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Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol

Protocol for the setup and use of a human choroid plexus endothelial-epithelial two-cell-type in vitro model

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SUMMARY

Choroid plexus, located in brain ventricles, is the site of blood-cerebrospinal fluid barrier that contains endothelial cells and an epithelial monolayer separated by stroma. We established a two-cell-type model of the human choroid plexus consisting of immortalized endothelial cells (iHCPEnC) and epithelial papilloma (HIBCPP) cells grown on opposite sides of filter supports. In this protocol, we describe the preparation of this model, the measurement of transepithelial electrical resistance (TEER), and immunofluorescence imaging-based analysis to determine the barrier function.

For complete details on the use and execution of this protocol, please refer to Muranyi et al. (2022).

BEFORE YOU BEGIN

⚠ CRITICAL: The barrier function of the endothelial-epithelial two-cell-type model strongly depends on the integrity of the HIBCPP cell layer which is represented at a TEER value of ~300 Ω cm². The starting point for seeding endothelial iHCPEnC on the opposite of the cell culture filter inserts is at TEER values of the HIBCPP cells between of 100–200 Ω cm². TEER measurements of the coculture start 24 h after endothelial cells are seeded.

Preparation of epithelial cells (HIBCPP cells)

⏰ Timing: 2 weeks

1. Preparation of HIBCPP medium.
   a. For cultivation of HIBCPP cells, supplement DMEM/F-12 (Ham) with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, 100 U/mL penicillin and 5 μg/mL insulin (in the following called HIBCPP medium).

Note: The cell culture medium can be stored at 4°C and does not have to be freshly prepared before experiments.
Note: We recommend preparing new medium every three months.

b. Pre-warm the medium before use.

2. Starting HIBCPP cell culture from frozen stocks.
   a. Thaw an aliquot containing $1 \times 10^6$ HIBCPP cells and transfer the cell suspension into a T-75 flask with 10 mL pre-warmed HIBCPP medium.
   b. Incubate the cells at 37°C and 5% (v/v) CO$_2$ for 24 h.
   c. Replace the media with fresh media and grow cell culture to 100% confluence. In the beginning of the culture this can take up to 2 weeks. The HIBCPP medium is replenished every two to three days. We suggest Monday, Wednesday and Friday.

3. Cultivation and passaging of ongoing HIBCPP cell culture (Schwerk et al., 2012).
   a. Trypsinize a confluent ongoing culture grown in a T-75 flask with 3 mL 0.25% trypsin-EDTA until cells detach, this takes approximately 15–20 min at 37°C. Trypsinization is inhibited by adding 17 mL HIBCPP medium.
   b. Verify by light microscopy whether cells have detached completely.
   c. Transfer the cell suspension into a 50 mL tube and centrifuge at 50,000 g for 10 min at room temperature (20°C - 23°C).
   d. Discard the supernatant by carefully aspirating the medium without touching the soft cell pellet and thoroughly resuspend the cell pellet in 1 mL HIBCPP medium.
   e. Add additional 9 mL medium and determine the cell density using a counting chamber (Neubauer improved 0.01 mm).
   f. Adjust the concentration to $1 \times 10^6$/mL and transfer $1 \times 10^6$ cells into a new T-75 flask containing 10 mL HIBCPP medium (seeding density of $13.3 \times 10^5$ cells/cm$_2$) and continue with incubation at 37°C and 5% (v/v) CO$_2$.
   g. The cells grow to confluence approximately within 1 week.

Preparation of endothelial cells (iHCPEnC)

© Timing: 2 weeks

4. Preparation of iHCPEnC medium.
   a. The iHCPEnC are cultivated in Complete Classic Endothelial Medium containing 10% FBS (Cell Systems, Kirkland, USA), 100 U/mL penicillin, 100 μg/mL streptomycin and CultureBoostTM (Cell Systems, Kirkland, USA) (in the following called iHCPEnC medium).
   b. Cells are grown in the absence of puromycin.

Note: To ensure the stability of the cell line 0.5 μg/mL puromycin can be added every fifth passage. The cell line was initially established by screening for puromycin resistant cells. As the cell line is considered stable, addition of puromycin would be an extra control.

c. Pre-warm the medium before use.

5. Starting iHCPEnC culture from frozen stocks.
   a. Thaw an aliquot containing $5 \times 10^5$ iHCPEnC and transfer the cell suspension into a 15 mL tube.
   b. Coat a T-25 flask with 2 mL of Attachment Factor™ (Cell Systems, Kirkland, USA) for 1 min, remove the Attachment Factor™. The coating factor can be stored at 4°C for 30 days and can be reused during that time.
   c. Centrifuge the cell suspension at 50 × g for 5 min.
   d. Discard the supernatant to remove DMSO present in the freezing medium.
   e. Resuspend the cell pellet in 1 mL fresh iHCPEnC medium and pipet cells into the coated T-25 flask containing 4 mL pre-warmed iHCPEnC medium.
f. Incubate the cells at 37°C and 5% (v/v) CO₂ and grow cell culture to 100% confluence, usually within 4–5 days.

6. Cultivation and passaging of iHCPEnC during ongoing culture (Muranyi et al., 2022).
   a. Routinely, cells are grown in T-25 flasks containing 5 mL iHCPEnC medium and passaged once a week. After passaging no medium change has to be made for one week and therefore until the next passaging or when they are used for experiments.
   b. Carefully wash the confluent cell layer once with 1 mL 0.25% trypsin-EDTA.
   c. Trypsinize iHCPEnC with 1 mL 0.25% trypsin-EDTA for approximately 2 min at room temperature and stop trypsinization reaction by adding 1 mL of iHCPEnC medium.
   d. Transfer the cell suspension into a 15 mL tube and centrifuge at 50 g for 5 min at room temperature.
   e. Discard the supernatant and resuspend the cell pellet in 2 mL iHCPEnC medium.
   f. Coat a T-25 flask with 2 mL of Attachment Factor™ (Cell Systems, Kirkland, USA) for 1 min, remove the Attachment Factor™. The coating factor is stored at 4°C and can be reused.
   g. Add 4.5 mL pre-warmed medium and 0.5 mL (approximately 2 x 10⁵ cells) of iHCPEnC suspension to the coated T-25 flask. Incubate the cells at 37°C and 5% (v/v) CO₂ until the cells grow to confluence, usually within 4–5 days.

**Measurement of transepithelial electrical resistance (TEER)**

- **Timing:** 20 min

7. Measurement of transepithelial electrical resistance (TEER).
   a. Start measuring TEER at day 4 after seeding when HIBCPP cells reach 100% confluency on filter inserts. For measurement, use commercially available equipment, e.g., a Millicell ERS-2 voltohmmeter with a MERSSTX01-electrode. Alternatively, a self-assembled reliable voltameter that can be used with a commercially available chopstick electrode which has previously been described (Theile et al., 2019).
   b. Sterilize the electrode in 10 mL of 80% ethanol in a 50 mL tube for 15 min to disinfect and to prevent cell culture contamination.
   c. Air dry the electrode from ethanol in sterile environment (sterile hood).
   d. Equilibrate the electrode for 5 s in 10 mL of HIBCPP medium w/o FBS in a 50 mL tube.
   e. Place the electrode with the long arm into the well of the plate, i.e., outside of the insert (usually 24-well), and the short arm into the reservoir of the filter insert. Do not touch the filter membrane.
   f. Read the TEER value.
   g. After measuring TEER, place the electrode back into a 10 mL of 80% ethanol for 15 min and subsequently store it protected in a dry 15 mL tube.

⚠️ **CRITICAL:** We used filter inserts with a TEER of ~100–200 Ω cm² for coculture with endothelial cells. If the TEER value of HIBCPP cell-cultured filter inserts remain below ~100–200 Ω cm² within a week, the filter inserts were excluded from the experiment.

**Note:** To calculate TEER values in Ω cm², a triplet of blank inserts (without cells), but filled with 0.5 mL HIBCPP medium in the insert and 1 mL HIBCPP medium in the well, is measured, initially for around 5 days, to determine an averaged blank TEER value of the inserts themselves. This blank value is subtracted from the measured TEER value of each HIBCPP cell cultured insert. To calculate the TEER value per cm², this blank corrected value is multiplied by the area of the insert membrane in cm² (0.336 for Greiner Bio-One 662641 inserts).

**Note:** It is recommended to seed cells on Wednesday (day 0) and flip them on Thursday (day 1), so that endothelial cells can be seeded on Monday (day 5) and the two-cell-type model of the BCSFB can develop from Monday (day 5) until Friday (day 9).
**Note:** For two-cell-type culture we grow HIBCPP cells on transparent filter inserts with 0.4 μm pore size and 2 x 10^6 pore density (Greiner Bio-One 662641). If different filter inserts are planned to be used, it would be advantageous to check the confluency of HIBCPP cells at a TEER of ~100–200 Ω cm^2 by fixing the cells at this step and staining the nuclei.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies** | | |
| Mouse anti-Ecad, dilution 1:200 | BD Biosciences | 610182 RRID: AB_397580 |
| Mouse anti-PECAM1, dilution 1:200 | Dako | M0823, RRID: AB_2114471 |
| Rabbit anti-ZO1, dilution 1:400 | Thermo Fisher Scientific | 61-7300; RRID: AB_2533938 |
| Donkey anti-rabbit Alexa 488, dilution 1:500 | Thermo Fisher Scientific | A21206, RRID: AB_2535792 |

| **Chemicals, peptides, and recombinant proteins** | | |
| DMEM/F-12 | Gibco | 11039-021 |
| Complete Classic Medium™ | Cell Systems | 420-500 |
| CultureBoost™ | Cell Systems | 4CB-500 |
| Attachment Factor™ | Cell Systems | 420-201 |
| Trypsin/EDTA 0.25% | Gibco | 25200-056 |
| Penicillin Streptomycin | Gibco | 15070-063 |
| Phalloidin Alexa 647 | Invitrogen | A21287 |
| Concanavalin A rhodamine | Vector Laboratories | RLK-2200 |
| Dulbecco’s PBS (1X) | Gibco | 14190-094 |
| Puromycin | Gibco | A11138-03 |
| Triton X-100 | Sigma | 8787 |
| ProLong antifade reagent | Invitrogen | P34930 |
| Paraformaldehyde, 4% in PBS | Thermo Fisher Scientific | J19943 |
| Paraformaldehyde solution 16% | Thermo Fisher Scientific | 28908 |
| Trypan blue, 0.4% | Sigma | 93595 |
| 4', 6-diamidino-2-phenylindol (DAPI) | Calbiochem | 268298 |
| Fetal bovine serum | Gibco | A4766 |
| Insulin, human recombinant | Sigma | I9278 |

| **Experimental models: Cell lines** | | |
| Human choroid plexus papilloma (HIBCPP) cells passage 20–32 | (Schwerk et al., 2012) | N/A |
| Immortalized human choroid plexus endothelial cells (HCPEnC) passage 18–50 | (Muranji et al., 2022) | N/A |

| **Software and algorithms** | | |
| ImageJ/FIJI | Schneider et al., 2012 | http://imagej.nih.gov/ij/ |
| Imaris 9.6.0 | EMBL | http://imaris.oxinst.com/ |
| Zen | Carl Zeiss | https://www.zeiss.de/mikroskopie/produkte/mikroskopsoftware/zen.html |

| **Other** | | |
| ThinCert, PET Membrane, pore 0.4 μm, transparent | Greiner Bio-One | 662641 |
| Millicell ERS-2, voltohmeter | Millipore | MERS00002 |
| Millicell ERS electrode | Millipore | MERSSTX01 |
| Counting chamber, improved 0.100 mm | Hecht Assistent | 120232 |
| Parafilm™ M sealing film | Merck | HS2345268 |
| Thermo cycler MX3005PTM | Agilent Technologies | N/A |
| Infinite M200 | Tecan | N/A |
| Olympus SpinSR10 spinning disk confocal | Olympus | N/A |
MATERIALS AND EQUIPMENT

**HIBCPP medium**

| Reagent                        | Final concentration | Amount |
|-------------------------------|---------------------|--------|
| DMEM / F-12                   | N/A                 | 500 mL |
| Fetal bovine serum (FBS)      | 10% (v/v)           | 50 mL  |
| Insulin (10 mg / mL)          | 5 µg / mL           | 250 µL |
| Pen / Strep (100 x)           | 1 x                 | 5 mL   |
| **Total**                     | N/A                 | 555 mL |

Store at +2°C to 8°C for 3 months.

**iHCPEnC medium**

| Reagent                        | Final concentration | Amount |
|-------------------------------|---------------------|--------|
| Complete Classic Medium™      | N/A                 | 500 mL |
| CultureBoost™                 | N/A                 | 10 mL  |
| Pen / Strep                   | 1 x                 | 5 mL   |
| **Total**                     | N/A                 | 515 mL |

Store at +2°C to 8°C for 3 months.

**Storage conditions (temperature and maximum time)**

| Reagent                                      | Storage temperature | Time            |
|----------------------------------------------|---------------------|-----------------|
| DMEM / F-12 supplemented [HIBCPP medium]     | +2°C to 8°C         | 90 days         |
| Complete Classic Medium™ w/o supplements     | +2°C to 8°C         | 30 days         |
| Complete Classic Medium™ supplemented       | +2°C to 8°C         | 90 days         |
| with CultureBoost™ [iHCPEnC medium]          | −80°C               | Up to 1 year    |
| Attachment Factor™                          | +2°C to 8°C         | 30 days         |
|                                              | −80°C               | Up to 1 year    |
| Complete Classic Medium: w/o CultureBoost and Pen / Strep. |               |                 |
| iHCPEnC medium: Complete Classic Medium with CultureBoost and with Pen / Strep. |               |                 |

STEP-BY-STEP METHOD DETAILS

**Preparation of the endothelial-epithelial two-cell-type model on filter inserts**

⊙ **Timing:** 2 weeks

This section describes the sequential preparation of the two-cell-type model consisting of an epithelial and endothelial cell layer grown on the opposite of filter inserts.

**Note:** We recommend the following steps. First, prepare inversely cultured HIBCPP cells. After 24 h, turn filter inserts to standard orientation. Start TEER measurement after 4 days and continue daily. Two-cell-type culture is prepared with transparent filter inserts with 0.4 µm pore size and 2 x 10⁶ pore density (Greiner Bio-One 662641). For practical handling see also (Dinner et al., 2016).

△ **CRITICAL:** As the background TEER value (insert without any cells) for different filter type varies, this value has to be determined for each individual filter type using separate filter inserts as described in the TEER measurement section, to calculate the corrected TEER values of cells mimicking the BCSFB in Ω cm².
1. Place a 12-well plate into a sterile, stainless steel box for surgical instruments.

   **Note:** When using 0.4 μm pore inserts the cell suspension needs some time to soak because of the small pore diameter. Do not close the 12-well plate containing the filters with its own lid, since the lid might touch the cell suspension placed on the membrane of the inverted filter insert. Instead, place the 12-well plate into a sterile, stainless steel box for surgical instruments. Close this box with its corresponding lid, thereby keeping a sufficient distance of the lid to the cell suspension on the membrane of the filter insert (see Figure 1).

2. Start the experiment in a 12-well plate with inversely flipped and medium filled filter inserts.

   **Note:** To fill the 0.4 μm pore filter inserts it is recommended to first place the insert in correct orientation in a 12-well plate using sterile forceps. First fill the insert with HIBCPP medium with a 10 mL serological pipet, and subsequently fill the whole well with HIBCPP medium. Flip the insert “under water” preventing air to enter the insert. Remove medium until the well is approximately half filled. Continue with next insert (see Figure 1).

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**Figure 1. Step-by-step protocol for the preparation of the inverted culture model using filter inserts with 0.4 μm pore size**

(A) Setup for preparation of filter inserts.
(B) 12-well plate is placed in stainless steel box without lid.
(C) Filter insert is placed into the 12-well plate using sterile forceps.
(D and E) Filter insert is filled with HIBCPP medium as well as the whole well (E).
(F and G) Using the forceps, the filter insert is turned upside down within the HIBCPP medium to avoid air bubbles getting into the filter compartment.
(H) Additional medium is aspirated until the well is filled halfway.
(I) A drop of medium is placed on the filter membrane to pre-wet the membrane.
(J) Stainless steel box is placed at 37°C, 5% CO₂ until the HIBCPP cell suspension is added to the filter membrane (where the drop was placed). Following the seeding of the cells, the filters remain in stainless steel box until they are flipped into the 24-well plate in correct orientation after 24 h incubation at 37°C, 5% CO₂ as the cell suspension would otherwise stick to the lid of the 12-well plate.
3. Pipet $8 \times 10^4$ HIBCPP cells (80 µL of HIBCPP cell suspension adjusted to $1 \times 10^6$/mL) on top (bottom side of the filter) of the inverted and medium filled filter inserts.

**Note:** With 0.4 µm pore inserts it is essential to first pre-wet the top (bottom side) of inverted filter insert with some extra HIBCPP medium before seeding the cells, otherwise the cells will not attach to the insert membrane due to remaining air in the pores preventing sufficient medium supply of HIBCPP cells. If filters with a pore size of 3 µm are used, the inserts can be filled as shown in (Dinner et al., 2016). Incubate for 30 min at 37°C. Before seeding, remove remaining medium from the top of the insert before pipetting the cell suspension onto the filter membrane.

4. Cover the seeded filter inserts with the lid of the box and incubate at 37°C, 5% CO2 (v/v) for 24 h.
5. At day one, add 1 mL HIBCPP medium into the wells of a 24-well plate and turn the filter inserts with sterile forceps to the standard orientation into the 24-wells. For practical handling see (Dinner et al., 2016).
6. Add 500 µL HIBCPP medium into the upper compartment of the filter inserts.
7. When TEER values reach ~100–200 Ω cm² (usually 4 days after seeding of HIBCPP cells), prepare iHCPEnC solution with $1 \times 10^5$/mL cells and add $4 \times 10^4$ iHCPEnC to the upper compartment of the filter insert containing the HIBCPP medium.
8. Measure TEER every day for the next 4–5 days. Usually, we observe a continuous increase of TEER values up to 600–1,000 Ω cm².
9. When TEER values start to increase continuously from ~100–200 to 600–1,000 Ω cm² the two-cell-type filter insert is considered to mimic the BCFSB in vitro and can be used for several analyses of the endothelial/epithelial interplay under healthy and pathologic conditions including adherence and migration of immune or cancer cells, infection with pathogens and stimulation with exosomes. Additionally, molecular transport or drug kinetic studies can be performed.

**Immunofluorescence of the two-cell-type model**

© Timing: 2 days

Immunofluorescence imaging allows analysis of the morphology of the endothelial and epithelial cells and to visualize protein distributions within the coculture.

⚠ CRITICAL: The handling of iHCPEnC on filter inserts is intricate, due to their flat morphology and weak adherence to synthetic membranes. This can be overcome with the following protocol.

10. The two-cell-type culture contains 500 µL iHCPEnC medium in the upper reservoir of the filter insert (basolateral side) and 1 mL HIBCPP medium in the 24-well of the plate.
11. Pre-warm 4% and 8% PFA in PBS to 37°C temperature.
12. For each insert to fix prepare a well with 1 mL 4% PFA in PBS in a 24-well plate.
13. Always start with the fixation of the endothelial cell layer.
   a. Carefully remove 250 µL of medium from the upper reservoir and very slowly add 250 µL of pre-warmed 8% PFA in PBS for a final concentration of 4% PFA.
   
   **Note:** This is done to not disturb the endothelial cell layer by removing the entire medium from the upper compartment.
   b. Fix iHCPEnC for 20 min at room temperature.

14. Subsequently, carefully transfer every insert to its 4% PFA well and fix HIBCPP cells for 20 min at room temperature.
15. Stop the fixation step by gently washing twice with PBS at room temperature, i.e., let the PBS slowly run down the wall of the insert to avoid disturbance of the iHCPEnC layer.

**Pause point:** Membranes can be stored in PBS at 4°C for 1 week.

16. Prepare a strip of Parafilm® with a consecutive row of 50 μL drops of the solutions required for the staining.
17. Cut out the membrane of the filter insert with a scalpel and place the membrane on a 50 μL drop PBS.
18. Lift the membrane with forceps from the PBS drop and place the membrane in a 50 μL drop of 0.1% Triton X-100 in PBS. Submerge the membrane for 5 min to permeabilize the cells.
19. To remove Triton X-100, wash the cells by placing the membrane in the next drop containing 50 μL PBS. Repeat this step.
20. Pipet a 50 μL drop of 2% BSA in PBS on the strip of Parafilm® and submerge the membrane to block the cells for 15 min at room temperature.
21. Prepare a further 50 μL drop of 2% BSA in PBS containing the primary antibody at the desired dilution (usually 1:100 or 1:200).
22. Incubate the membranes for 1 h at room temperature in the dark.
23. To wash the cells twice, place the membrane to the next 50 μL PBS drop and repeat.
24. Prepare a 50 μL drop of 2% BSA in PBS containing the secondary antibody at the desired dilution (usually 1:500) and DAPI at a final concentration 1:10,000 for staining of nuclei. Incubate for 1 h at room temperature in the dark.
25. Again, wash cells twice with PBS and once with ddH2O. Aspirate the remaining fluid and let the membranes air dry for 2–3 min.
26. Mount the filter membrane within two microscope cover glasses (24 × 50 mm) with mounting medium ProLong antifade to allow imaging from both sides (iHCPEnC on the upper, HIBCPP on the lower membrane side).

**Note:** Importantly, mounting medium has to be on both sides of the filter membranes. This can be achieved by placing a drop of mounting medium on the cover glass and placing the filter membrane onto the drop. Once all membranes are placed on the cover glass (up to eight membranes can fit), mounting medium is added to the other side of the membranes to ensure they are covered from both sides, followed by placing the second cover glass.

**Note:** HIBCPP cells can clearly be identified on the filter membrane in contrast to iHCPEnC. It is recommended to mount all membranes in the same orientation.

27. Store overnight (~24 h) at room temperature in dark before analyzing the membranes using a fluorescent microscope.

**EXPECTED OUTCOMES**

**Barrier characteristics based on TEER measurement**

The scheme in Figure 2 summarizes the development of the two-cell-type BCSFB in vitro model. iHCPEnC are grown on the upper side, HIBCPP cells on the opposite, meaning the bottom side, of the membrane. To compare the individual cell lines to the two-cell-type model, they are cultured separately on the corresponding sides of filter membranes (i.e., HIBCPP in the inverted culture and iHCPEnC in a standard culture). To compare data from different types and sizes of filter inserts, the blank values of the filter inserts are subtracted from the measured TEER values and extrapolated to Ohm cm². TEER values are measured starting from day five after iHCPEnC were seeded. Co-culturing of HIBCPP cells together with endothelial iHCPEnC (600–1,000 Ω cm²) led to significantly higher TEER values compared to HIBCPP cells (350–500 Ω cm²) and iHCPEnC (10–50 Ω cm²) that were cultured separately. The increase in TEER does not result from simple addition of TEER values
generated of HIBCPP cells and iHCPEnC alone. Instead, a synergistic interplay affects HIBCPP cells to form a barrier with strengthened properties compared to HIBCPP cells alone.

Visualization of the two-cell-type BCSFB model based on immunofluorescence staining

Figure 3A shows the endothelial and epithelial cell layer of cells grown in the coculture model analyzed by spinning disc confocal microscopy at low resolution. As mentioned above and as can be seen from the image, the iHCPEnC form layers with very flat morphology. A clear staining of the endothelial marker PEACAM1 in iHCPEnC when grown in the coculture is shown in Figure 3B. Simultaneously, HIBCPP cells grown on the opposite side of the membrane show a characteristic staining for the adherence and tight junction proteins E-cadherin (Ecad) and Zonula occludens-1 (ZO1) (Figure 3C).

LIMITATIONS

To our knowledge, this coculture system is the first human endothelial-epithelial in vitro coculture model of the BCSFB. Due to its simplicity and easy to prepare method it readily enables analysis of endothelial/epithelial interplay under healthy and pathologic conditions, including immune or cancer cell adherence, infection with pathogens or stimulation with exosomes, as well as to perform molecular transport and drug kinetic studies. In vivo, the choroid plexus contains connective tissue
stroma and immune cells, which are lacking in the two-cell coculture model. Additionally, immortalized and papilloma cell lines, even if they retain many properties of the corresponding primary cells, can deviate from in vivo conditions. Due to the complexity of in vivo systems, in vitro models such as our coculture model cannot completely mimic the BCSFB and replace in vivo models. However, data gathered using our enhanced in vitro model can provide a prognosis of the in vivo situation.

TROUBLESHOOTING

Problem 1
Due to variation of HIBCPP cell growth, the number of seeded cells on the filter membranes might need to be adjusted (see step 3). The number of cells seeded to achieve a functional BCSFB model range between 5 x 10^4 to 1.5 x 10^5. For valuation, HIBCPP cells seeded at a density of 7 x 10^4 on filter inserts usually reach a TEER value of ~70–150 Ω cm² within 4 days. If this range of TEER values is not reached within this time frame, the number of cells seeded on the inserts can be adjusted.

Potential solution
If TEER values higher than ~250 Ω cm² are measured in this period, a reduction of the number of seeded cells to 5 x 10^4 should be considered. If a TEER value lower than ~20 Ω cm² is measured, the amount of HIBCPP cells should be increased to 1 x 10^5.

Problem 2
Endothelial cells detach easily when seeded on filter inserts.

Potential solution
Careful handling of the co-culture is advised as specified in the immunofluorescence protocol (see steps 10–27).
Problem 3
Filter inserts with small pore sizes of 0.4 μm can be difficult to fill with cell culture medium for the inverted culture of HIBCPP cells.

Potential solution
Handling of these filter inserts is described in note of this protocol (see notes of steps 1–3).

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be addressed to and will be fulfilled by the lead contact, Walter Muranyi (walter.muranyi@medma.uni-heidelberg.de).

Materials availability
General materials used in the methods are commercially available with no restrictions. HIBCPP cells and iHCPEnC are available upon request following Material Transfer Agreement (MTA), due to our institutional recommendations from C.S. and H.S.

Data and code availability
This study did not generate code.

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AUTHOR CONTRIBUTIONS
H.S. conceived the project. W.M., R.H., C.S.-G., and M.L. performed the methods, experiments, and data analysis. W.M., J.B., C.S., and H.S. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES
Dinner, S., Borkowski, J., Stump-Guthier, C., Ishikawa, H., Tenenbaum, T., Schroten, H., and Schwerk, C. (2016). A choroid plexus epithelial cell-based model of the human blood-cerebrospinal fluid barrier to study bacterial infection from the basolateral side. J. Vis. Exp. 54061. https://doi.org/10.3791/54061.

Muranyi, W., Schwerk, C., Herold, R., Stump-Guthier, C., Lampe, M., Fallier-Becker, P., Weiss, C., Sticht, C., Ishikawa, H., and Schroten, H. (2022). Immortalized human choroid plexus endothelial cells enable an advanced endothelial-epithelial two-cell type in vitro model of the choroid plexus. iScience 25, 104383. https://doi.org/10.1016/j.isci.2022.104383.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089.

Schwerk, C., Papandreou, T., Schuhmann, D., Nickol, L., Borkowski, J., Steinmann, U., Quednau, N., Stump, C., Weiss, C., Berger, J., et al. (2012). Polar invasion and translocation of Neisseria meningitidis and Streptococcus suis in a novel human model of the blood-cerebrospinal fluid barrier. PLoS One 7, e30069. https://doi.org/10.1371/journal.pone.0030069.

Theile, M., Wiiora, L., Russ, D., Reuter, J., Ishikawa, H., Schwerk, C., Schroten, H., and Mogk, S. (2019). A simple approach to perform TEER measurements using a self-made voltamperemeter with programmable output frequency. J. Vis. Exp. https://doi.org/10.3791/60887.