bio-protocol

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An *in vitro* Blood-brain Barrier Model to Study the Penetration of Nanoparticles

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Abstract

The blood-brain barrier (BBB), a crucial protection mechanism in the central nervous system (CNS), is a selective barrier comprised of endothelial cells. It hampers the development of therapeutic and diagnostic tools for neurological diseases due to the poor penetration of most of these agents. Rationally engineered nanoparticles (NP) can facilitate the transport of therapeutic and diagnostic agents across the BBB. However, evaluating BBB penetration by NP majorly relies on the use of expensive and time-consuming animal experiments with low throughput. *In vitro* BBB models composed of brain endothelial cells can be a useful tool to rapidly screen multiple NP formulations to compare their BBB penetration ability and identify optimal formulations for *in vivo* validation. In this protocol, we present an *in vitro* model of BBB developed using murine cerebral cortex endothelial cells (bEnd.3). bEnd.3 is a commercially available, easy to manipulate cell line that forms tight junctions with potent paracellular barrier property. The protocol includes culturing of bEnd.3 cells, establishment of the *in vitro* model, and assessing NP permeability. We believe that, due to its simplicity and consistency, this step-by-step protocol can be easily used by researchers to screen NP-based drug delivery systems for BBB penetration.

Keywords: Blood-brain barrier, *In vitro*, Nanoparticles, Central nervous system, Permeability, TEER

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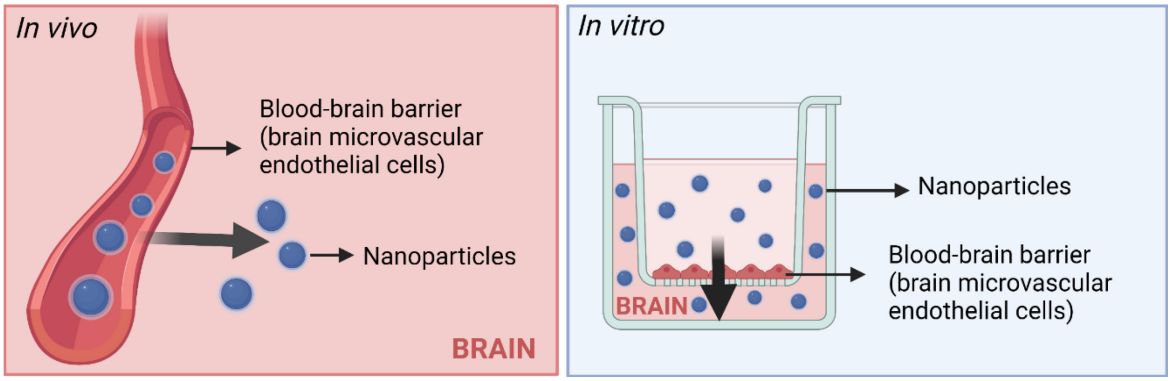
Graphic abstract:



Background

The diagnostic and therapeutic approaches targeting brain pathologies, such as Parkinson's disease, Alzheimer's disease, and glioblastoma, require delivery of imaging contrast agents and drugs across the blood brain barrier (BBB) (van Rooy *et al.*, 2011; Pehlivan, 2013; Aday *et al.*, 2016). Only a limited number of small molecules can cross the BBB (Pardridge, 2003), with more than 98% of drugs developed for CNS pathologies not able to cross the BBB, which hampers their clinical development (Pardridge, 2005). Rationally engineered nanoparticles (NP) can facilitate the transport of therapeutic and diagnostic agents across the BBB (Li *et al.*, 2021). Development of such NP formulations typically requires their screening in rodent models to assess their BBB penetration and to gain a better understanding of the expression and functionality of transporters in the BBB (Cecchelli *et al.*, 2014; Aday *et al.*, 2016). However, rodent studies can be expensive and time-consuming, with low throughput, especially when a large library of NPs needs to be screened. *In vitro* BBB models composed of brain endothelial cells can be a useful tool to rapidly screen multiple NP formulations to compare their BBB penetration ability and identify optimal formulations for *in vivo* validation. These models can also be used to gain mechanistic understanding of BBB penetration by NPs. Although *in vitro* models of rodent BBB have been widely used in the literature for evaluating the brain penetration mechanisms of different drug and NP formulations, there is no detailed protocol that can be easily used by researchers with different backgrounds. Here, we present detailed protocols to establish a simple and consistent *in vitro* BBB model using murine brain microvascular endothelial cells (bEnd.3), and to study *in vitro* penetration of NP formulations using this model. Our step-by-step protocol can be easily used by researchers to screen NP-based drug delivery systems for BBB penetration.

Materials and Reagents

1. 15-mL centrifuge tubes (CellTreat, catalog number: 229411)
2. T-75 flasks (CellTreat, catalog number: 229341)
3. bEnd.3 cells (ATCC®, catalog number: CRL-2299™), expand and store cell stocks in liquid nitrogen
4. Gelatin (porcine skin type A; Sigma, catalog number: G1890), store at room temperature
5. Dulbecco's modified Eagle's medium (DMEM) (Gibco, catalog number: 11965118), store at 4°C
6. Fetal bovine serum (FBS) (Gibco, catalog number: 26140079), aliquot and store at -20°C
7. Penicillin-streptomycin (P/S; 10,000 U/mL; Gibco, catalog number: 15140122), aliquot and store at -20°C
8. Trypan blue solution, 0.4% (Gibco, catalog number: 15250061), store at room temperature
9. Transwell® inserts (Corning, catalog number: 3401)
10. Growth factor reduced (GFR) Matrigel® (Corning, catalog number: 354230), aliquot and store at -20°C. Follow the instructions given in the procedure section.

11. DPBS (Ca^{2+}/Mg^{2+} free) (Gibco, catalog number: 14190359), store at room temperature
12. TrypLE™ Express (Gibco, catalog number: 12604-021, store at room temperature) or 0.05% Trypsin/EDTA (Gibco, catalog number: 15400-054, aliquot and store at -20°C)
13. Trypsin Neutralizing solution (Lonza, catalog number: CC-5002), use it only if you use trypsin as detachment enzyme, aliquot and store at -20°C
14. Phenol red-free medium (Gibco, catalog number: 21063029), store at 4°C
15. HEPES-buffered Krebs-Ringer solution (optional; Alfa Aesar, catalog number: J67795-AP), store at 4°C
16. 50–100 nm sized nanoparticles prepared from poly(lactic-co-glycolic acid) (PLGA) and labeled with Dy677 fluorescent dye [Lab-made; please check Li *et al.* (2021)], freshly prepared before use
17. Mouse serum (Sigma, catalog number: M5905), aliquot and store at -20°C

Equipment

1. Laminar flow cabinet (Baker, SterilGard SG403-Class II Type A/B3)
2. CO_2 incubator (Thermo, Forma Series 3110 water-jacketed incubators)
3. Benchtop centrifuge (Eppendorf, model: 5810R)
4. Improved Neubauer cell counting chamber (EMS, catalog number: 68052-16)
5. EVOM-2 Volt/Ohm meter (World Precision Instruments, catalog number: 300523)
6. LSE™ Low Speed Orbital Shaker (Corning™, catalog number: 6780FP)
7. Plate reader (Tecan, model: Infinite Pro 200)

Software

1. Tecan i-Control plate reader software, 2.0.10.0
2. GraphPad Prism, 9.2.0

Procedure

A. Defrosting and maintenance of cultures

1. Coat T-75 flasks with 1% gelatin (use 5–6 mL per T-75 flask) and incubate them at 37°C for 15 min.
2. After 15 min, aspirate gelatin and add 10 mL of complete medium (DMEM, with 10% FBS and 1% P/S) per flask.
3. Recover bEnd.3 cells from liquid nitrogen and dip the lower half of the vial into the 37°C water bath to thaw.
4. Before completely thawing the whole content of the vial (when you have a small piece of ice inside the vial), wipe the outside of the vial with disinfecting solution (70% ethanol) and move to a laminar flow culture hood.
5. Open the vial and add 0.5–1 mL of pre-warmed complete medium drop by drop onto the cells.
6. Pipette the suspension up and down slowly, to disperse the cells.
7. Seed them as 0.5×10^6–1×10^6 bEnd.3 cells/flask in a total volume of 15 mL.
8. Following inoculation, swirl the medium in the flasks to distribute the cells, and place T-75 culture flasks in a 37°C, 5% CO_2, humidified cell culture incubator.
9. After cell attachment (3–4 h), replace the freezing medium containing DMSO with 15 mL of fresh complete medium.
10. Incubate the cultures in a 37°C, 5% CO_2, humidified cell culture incubator.
11. Replace the media with fresh media every 2–3 days.

B. Cell seeding into Transwell® inserts

12. When the cells reach 80–90% confluency, start permeability experiments.
13. On the day of cell seeding to Transwell® inserts (Corning), remove GFR Matrigel aliquots from the freezer and thaw them on ice.

CAUTION: Matrigel® jellifies quickly at room temperature. Prepare Matrigel aliquots using pre-chilled pipette tips and pre-chilled microcentrifuge tubes prior to experiments. Work on ice and avoid multiple freeze-thaw cycles.

14. Dilute GFR Matrigel in cold DMEM (without FBS and antibiotics) and coat 12 mm polycarbonate Transwell inserts with 2% GFR Matrigel (200 µL/insert).
15. Incubate Transwell inserts at 37°C for 1 h.

CAUTION: Cells must be seeded immediately at the end of this period.

16. Remove the media from T-75 flasks by aspiration.
17. Wash cells with 5 mL of DPBS (Ca^{2+}/Mg^{2+} free) and remove the solution by aspiration.
18. Pipette 3 mL of pre-warmed TrypLE Express or 0.05% Trypsin/EDTA solution into the T-75 flask.
19. Swirl the flask to ensure the solution to cover all the cells.
20. Monitor the trypsinization process at room temperature under a microscope.
21. Release the rounded cells from the culture surface, by hitting the side of the flask against your palm until most of the cells are detached.
22. If you are using Trypsin, pipette 6 mL of Trypsin Neutralizing solution or 10% of FBS-containing DMEM to the flask, to inhibit tryptic activity. If you are using TrypLE Express, use DMEM to dilute the enzyme.
23. Transfer the cell suspension into a 15-mL centrifuge tube and centrifuge at 250 × *g* for 5 min to pellet the cells.
24. Aspirate the supernatant from the tube without disturbing the cell pellet.
25. Resuspend the cells in 2 mL of complete medium by gently pipetting the cells up and down, and count the cells using a cell counting chamber. Use trypan blue to exclude dead cells.
26. Aspirate Matrigel from the Transwell inserts and wash inserts once with DMEM (without FBS and antibiotics; use 300 µL/insert).
27. Plate cells immediately into the coated inserts at a density of 80,000 cells/insert in 500 µL.
28. Add 1.5 mL of medium to the basolateral side, and replace media in apical/basolateral sides, every 2–3 days.
29. Monitor Transendothelial Electrical Resistance (TEER) of endothelial cells using an EVOM-2 Volt/Ohm meter (World Precision Instruments). TEER is a widely used method to measure the integrity of a cell monolayer.
30. To measure TEER, sterilize the EVOM-2 with 70% isopropanol (IPA) before taking it into the laminar flow hood.
31. Connect the probe into the EVOM-2 and sterilize it by placing into 70%IPA for 15 min.
32. After 15 min, wash the probe by placing it into DPBS.
33. Measure TEER by inserting the long prong into the break in the Transwell hangar, and lower it until it touches the bottom of the well (see Figure 1).
34. Read and record the values on the screen of EVOM-2.
35. To calculate TEER (ohm*cm^2), multiply the resistance value with the surface area (1.12 cm^2 for the Corning 3401).
36. Repeat measurements every 1–2 day(s).
37. Once TEER reaches ≥50 ohm*cm^2 (~ after 1 week), perform permeability experiments.

C. Nanoparticle permeability experiments

38. Prior to experiments, prepare a new 12-well plate with 1.5 mL of DMEM (without serum and antibiotic solution)/well.

> *CAUTION: It is recommended to use a phenol red-free medium to prevent problems with fluorescence measurements. If phenol red-free medium is not commercially available, prepare calibration curves using labelled nanoparticles in phenol red-containing media, to see the effect of phenol red in the measurements. Alternatively, HEPES-buffered Krebs-Ringer solution can be used for nanoparticle permeability experiments.*

39. Dilute fluorescence-labelled nanoparticles in DMEM (or HEPES-buffered Krebs-Ringer solution—see Step 38) at the desired concentration.

> *CAUTION: The concentration range for different nanoparticle formulations must be determined prior to experiments. Calibration curves or prior in vitro experiments can help to find the best concentration range for each nanoparticle formulation.*

40. Transfer Transwell inserts containing cells into the new 12-well plate prepared in Step 38.

> *CAUTION: Include at least two empty filters (per nanoparticle formulation), without cells but with Matrigel coating, to calculate the maximum (100%) nanoparticle penetration.*

41. Aspirate the media from the apical part of the inserts and add 500 µL of nanoparticle solution to the apical compartment in DMEM (or HEPES-buffered Krebs-Ringer solution). Collect aliquots from apical and basolateral sides at time zero.

> *CAUTION: If you aim to find the effect of serum on nanoparticle penetration through the BBB, dilute nanoparticles in DMEM with mouse serum and incubate 30 min before the permeability experiments.*

42. Keep the Transwell plates on an orbital shaker, at 37°C, 5% CO_2.
43. After 4 h, withdraw the Transwell inserts from the receiver compartment.
44. Collect aliquots from the apical and basolateral compartments and measure volumes in these compartments, for the penetration and mass balance calculations.
45. Prepare different concentrations of nanoparticle formulations in the solution you use in permeability studies (see Steps 38 and 39) to plot calibration curves.
46. Quantify fluorescence using a plate reader at room temperature with the appropriate excitation/emission values for your fluorescent nanoparticles. Use at least three inserts with cells in each permeability measurement. Use empty filters without cells to determine the maximum nanoparticle penetration (100% penetration) *in vitro*.

Data analysis

1. Use calibration curves to calculate the concentration of NPs in the apical and basolateral sides (Figure 1).
2. Using volume values in basolateral/apical sides at the end of the experiment, calculate the amount (mass) of the nanoparticles in both compartments (Mass of NPs = Concentration of NPs × Volume).
3. To assess possible adsorption to plastics and non-specific binding to cells, calculate the mass balance (%)—

divide the amount of NPs recovered in both compartments at the end of the experiment by the total amount added in the donor compartment at time zero. Mass balance value should be between 80–120% for reliable calculations.

4. Calculate the amount of NPs that have penetrated the basolateral compartment in empty filters (without cells; see Step 40). Consider this value as 100% penetration (penetration through filters, without endothelial cell barrier).
5. Normalize the data (% penetration) for different conditions using the value from empty filters (100% penetration).
6. Perform statistical analysis and graphing with GraphPad Prism. Consider a value of $P < 0.05$ as statistically significant.
7. For an example of the results that can be obtained using this protocol, please see Li *et al.* (2021).

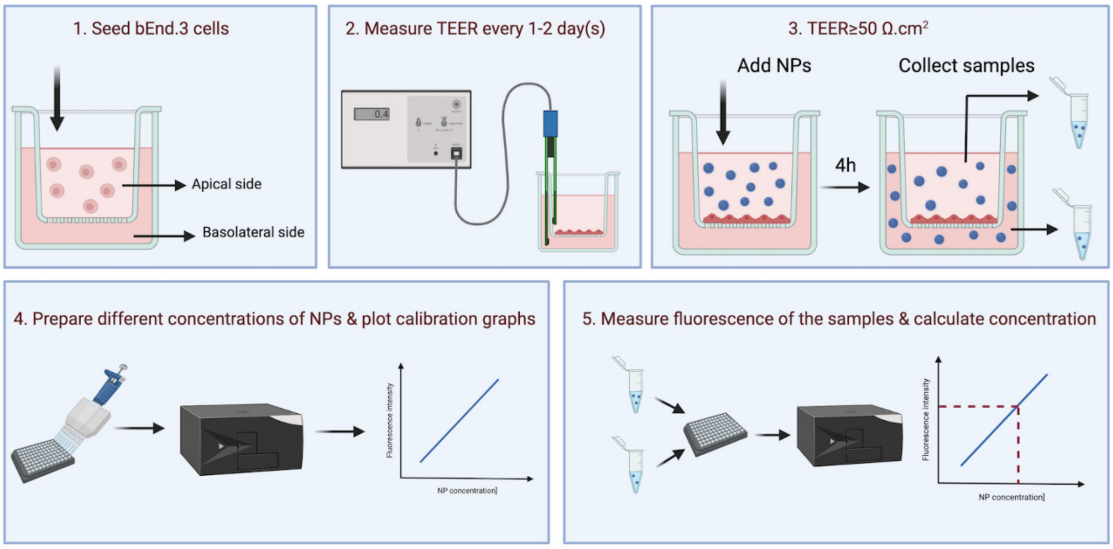


Figure 1. Schematic of the protocol used to calculate NP concentration in apical and basolateral sides.

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Competing interests

J.M.K. has been a paid consultant and or equity holder for multiple biotechnology companies (listed here: https://www.karplab.net/team/jeff-karp). The interests of J.M.K. were reviewed and are subject to a management plan overseen by his institutions in accordance with its conflict of interest policies. J.M.K, N.J., S.A. and W.L. have one pending patent based on the nanoparticle work presented in this manuscript.

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