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1 **TITLE:**

2 A Triple Culture Cell System Modeling the Human Blood-Brain Barrier

3

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33

34 **SUMMARY:**

35 This protocol describes a method to establish a human blood-brain barrier (BBB) *in vitro* model.
36 The endothelial cells and pericytes are seeded on each side of an insert filter (blood
37 compartment), and astrocytes are seeded in the bottom well (brain compartment). The model
38 characterized was used for nanoparticles transport experiments.

39

40 **ABSTRACT:**

41 The delivery of drugs to the brain remains a challenge due to the blood-brain barrier's (BBB)
42 highly specific and restrictive properties, which controls and restrict access to the brain
43 parenchyma. However, with the development of nanotechnologies, large panels of new
44 nanomaterials were developed to improve drug delivery, highlighting the need for reliable *in vitro*

45 microsystems to predict brain penetration in the frame of preclinical assays. Here is a
46 straightforward method to set up a microphysiological system to model the BBB using solely
47 human cells. In its configuration, the model consists of a triple culture including brain-like
48 endothelial cells (BLECs), pericytes, and astrocytes, the three main BBB cellular actors necessary
49 to induce and regulate the BBB properties in a more physiological manner without the
50 requirement of tightening compounds. The model developed in a 12-well plate format, ready
51 after 6 days of triple culture, is characterized in physical properties, gene, and protein expressions
52 and used for polymeric nanogel transport measurement. The model can be used for an extensive
53 range of experiments in healthy and pathological conditions and represents a valuable tool for
54 preclinical assessments of molecule and particle transport, as well as inter-and intracellular
55 trafficking.

56

57 **INTRODUCTION:**

58 The BBB, localized at the level of brain capillary endothelial cells (ECs), controls and regulates
59 access to the brain parenchyma, which is crucial for maintaining brain homeostasis and the
60 function of neural cells^{1,2}. However, in the case of brain pathology, the lack of access to the brain
61 parenchyma represents a real obstacle to developing therapeutic strategies.

62

63 The BBB ECs possess a complex set of properties, including tight junction (TJ) proteins, which seal
64 the intercellular space, associated with a system of efflux pumps, specific transporters, and
65 receptors, which control the transcellular pathway¹⁻³. Moreover, all these properties are induced
66 and maintained, thanks to communications with the pericytes embedded in the BBB EC basement
67 membrane and the astrocytes, whose end-feet surround the brain capillaries¹⁻³. Hence, studying
68 the BBB *in vitro* is a challenge considering the complexity of its architecture and the
69 communications among the different cell types constituting the neurovascular unit (NVU)².
70 Moreover, the different cell types are crucial for the induction and maintenance of BBB
71 properties and consequently impact the prediction of the crossing through the BBB. Different
72 strategies for drug delivery to the brain were already tested using a large panel of tactics to
73 bypass the BBB restricted properties⁴. More recently, with the progress of nanotechnologies,
74 new materials are being developed for applications as drug carriers^{5,6}. In addition to their higher
75 load, reduced toxicity, and increased drugs' bioavailability, these new nanomaterials can be
76 functionalized for a Trojan horse strategy to cross the BBB and specifically target cells in the
77 parenchyma^{5,6}. Among the different types of nanomaterials being evaluated, nanogels have
78 attracted considerable attention, mainly due to their colloidal properties and ability to tailor the
79 chemical structure to introduce stimuli-responsive properties⁷⁻¹⁵.

80

81 *In vitro* models are now developed for preclinical studies using human cells to predict brain
82 penetration of drugs¹⁶. Different settings of these models are available, from monolayers of brain
83 ECs to multiple cell systems¹⁶. Considering the importance of the NVU cells in the BBB induction
84 and maintenance and the coordinated response to the pathological environment, BBB *in vitro*
85 models need to consider all these protagonists to improve the relevance of the prediction^{2,17}.

86

87 The current method describes setting up a triple culture *in vitro* model of the human BBB, which
88 is fully developed with human cells to study specific cellular and human molecular mechanisms.

89 To be physiologically relevant, the model consists of the main three cellular actors of the BBB
90 (ECs, pericytes, and astrocytes) necessary to induce and maintain the BBB properties, without
91 the use of tightening compounds and displaying a set of properties required to be considered as
92 an *in vitro* BBB model^{16,18}. The model is set up in a configuration delimiting the blood and brain
93 compartment, suitable for preclinical studies of drug and particle transport to predict brain
94 penetration. The usefulness of the model is illustrated by measuring the transport of polymeric
95 nanogels.

96

97 **PROTOCOL:**

98 The protocol was approved by the French Ministry of Higher Education and Research (reference:
99 CODECOH DC2011-1321) and by the local investigational review board (Béthune Maternity
100 Hospital, Beuvry, France). For obtaining the endothelial cells (ECs), written and informed consent
101 from the donor's parents was obtained to collect umbilical cord blood, in compliance with the
102 French Legislation. The pericytes are provided by Professor Takashi Kanda (Department of
103 Neurology and Clinical Neuroscience, Yamaguchi University Graduate School of Medicine, Ube,
104 Japan) that were isolated as per Reference¹⁹. Primary human brain cortex astrocytes are
105 purchased from a commercial provider (see **Table of Materials**).

106

107 **1. Cell culture**

108

109 **1.1. Culturing of endothelial cells**

110

111 NOTE: Endothelial cells (ECs) are derived from CD34⁺ hematopoietic stem cells isolated from
112 human umbilical cord blood, according to the method described by Pedroso et al.¹⁸. The
113 endothelial phenotype of the ECs is described in Pedroso et al.²⁰.

114

115 1.1.1. Cultivate human ECs using endothelial cell basal medium (ECM) supplemented with 5%
116 of fetal calf serum (FCS), 0.5% of gentamicin, and 1% of endothelial cell growth supplement (see
117 **Table of Materials**).

118

119 1.1.2. For sub-culturing ECs, two days before the setting of the model, coat one dish with 10 mL
120 of 2% gelatin for 15 min at 37 °C and then replace it with 20 mL of warm ECM. Thaw one vial of
121 ECs containing 1 million cells (cells were manually counted as described in step 2.3.3 before cell
122 freezing) in the pre-coated cell culture dish.

123

124 1.1.3. After 3 h at 37 °C, renew the medium and maintain the cells until the setting of the
125 triculture in a humidified atmosphere inside an incubator at 37 °C under 5% CO₂ and 21% O₂.

126

127 **1.2. Culturing of the pericytes**

128

129 NOTE: The pericytes are isolated from the human brain according to the protocol published by
130 Shimizu et al.¹⁹, whose isolation procedure follows the method published by Kanda et al.²¹ with
131 modifications.

132

133 1.2.1. Cultivate the pericytes using Dulbecco's modified Eagle medium supplemented with 4.5
134 g/L of glucose (DMEM HG), 20% of FCS, 0.5% of gentamicin, and 1% of glutamine (see **Table of**
135 **Materials**).

136
137 1.2.2. For pericyte sub-culturing, five days before the setting of the model, coat two dishes with
138 8 mL/dish of 100 µg/mL of collagen type I solution in 0.02 N acetic acid for 1 h at room
139 temperature (RT) and then wash twice with RT DMEM HG. Thaw one vial of pericytes containing
140 1 million cells in a conical tube containing 10 mL of warm medium and centrifuge the suspension
141 for 5 min at 190 x *g* at 20 °C.

142
143 1.2.3. Resuspend the pellet in 10 mL of warm medium and seed in the pre-coated cell culture
144 dishes prefilled with 15 mL of warm medium/dish. The medium is renewed after 3 days, and the
145 cells are maintained until the setting of the triculture in a humidified incubator at 37 °C under 5%
146 CO₂ and 21% O₂.

147
148 1.3. Culturing of the astrocytes

149
150 1.3.1. Cultivate the astrocytes using a basal astrocyte medium supplemented (AM) with 10% of
151 FCS, 1% of astrocyte growth supplement (AM), and 1% of penicillin/streptomycin solution (see
152 **Table of Materials**).

153
154 1.3.2. For sub-culturing the astrocytes, one week before the setting of the model, coat one T75
155 cell culture flask with 10 mL of 2 µg/cm² poly-L-lysine (PLL) for 1 h at 37 °C and wash twice with
156 RT sterile water. Thaw one vial of astrocytes containing 1 million cells in 20 mL of warm medium
157 and seed in the pre-coated T75 cell culture flask.

158
159 NOTE: The commercially obtained cell vials confirm the presence of ~1 million cells, so the
160 counting of cells was not performed here.

161
162 1.3.3. Maintain the cells in a humidified incubator at 37 °C under 5% CO₂ and 21% O₂. The
163 medium is renewed after 24 h and then every 2 days until the setting of the triculture.

164

165 **2. Triple culture model setting**

166
167 NOTE: The assembly of the three cell types is performed on the same day. The day before the
168 setting of the triculture, perform the collagen type I coating on the reverted insert filters (see
169 **Table of Materials**) and seed the astrocytes in the PLL-precoated wells of a 12-well plate.

170

171 **2.1. Seeding of astrocytes in the wells**

172

173 **2.1.1. Coat the wells with 500 µL of 2 µg/cm² PLL solution as described in step 1.3.2.**

174

175 **2.1.2. Wash the cells once with 10 mL of warm phosphate buffer saline - calcium and**
176 **magnesium-free 1x (1x PBS-CMF) (Table 1) before incubating for 3 min at 37 °C with 10 mL of**

177 warm 20% trypsin/EDTA (T/E) solution and mechanically detach the cells from the flask. Transfer
178 the suspension to a conical tube containing 5 mL of warm non-diluted FCS.

179

180 NOTE: According to the provider's protocol²², the collection of astrocytes can be optimized by
181 placing the flask in the incubator for 1 min and tapping the flask to help complete the
182 detachment. The remaining cells should be collected with 5 mL of T/E neutralization solution and
183 placed in the FCS-containing conical tube.

184

185 2.1.3. Centrifuge the suspension for 5 min at 20 °C at 190 x *g*.

186

187 2.1.4. Resuspend the cell pellet in 5 mL of warm AM. Count the cells by diluting 20 µL of the cell
188 suspension in 80 µL of 1x PBS-CMF using a manual counting chamber under a microscope (see
189 **Table of Materials**). Plate around 40,000 cells/cm² in each PLL-precoated well in a volume of 1.5
190 mL of warm AM.

191

192 2.2. Seeding of pericytes on the reverted insert filters

193

194 2.2.1. Add 250 µL of collagen type I solution (100 µg/mL) on the reverted insert filters, placed
195 at the periphery of a covered 25 mm high dish (see **Table of Materials**) using sterile tweezers.
196 Leave the coating for 1 h at RT under sterile conditions.

197

198 NOTE: The used dish needs to be high enough to ensure the maintenance of sterility when
199 outside the hood and avoid contact between the solutions on the reverted filter and the cover of
200 the dish.

201

202 2.2.2. Remove carefully the collagen type I solution with a glass pipette connected to an
203 aspirating system. Wash twice with 250 µL of RT DMEM HG and then carefully remove all the
204 solution from the insert filters. Leave the coated insert filters at RT under sterile conditions until
205 the seeding of the cells.

206

207 NOTE: During the coating procedure, be careful not to touch the filter to avoid membrane
208 damage. Once coated with collagen type I, the insert filters can be stored overnight at RT.

209

210 2.2.3. On the day of the triculture setting, wash the pericytes twice with 10 mL of warm 1x PBS-
211 CMF and incubate the cells with 2 mL of warm trypsin. Monitor the action of the trypsin by
212 observing the cells under the microscope. Once the cells start to detach, remove the trypsin and
213 add 5 mL of warm ECM before mechanical dissociation.

214

215 2.2.4. Count the cells by diluting 20 µL of the cell suspension in 80 µL of 1x PBS-CMF using a
216 manual counting chamber under a microscope, and seed 44,500 cells/cm² on the pre-coated
217 reverted insert filters in a volume of 250 µL. Keep the insert filters in a humidified incubator at
218 37 °C for 3 h under 5% CO₂ and 21% O₂.

219

220 2.2.5. Carefully revert the insert filters using sterile tweezers in a 12-well plate containing 1.5

221 mL of warm ECM/well. The insert filters are now ready to be coated on the other side.

222

223 2.3. Seeding of endothelial cells on the insert filters

224

225 2.3.1. Coat the upper side of the insert filters with 500 μ L of extracellular matrix-based hydrogel
226 (1/48 v/v) (see **Table of Materials**). After 1 h, in a humidified incubator at 37 °C under 5% CO₂
227 and 21% O₂, wash once with 500 μ L of RT DMEM HG.

228

229 2.3.2. Wash once with 10 mL of warm 1x PBS-CMF and incubate the cells with 2 mL of warm
230 trypsin. Once the cells start to detach, remove the trypsin and add 5 mL of warm ECM before
231 mechanical dissociation.

232

233 2.3.3. Count the cells by diluting 20 μ L of the cell suspension in 80 μ L of 1x PBS-CMF using a
234 manual counting chamber under microscope and seed the ECs at a density of 71,500 cells/cm²
235 on the pre-coated insert filters in a volume of 500 μ L of warm ECM.

236

237 2.3.4. Replace AM with 1.5 mL of warm ECM/well and then transfer the seeded insert filters
238 (ECs + pericytes) upon the wells containing the astrocytes.

239

240 2.3.5. Place the triculture cell systems in a humidified incubator at 37 °C under 5% CO₂ and 21%
241 O₂.

242

243 2.4. Maintenance of the triple cell culture for the induction of the BBB properties

244

245 NOTE: For the induction of the BBB properties in the ECs, 6 days of triple culture are necessary.

246

247 2.4.1. Renew the medium every other day until day 6, taking off carefully the medium from the
248 upper and bottom compartment using a glass pipette connected with an aspiration system.

249

250 2.4.2. Quickly replace with warm ECM in a volume of 500 μ L in the upper compartment and 1.5
251 mL in the bottom compartment, and put back the cells in a humidified incubator at 37 °C under
252 5% CO₂ and 21% O₂.

253

254 3. BBB phenotype validation

255

256 NOTE: After 6 days of triple culture, the time necessary to induce the BBB phenotype in the ECs,
257 the human BBB model is ready for experiments. The physical integrity of the brain-like
258 endothelial cells (BLECs) is visualized by immunofluorescence staining of TJ proteins evaluated
259 using permeability assay to BBB integrity markers. The BBB phenotype validation also includes
260 genes/proteins expression analysis and efflux pumps functionality according to the procedure
261 described in Deligne et al.,²³. Pericytes and astrocytes are visualized by respective staining
262 markers according to the procedure described in Deligne *et al.* 2020²³.

263

264 3.1. Immunofluorescence staining

265
266 3.1.1. Fix the insert filters and astrocytes in ice-cold methanol/acetone (50/50 v/v) for 1 min and
267 wash twice with RT 1x PBS-CMF.

268
269 3.1.2. Carefully separate the filter from the insert by cutting the membrane using a scalpel.
270 Perform immunocytochemistry on the membrane and bottom wells according to Deligne et al.²³.

271
272 NOTE: For the blocking step, use 250 µL of SEA BLOCK Blocking buffer (see **Table of Materials**)
273 for 30 min at RT.

274
275 3.2. BBB integrity assay

276
277 3.2.1. Assess the physical integrity of the BLECs by a permeability assay using BBB integrity
278 markers with different molecular weights, like Sodium fluorescein (NaF) and 20 kDa Dextran
279 (FD20) (see **Table of Materials**).

280
281 NOTE: The experiment can be performed according to the procedure described in Deligne et al.²³.

282
283 3.3. Efflux pump functionality

284
285 3.3.1. Assess the functionality of P-glycoprotein (P-gp) and breast cancer resistance protein
286 (BCRP) by measuring the intracellular accumulation of Rhodamine 123 (R123) with and without
287 Elacridar, a P-gp and BCRP inhibitor (see **Table of Materials**).

288
289 NOTE: The experiment can be performed according to the procedure described in Deligne et al.²³.

290
291 3.4. Gene and protein expressions

292
293 3.4.1. Perform gene and protein sample collection on ice after a quick wash with cold Ringer
294 HEPES (RH) (**Table 1**) of the cells. Before EC sample collection, scrape off the pericytes from the
295 inverted insert filters²⁰.

296
297 **4. Nanogel transport**

298
299 NOTE: To estimate the passage of polymeric nanogels (NGs) from the luminal to the abluminal
300 compartment of the triculture BLEC model, 0.1 mg/mL of NG solution was added at day 6 on the
301 luminal compartment for 24 h. Studied NGs were fluorescently tagged N-Isopropylacrylamide
302 (NIPAM)-based hydrogels with an average size of 8-10 nm (see **Table of Materials**).

303
304 4.1. Weigh the nanogel powder and solubilize it in ECM at a concentration of 1 mg/mL.
305 Sonicate the solution for 10 min and filter using a 0.2 µm PTFE filter.

306
307 NOTE: Prepare a fresh NG solution the day of the experiment.

308

309 4.2. Change the medium in the luminal compartment and add 50 μL of NGs solution in the
310 upper compartment for a final concentration of 0.1 mg/mL.

311
312 NOTE: Perform a dilution of 1:10 from the original solution.

313
314 4.3. After 24 h of incubation, collect aliquots from luminal (20 μL) and abluminal (200 μL)
315 compartments and place them in a black 96-well plate.

316
317 4.4. Quantify the fluorescence using a fluorescent multiplate reader (see **Table of Materials**)
318 with a black 96-well plate using the setting of excitation/emission wavelengths at 477/540 nm.
319 Calculate the percentage of crossing referred to the initial working solution added at time = 0 h
320 (t_0)^{6,15}.

321
322 NOTE: To prepare the 96-well plate for the fluorescence measurement, add 200 μL of solution
323 from the abluminal compartment and 20 μL of solution from the luminal compartment and t_0
324 preparation (add 180 μL of ECM to reach a final volume of 200 μL). Include also a calibration
325 curve and a blank to the reading plate. Instrumental parameters: Detection method -
326 Fluorescence, Optical Position - Top, Read Type - Endpoint, Excitation wavelength - 477 nm,
327 Emission wavelength - 540 nm, Sensitivity - 100, Shake - Double Orbital for 5 s.

328
329 **REPRESENTATIVE RESULTS:**

330
331 **Setting of the human triple culture BBB model**

332 The protocol required for the setting of the human BBB *in vitro* model is described in **Figure 1**
333 and includes successive steps whose order must be strictly respected. First, the three cell types
334 are cultivated individually in cell culture dishes (**Figure 1A**) before being assembled in an insert
335 filter system. The triple culture setting begins with seeding the first cell type, astrocytes, in the
336 pre-coated bottom well. The following day, pericytes and ECs are seeded on the insert filter's
337 pre-coated abluminal and luminal surfaces, respectively. The insert filter is then transferred over
338 the astrocytes. The model is maintained in culture for 6 days, the time necessary to induce the
339 BBB properties in ECs, with a renewal of medium every other day according to the patented co-
340 culture model²⁴. The ECs are then renamed as BLECs (**Figure 1B**).

341
342 **Characterization of the human BBB model**

343 The triple cell culture model has been characterized for the presence of a set of BBB-specific
344 properties. First of all, immunocytochemistry data confirmed the expression of conventional
345 markers such as platelet-derived growth factor receptor β (PDGFR- β)^{25,26} and desmin for
346 pericytes and glial fibrillary acidic protein (GFAP)²⁶ for astrocytes (**Figure 2A**). Hence, after the 6
347 days of culture with the pericytes and astrocytes, the monolayer of BLECs, visualized with the
348 adherent junction staining of VE-Cadherin, displays a continuous localization of TJ proteins,
349 Claudin-5 and ZO-1, at the cell borders (**Figure 2A**). The setting-up of the TJs is correlated with
350 low paracellular permeability coefficients measured using BBB integrity markers of low molecular
351 weight, i.e., NaF (376 Da)^{16,27} and high molecular weight, i.e., FD20 (20 kDa)²⁷, as shown in **Figure**
352 **2B**. The values measured are comparable with validated BBB *in vitro* models using the exact

353 source of ECs^{23,24,28}. Altogether, these results highlight the low paracellular permeability of the
354 triple culture BLEC monolayer, which is characteristic of the *in vivo* BBB. Additionally, R123
355 intracellular accumulation in BLECs exhibited a significant increase in the efflux pump inhibitor
356 Elacridar^{23,24} compared to the control condition with its absence (**Figure 2C**). This indicates the
357 presence of active efflux pump molecules, namely P-gp and BCRP, in the BLECs.

358
359 To further characterize the BLECs, gene expression and protein level of key BBB features were
360 studied (**Figure 3**). The data obtained with the triple culture model were compared with the
361 validated and patented co-culture model consisting of ECs and pericytes²⁴ used as a control
362 model. The astrocytes represent the third cell type added in the initial co-culture model in the
363 triple culture model. Hence, the gene expression analysis (**Figure 3A**) of triple culture BLECs,
364 compared with co-culture BLECs, showed the maintenance of expression of key BBB features
365 such as TJ proteins (claudin-5 and zonula occludens-1) and efflux pumps (P-gp and BCRP), and
366 the upregulation of most studied BBB transporters (glucose transporter 1) and receptors
367 (transferrin receptor). Protein quantification data (**Figure 3B**) were found to be in line with the
368 transcriptional results. Overall, these data support the positive induction of BBB properties in the
369 triple culture BLEC layer similar to the validated co-culture model. Altogether, the triple culture
370 model displays the required physical and metabolic properties for an *in vitro* microphysiological
371 system to model the BBB.

372
373 **Applicability to drug delivery strategies - measurement of nanogel transport**
374 To assess the possibility of using the triple culture model to study new brain delivery strategies,
375 the transport of fluorescently-tagged NIPAM-based neutral NGs was evaluated^{6,15}. At time 0, NGs
376 were placed in the luminal compartment at a concentration of 0.1 mg/mL (**Figure 4A**). After 24 h
377 of incubation, 5.82% of the NGs were found in the abluminal compartment (**Figure 4B**), proving
378 their ability to cross the BLECs.

379
380 The results demonstrate the suitability of the model to measure the permeability of small and
381 larger compounds, as described with the integrity markers, and evaluate the transport of
382 nanomaterials such as polymeric NGs.

383
384 **FIGURE AND TABLE LEGENDS:**

385
386 **Figure 1: Representation of critical steps for the setting of the triple culture *in vitro* model of**
387 **the human BBB. (A)** Phase-contrast images of the three cell components of the BBB model:
388 endothelial cells (EC), pericytes (PC), and astrocytes (AC). Scale bar = 250 μ m. **(B)** Schematic and
389 illustrative timeline for the setting of the triple culture human BBB *in vitro* model. The highlighted
390 box represents the coating procedure for the inverted insert filter.

391
392 **Figure 2: Assessment of the properties of the triple culture BBB model. (A)** Representative
393 immunostaining images of the distinctive markers for BLECs (Claudin-5: CLD5, Zona Occludens-1:
394 ZO1 and VE-Cadherin: Ve-Cadh), pericytes (Platelet-Derived Growth Factor Receptor- β : PDGFR- β
395 and desmin), and astrocytes (Glial Fibrillary Acidic Protein: GFAP). Scale bar = 10 μ m. **(B)**
396 Paracellular permeability of BLECs to fluorescent BBB integrity markers, Sodium Fluorescein (NaF,

397 376 Da, Pe: 0.61 ± 0.062) and FITC-Dextran (FD20, 20 kDa, Pe: 0.04 ± 0.005). N = 3; n = 9. Mean \pm
398 SEM. (C) P-gp and BCRP functionality in ECs was assessed by quantifying intracellular R123 with
399 ($124.2\% \pm 3.39\%$) and without ($100\% \pm 8.79\%$) Elacridar. N = 4; n = 12. Mean \pm SEM. $p = 0.017$
400 using an unpaired t-test.

401

402 **Figure 3: Evaluation of BLEC gene expression and protein level of distinctive markers in the**
403 **triple culture model compared with the co-culture BBB model. (A)** Gene expression of tight
404 junction proteins (Claudin-5, CLD5, and Zona Occludens-1, ZO1), transporters (Glucose
405 Transporter, GLUT1, P-glycoprotein, PGP, and Breast Cancer Resistance Protein, BCRP), and large
406 molecule-receptors (Transferrin Receptor, TRFR), normalized by the expression of RPLP0. N = 3;
407 n = 9. (B) Protein level of tight junction proteins (CLD5 and ZO1), transporters (GLUT1, PGP, and
408 BCRP), and large molecule-receptors (TRFR), normalized by the expression of β -actin. N = 3; n =
409 9. Mean \pm SEM. For (A) and (B), values >1 correspond to higher gene expression or protein levels
410 in the triple culture model. The red line corresponds to a value of 1 where the expression level
411 (genes or proteins) of the two models is equivalent.

412

413 **Figure 4: Measuring nanogel transport in the triple culture model. (A)** Schematic representation
414 of the nanogel transport assay. (B) Percentage of nanogel transport after 24 h of incubation in
415 the triple culture model ($5.82\% \pm 0.09\%$). N = 2; n = 6. Mean \pm SEM.

416

417 **Table 1: Composition of the different buffers used in the protocol.**

418

419 **DISCUSSION:**

420 Treatment of brain diseases remains a challenge considering the difficulty of the drugs to hurdle
421 over the BBB to reach their cellular and molecular targets in the brain parenchyma.

422

423 Drug development for brain diseases currently exhibits a low success rate since most drugs
424 displaying promising results in preclinical models failed to show any benefit when used in the
425 clinic. Following the "3R rule," which aims at reducing the number of animals used for
426 experimentation, *in vitro* models of the BBB are developed to study brain pathologies and to
427 predict brain penetration of drugs²⁹. *In vitro* models of BBB have mainly been developed using
428 animal cells and have become more sophisticated to improve the relevance of the results
429 obtained¹⁶. One of the significant advances in the use of human cells, which brings undeniable
430 new insight and more specificity, at the cellular and molecular levels, to study human disease
431 mechanisms¹⁶. However, the development of relevant models requires considering the
432 improvement of the BBB *in vitro* model settings and the knowledge arising, thanks to animal
433 models. Hence, it needs to consider the complexity of the BBB architecture and the importance
434 of the cell-cell communications to study the BBB under physiological and pathological
435 conditions³⁰.

436

437 The protocol presented here describes a method to set up a full human BBB *in vitro* model
438 comprising the three main cell types of the BBB, without limitation of access to brain tissue. As a
439 multiple cell system, the induction and the maintenance of BBB properties, without the artificial
440 use of tightening compounds, but instead induced by cell-cell communications is more

441 physiologically relevant and in line with the *in vivo* induction of the BBB properties³¹. Hence, the
442 respect of the chronology of the protocol is prime of importance for the success of the protocol.
443 Moreover, the incubation times during the setting of the triple culture and once the three cell
444 types are assembled represent the main critical steps of the protocol.

445
446 The BBB properties in ECs are induced by the co-culture with pericytes, as described for the co-
447 culture model²⁴. Hence, the culture of pericytes at the reverse side of the insert filter is the most
448 critical point and requires strictly following the protocol at the risk of not having enough pericytes
449 for the induction of the BBB properties. First of all, during the coating procedure and also cell
450 seeding, attention has to be put not to have the cover of the Petri dish in contact with the coating
451 and also the medium once the cells are seeded to ensure a good coating of the filter and not to
452 lose cells (steps 2.2.1 and 2.2.4). Moreover, once the pericytes are seeded, it is essential to wait
453 the indicated time for the attachment of the pericytes (step 2.2.4) before reverting the insert
454 filter for the coating and seeding of ECs on the other side (steps 2.2.5 and 2.3). Once seeded, six
455 days are required to induce the BBB properties through cell-cell communications (step 2.4).

456
457 The model is validated in terms of restricted permeability (associated with the setting of the tight
458 junctions) since the ECs of the triple culture model display permeability values to BBB integrity
459 markers similar to the validated co-culture model and also measured in validated animal or
460 human models^{16,27,32}. Moreover, the validation of an *in vitro* BBB model requires, in addition to
461 the restricted permeability, the responsiveness to other cell types of the NVU and the expression
462 of functional receptors and transporters¹⁶. In addition, the model is reproducible and produces
463 multiple insert filters and wells to perform numerous analyses (gene and protein expression,
464 fluorescent staining, toxicity tests) on each cell type separately without requiring a cell sorting
465 method.

466
467 The model was developed using a 0.4 μm pore size filter to have one cell type on each side of the
468 insert filter. The insert filter system allowed the study of cell-cell communications in physiological
469 conditions by transferring it upon well-containing astrocytes. The presence of astrocytes in the
470 system represents a plus value compared to the initial co-culture *in vitro* model²⁴. Indeed,
471 considering the importance of astrocytes in the physiology of the BBB, this third cell type allows
472 further understanding of the cell-cell communications within the BBB. Moreover, the triple cell
473 culture system can also be studied in pathological conditions such as stroke, in which the
474 astrocytes play an essential role³³⁻³⁵. In addition, the design of BLECs/ pericytes on both sides of
475 the insert filter can easily be placed upon other cell types to mimic pathological conditions such
476 as brain cancer²³.

477
478 The pore size of the insert filter can bring limitations with some experiments, such as cell
479 transmigration across the BBB. However, the development of the model with a larger pore size
480 requires the adaptation of the protocol to ensure the formation of a physiological monolayer of
481 ECs and not multiple layers, which is not physiologically relevant to mimic the BBB³⁶.

482
483 The model's applicability has been demonstrated using NGs transport experiment exhibiting the
484 possibility to do transport experiment using a multicellular system. Nevertheless, one should be

485 aware of the difficulties in having a control compound or molecule for the transport experiment,
486 sharing comparable properties with NGs since each nanostructure exhibits a unique set of
487 properties (molecular weight, charge, shape, physical properties, protein corona formation).
488

489 One limitation of the model is the absence of shear stress, which was demonstrated to influence
490 the differentiation of ECs and the expression of TJ proteins³⁷. However, developing a fluidic
491 system mimicking the brain capillary is challenging considering the complexity of adding a fluidic
492 part, requiring a specific device, in a multiple cell system. Moreover, the particular device is
493 usually not commercially available and does not allow many replicates, thus making fluidic
494 systems less adapted for high-throughput use.

495
496 In summary, this triple culture system consisting of human cells reproduces *in vitro* the
497 architecture of the BBB. It allows the generation of many inserts that can be used for extensive
498 screening of compounds.

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509 The authors declare that they have no conflict of interest.

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