 not 5, modify across the manuscript (example line 429)

Thank you, this has now been changed.

2. Revise figures as the numbering is not accurate within the text mainly under section "Transfection activity of DNA NPs in hCMEC/D3 monolayers" (Fig. 3 & 4).

Thank you, this has now been fixed.

3. Scheme 1 a & b do not exist. Only scheme 1.

Thank you, this has now been changed.

4. The equation (line 312) is not accurate: VA below should be VB: =/(0) × (Δ/Δ)

Thank you; this has now been changed on line 331.

$$P_{app}\left(\frac{cm}{min}\right) = V_A/(AM_{A0}) \times (\Delta M_B/\Delta t) \textbf{ or } P_{app}\left(\frac{cm}{min}\right) = V_B/(AC_{A0}) \times (\Delta C_B/\Delta t)$$

5. Lines 319-320: the authors description of Δ/Δ is not correct. They described it as the change of mass over time in the basolateral compartment the change of mass over time in the apical compartment. This is not clear, C is not a mass and there is no apical in this term.

Thank you, this has now been changed.

6. The authors said (lines 368-369): for each Transwell insert/each well in a 48-well plate/each well in a 96-well plate, prepare DNA NP at N/P…etc. What is the surface area of the transwell?

The surface area of the Transwell is 0.3cm^2, this information is now included on line 394.

7. LY permeation is not "inhibited" rather "restricted", revise accordingly.

Thank you for the suggestion, we have changed this in the text on line 541.

Reviewer 4

Major Concerns:

1. In order to demonstrate that the described Lucifer Yellow assay is indeed a "robust permeability marker", the authors should consider testing a different cell source side-by-side, either using another brain endothelial cell line, or a peripheral (non-brain) endothelial cell line which is expected to be leakier, or an entirely unrelated cell type that does not form tight junctions. Alternatively, if such cell sources are not accessible to the authors, manipulation of hCMEC/D3 cells of some sort could be considered, such as changing the media composition or treating cells with a physiological stimulus such as pro-inflammatory cytokines.

Thank you for your suggestion. We would like to point out that we had originally included data on calcium-depleted cells that was performed by changing the media composition (Figure 4a). Extracellular calcium is a critical component for the maintenance of cell-cell junctions in various cell types [11-13], including the brain microvessel endothelial cells. It has been reported that the junctional permeability in native rabbit esophageal epithelium was increased during calcium depletion [14]. In figure 4a, the LY P_{app} value in calcium-depleted cells was significantly higher than cells treated with regular growth medium and close to the P_{app} value of cell-free inserts, suggesting that the calcium-depleted cells had lost their barrier properties resulting in higher LY P_{app} values. The results of the western blot (newly included in Figure 3) also corroborate that the calcium-depleted cells had lost their barrier properties resulting in lower expression of the tight junction marker ZO-1, compared to cells treated with regular growth medium.

2. It is concerning that a fully functional hCMEC/D3 monolayer cuts Papp values only in half compared to Transwells without cells (Fig. 3a). This would imply that hCMEC/D3 cells are a very leaky BBB model. Please provide insights, potentially from comparative studies in the literature.

This is a valid concern and we would like to clarify. The P_{app} value we reported is within the typical range (2.7-11.7 × 10^{-6} cm/s) for hCMEC/D3 cells cultured on a PET membrane with a 3.0 μm pore size [6, 15, 16]. It is also worth mentioning that molecular mass of the permeability marker, (LY here) and stirring speed are critical factors that influence the measured P_{app} value. While the reviewer is right that the hCMEC/D3 cells are not the tightest BBB model, it is still an acceptable and useful BBB model [17, 18]. The hCMEC/D3 cell line is considered as an appropriate immortalized human endothelial cell line [1] because this cell line retains most of the morphological and functional properties of brain endothelial cells, even without

cocultured glial cells [18, 19], and they express multiple BBB markers including active transporters and receptors [18, 20].

3. The authors often emphasize that monitoring cell morphology is key, and that formation of tight junctions mediates the decrease of Papp values over time (Fig. 2), or its increase when calcium is depleted from the medium, thereby de-stabilizing tight junctions (Fig. 3a). Accompanying immunocytochemistry images of a representative tight junction molecule, for instance Occludin or Claudin-5, would further strengthen the authors' conclusion, and validate the hCMEC/D3 as BBB model. This is particularly important for this study since the authors frequently refer to proper tight junction formation as mediators for Papp values, but no direct evidence is provided.

Thank you and we agree that this is a very good point. We have now added data from a western blotting study (Figure 3) demonstrating changes in expression of a tight junction marker, ZO-1, to complement data from the LY P_{app} studies. We believe that the newly included data using an orthogonal technique concurs well with the changes in LY P_{app} values over culture time and overall, strengthens the paper's conclusions. While immunocytochemistry (ICC) images could show the expression of ZO-1 on the cell membrane, ICC data is not amenable for quantification. We therefore used western blotting to detect changes in ZO-1 protein expression and have used band densitometry analysis to quantify the data.

4. Some steps in the procedures are overexplained, particularly steps 1 and 2 which simply describe standard cell culture techniques, while other steps require more information: volume of the bottom well, molecular size of Lucifer Yellow, pore size and material of transwells. Additional clarification is needed about the sampling procedure: were samples taken as end points from separate transwells per time point, or were the same transwells sampled daily? Does the passage of hCMEC/D3 matter?

Thank you for raising these questions. We agree that this is important information that should have been included and we have now included the information on the volume of the companion well, molecular size of lucifer Yellow, pore size and Transwell insert material on lines 391-392. Yes, the samples were taken from separate Transwells at each indicated time point. We have included this information in the methods section. Yes, the passage number of hCMEC/D3 does matter. It has been reported that hCMEC/D3 cells undergo dedifferentiation to unstable phenotypes over long periods of passaging and the it has been recommended to use cells within passage number 35 [15]. We would like to point out that all our studies were conducted on cells under passage 35.

Minor Concerns:

1. Although the Transwell BBB model is still considered the gold standard in the field, the authors should acknowledge in their introduction or discussion that significant advances in the field have been made over the last years, including co-culture systems, microfluidic systems, and 3D cell culture systems, which represent physiologically more relevant mimics of the BBB in vitro with significantly improved barrier function. I am not expecting a comprehensive review of all these novel platforms, but some wording around these advances in the field should be

included, discussing some critical limitations of the Transwell setup in this context, but then highlighting some of the advantages the authors see in in the Transwell system.

We have added a brief section discussing some of the novel platforms in the introduction section (lines 88-92) and have highlighted the merits of limitations of the Transwell system in the discussion section (lines 728-732).

2. On a similar note, limitations of the hCMEC/D3 cell line should be briefly discussed, in light of recent advances in stem cell derived BBB models, which demonstrate extraordinary tightness. Then, the authors should explain why the hCMEC/D3 cell line might still be a useful model for BBB, despite the emerging use of brain endothelial cells generated from human pluripotent stem cells.

Thank you for your suggestion. We have information in the introduction section addressing this comment (lines 56-71 and 88-92). Briefly, the hCMEC/D3 cell line is an appropriate immortalized endothelial cell line [1] as they form confluent monolayers with properties representative of the human BBB and do not require co-culturing with other cell types [21]. It should be noted that although stem cell-derived BBB models showed higher permeability in many studies compared with hCMEC/D3 cell line and they do express some BBB markers, they are yet to evolve as the most common BBB cell model [1]. Importantly, stem-cell derived BBB models remain to be characterized with respect to maximum passage numbers that allow the cells to maintain stable BBB phenotypes [22].

3. Introduction: the authors mention coating and media composition being an issue for reliably measuring TEER, however, this can be resolved by the addition of blank transwells, like the authors do for their Lucifer Yellow permeability assay in order to calculate Papp.

Thank you for making this point, we have now revised the text accordingly on lines 78-80.

4. The authors are encouraged to add Mantle et al. Molec. Pharm. 2016 (https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.6b00818) when comparing TEER and size exclusion assays.

Thank you, we have now added this reference.

5. Please provide evidence from the literature that hCMEC/D3 cells are "hard-to-transfect" cells.

The morphologic and molecular features of cerebral endothelial cells allow these cells to prevent the extravasation of large and small solutes due to the presence of tight junctions that reduce paracellular transport. Of particular relevance to transfection, the low rate of pinocytotic vesicle formation in these cells limits transcellular transport [23]. There are multiple published reports that explored liposomes and nanoparticles as vehicles for crossing the BBB and deliver drugs such as doxorubicin, loperamide and tubocurarine. However, only the surfactants polysorbate (Tween) 20, 40, 60 and 80, and some poloxamers (Pluronic F 68) can have been able to mediate moderate drug uptake [24]. A few studies have reported modification of liposomes with antibodies to increase

transfection efficiency [18, 21]. Based on the inherent characteristics of these cells and due to limited choices of excipients, hCMEC/D3 can be considered as "hard-to-transfect" cells.

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