Supplementary Material

Drug Transport across the Human Placenta: Review of Placenta-on-a-Chip and Previous Approaches

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Contents

S.1. Fabrication of microfluidic devices

Miura et al. [1] constructed their microfluidic device through standard photolithographic methods. The microfluidic channels were prepared through the mixing of polydimethylsiloxane (PDMS) and a crosslinking agent, using an SU-8 mold. Once the channels were thermally cured, a biopsy punch was used to reveal the center of the maternal channel. Next, the vitrified collagen (VC) membrane was assembled with fetal and maternal PDMS channels. Next, both PDMS layers were O_2 plasma treated. Afterward, the microfluidic device was incubated for 90 min at 75^0C to promote adequate bonding. On a side note, the stamping of the two PDMS slabs, corresponds to the intervillous space and fetal capillary compartment; as a result, there is independent flow between the lower and upper channels.[2] [3] The PDMS is a crucial component of the microfluidic device due to the PDMS's ability to control mass transport across the system's semiporous membrane.[4] After the chip was successfully fabricated, cell culturing and seeding within the microfluidic device was performed.[1]

Lee et al. [2] manufactured their placenta-on-a-chip's microfluidic device by bonding together two PDMS slabs with a VC membrane. This process began with the fabrication of the PDMS microchannels. Their PDMS microchannels were produced using a soft-lithography technique similarly with *Miura et al*. This technique involves the spin-coating a negative photoresist on a clean silicon wafer, and then baked through two stages concurrently: 65^0C for 7 min and 95^0C for 40 min. Next, a mask film containing the desired microchannel patterns was placed over the photoresist layer, and then exposed to ultraviolet (UV) light. Afterward, the wafer was baked again at 65^0C for 5 min and 95^0C for 18 min. Subsequently, the desired mold containing the microchannel photoresist was rinsed with a developer solution and isopropyl alcohol. Following the production of the SU-8 mold, the fabrication of the PDMS channels was

carried out. The following process began with the mixing (10:1) ratio of a curing agent with a PDMS prepolymer, respectively. Next, the PDMS mixture was poured on the previously fabricated channel mold and placed in a vacuum chamber before incubated at 65^0C for 4 hr. Once PDMS mixture finished curing, the solid model was removed from the mold and cut to the desired form. The final dimensions of the cross-sectional area of the microchannels were 500 μm × 200 μm concerning width and height. After the process was finished, *Lee et al.* repeated the procedure for the upper PDMS slabs.

Once the channels were constructed, the creation of the VC membrane was carried out. Collagen type I was mixed with distilled water and Dulbecco's Modified Eagle Medium (10X) (DMEM) at a final concentration of 2.43 mg ml^{-1}. Subsequently, using Sodium Hydroxide, the pH level was adjusted to 7.4. Succeeding after pH level adjustment, the gel solution was introduced to the lower microchannel and incubated at 37^0C for 40 min. For final constructive measures, to produce the VC membrane, the collagen gel was left to dry overnight. After the formation of the VC membrane, both upper and lower PDMS layers were treated with a plasma cleaner and bonded together. Finally, the incubation of the bonded channels was carried out under the following conditions, 65^0C for 30 min.[2]

Blundell et al. [5] chip fabrication process shared similar methods with *Miura et al*. and *Lee et al*. For example, *Blundell et al*. microfluidic device's upper and lower layers were fabricated through standard soft lithography techniques. The group began their process by mixing a PDMS base with a curing agent at a weight ratio of 10:1, and then placed on an SU-8 mold that contained microchannel features. The dimensions of the microchannel were 1 mm (width) × 1.5 cm (length) × 135 μm (height). Next, to secure fluidic access for both the lower and upper microchannels, 1 mm diameter holes were created from a biopsy punch. After the two

PDMS slabs were fabricated, *Blundell et al*. bonded the two PDMS layers to a semipermeable polycarbonate membrane with an adhesive PDMS mortar. This mortar film layer was created by mixing a PDMS cursor with a curing agent at a 10:3 weight ratio. Next, at 2500 rpm the mixture was spin-coated on a 100 mm Petri dish for 5 min. Subsequently to transfer the mortar film to the two PDMS slabs, both PDMS layers were placed on the petri dish. Following the movement of mortar film onto the two PDMS surfaces, a semipermeable polycarbonate membrane was bonded to the upper PDMS slab. Afterward, the two layers were attached to the lower slab and then cured at room temperature overnight. The following microfluidic fabrication was carried out similarly for *Blundell et al.* [6] study for examining glyburide and heparin transport across their placenta-on-a-chip.

Zhu et al. [7] carried out the fabrication process of their microfluidic device in a similar manner to *Miura et al*., *Lee et al.* and *Blundell et al.*, using standard soft lithography techniques. A PDMS monomer was mixed with a curing agent at a weight ratio of 10:1. The mixture was then prepared for the creation of the PDMS microchannels, which included the casting of the PDMS mixture on the molds made of SU-8. The microchannels were constraint through the following dimensions: 1.5 mm (width) × 1.5 cm (length) × 400 μm (height). Next, through electrostatic interaction, a transparent semipermeable membrane adhered to the bottom of the upper PDMS layer. Subsequently, the PDMS mixture (PDMS base and curing agent at 50:1 (w/w)) was spread evenly on the transparent semipermeable membrane. Once the PDMS mixture cured on the upper surface, the upper and lower slabs were bonded together following a plasma treatment. Then, the microfluidic device was cured at 80^0C for 30 min to ensure adequate bonding. In another study, *Yin et al.* [8] used the same methodology for fabricating their placenta-on-a-chip. However, their microfluidic device was composed of two PDMS layers that

contained three microchannel patterns on the upper layer. The two cell microchannel and center matrix channel dimensions were 350 μm (width) × 2 mm (length) × 200 μm (height) and 300 μm (width) × 2 mm (length) × 50 μm (height), respectively.[8]

Pemathilaka et al. [9] fabricated their microfluidic device similarly with the previously elaborated studies, using standard soft lithography techniques. The fabrication process began by creating an SU-8 mold for the chip. The wafer mold was placed in a petri dish, and then a PDMS base and curing agent solution were introduced into the mold at a 10:1 weight mixture. After the PDMS solidified in room temperature, the PDMS was removed from the mold and separated into their upper and lower layers. Next, a biopsy punch was used to create the inlet and outlets holes for the PDMS channels. Subsequently, to represent the barrier between the fetal and maternal interfaces, a 0.4-micron pore-sized polyester track etched (PETE) membrane was placed over the mid-section of the lower channel. Afterward, both PDMS layers were treated with plasma for 1 min and then aligned and attached seamlessly. Once the two PDMS slabs and PETE membrane were assembled, the microfluidic device was left to cure overnight. Following the fabrication of *Pemathilaka et al*. microfluidic device, cell culturing and seeding in the placenta-on-a-chip was executed.

S.2. Cell culturing

Miura et al. [1] cultured human trophoblast cells (BeWo) at 37^0C in a humidified incubator with 5% CO_2. The BeWo cells were cultured in Ham's F12 Nutrient Mixture, containing 10% fetal bovine serum (FBS) and 50 μg ml^{-1} kanamycin sulfate. Human villous trophoblasts cells were cultured and prepared for experiments at passage 3.

Lee et al. [2] cultured human trophoblast cells (JEG-3) and green fluorescent protein (GFP)-stained human umbilical vein endothelial cells (HUVECs) for their placenta-on-a-chip. JEG-3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with FBS, and 1% penicillin/streptomycin. HUVECs were cultured and maintained in Endothelial Cell Growth Basal Medium-2 (EBM^{TM}-2), accompanied by Endothelial Cell Growth Medium-2 (EGM^{TM}-2) MV Bullet Kit. Both cell cultures were conducted in their flasks and maintained at 37^0C in a humidified incubator under 5% CO_2 in air.

Blundell et al. [5] culturing process included human trophoblast cell line BeWo b30 and human primary placental villous endothelial cells (HPVECs). The trophoblast cells were cultured and maintained in DMEM/F-12K medium, contained 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. HPVECs were maintained and grown in EGM^{TM}-2, supplemented with 2% FBS. Both culturing processes took place in flasks and were kept at 37^0C in a humidified incubator with 5% CO_2. The same cells and cultivation procedures were used in *Blundell et al.* [6] experiment.

Zhu et al. [7] study included the cell culturing of human BeWo, HUVEC and THP-1 cells. The BeWo cells were cultured in DMEM/F-12K medium, supplemented with 20 % FBS, 1% L-glutamine, and 1% penicillin/streptomycin. The HUVECs were cultured on a Collagen I-coated plate in ECM medium containing 5% FBS, while the THP-1 cells were cultured in RPMI 1640 containing 1% penicillin/streptomycin, 10% FBS. And 1% L-glutamine. Briefly, THP-1 is a human monocytic cell line that originates from a monocytic leukemia patient. In this case, these cells are included in this study because the differentiation of THP-1 cells into macrophages (white blood cells) is essential for examining the signals between trophoblast and maternal white blood cells when confronted with bacterial infections. Nevertheless, all cells were cultured and

maintained at 37^0C in a humidified atmosphere of 5% CO_2 in the air. Likewise, with *Yin et al.* [8] chip fabrication, cell culturing was done similarly with *Zhu et al.*, with the absence of TH-1 cells.

Pemathilaka et al. [9] used human BeWo cells and HUVECs to replicate their microengineered placental barrier. The BeWo cells were cultured in F-12K medium, containing 10 % FBS. The HUVECs were cultured in an endothelial basal medium (EBM), supplemented with FBS. Till cells were 80-90% confluent, both cell lines were kept in incubation at 37 ^{0}C with 5% CO_2 in air.

S.3. Cell seeding

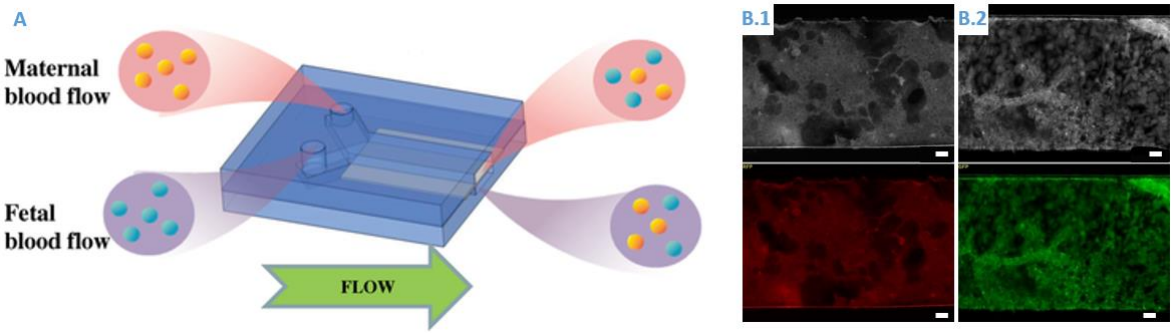

Figure S1. Cell seeding in *Pemathilaka et al.* microchip: (A) Placenta-on-a-chip schematic shows maternal and fetal bloodstreams perfused through the channels. Two PDMS layers are separated by a porous membrane and a channel on each side. (B.1-B.2) BeWo cells imaged for Red Fluorescent Protein and HUVECs imaged for Green Fluorescent Protein in grayscale and color imaging. [9] Scale bars, 50 µm. Image reproduced from Pemathilaka et al (2019) / CC BY

After *Miura et al*. [1] fabricated their microfluidic device, and finished cell culturing, cell seeding in the microfluidic device was carried out. Cell seeding began with the rehydration of the

VC membrane by filling the microchannels with culture medium. Next, 20 μl of BeWo cell suspension (3×10^4 cells) were added to the maternal compartment. Once the BeWo cells were added, the maternal chamber was sealed with a thin PDMS membrane, and then incubated at an inverted position in a CO_2 incubator. When microfluidic device finished culturing for 1-2 hr, a peristaltic pump was connected to the channel to control the flow rate.

Following the fabrication and design of *Lee et al.* [2] microfluidic device and cell culture, their chip underwent cell seeding. Initially, the chip was sterilized by UV. Then, the vitrified membrane was coated with 40 μg ml^{-1} fibronectin and 1.5% gelatin. Next, to bond cells to the membrane's lower surface, JEG-3 cells suspended in growth medium at 5×10^6 cells ml^{-1} were added to the lower channel with a 1 ml syringe and incubated at an inverted position. After the JEG-3 cells were attached to the membrane in the lower channel, HUVECs were injected in the upper channel and incubated at an inverted position for 2 hr to adhere to the top side of the VC membrane. Finally, the microfluidic device underwent cell seeding, the lower and upper channels were perfused with their respective media at a constant flow rate of 30 μl h^{-1}.

After *Blundell et al.* [5] fabricated their microfluidic device and performed cell cultivation, microfluidic cell seeding was carried out. This process began when the microfluidic device was sterilized with UV irradiation. Following UV irradiation, human fibronectin solution (0.1 mg ml^{-1} in Phosphate-buffered saline (PBS)) was introduced into the chip's microchannels and incubated at 37^0C for 4 hr to prepare for cell seeding. Next, HPVECs (4×10^6 cells ml^{-1}) were injected into the lower microchannel and incubated for 1 hr at 37^0C. Similarly, BeWo cells were introduced into the upper microchannel at a concentration of 4×10^6 cells ml^{-1} in DMEM/F-12K. The microfluidic device was then incubated at an inverted position for 1 hr at 37^0C to promote cell attachment. Subsequently, a syringe pump was attached to the two inlets, in which

continuous perfusion at a flow rate of 100 μl h^{-1} was pumped through the upper and lower microchannels. The constant flow rate of 100 μl h^{-1} mimics the continual diffusion of fluids and minerals across the placenta between the mother and fetus. Similar cell seeding process was used in place for *Blundell et al.* [6] study.

Zhu et al. [7] cell seeding procedure began with the sterilization of their fabricated microfluidic device with UV irradiation, and then coating the microchannels with Collagen I (0.1 mg ml^{-1} in ddH_2O). Initially, at 2×10^6 cells ml^{-1}, the harvested HUVECs were resuspended in ECM medium. Subsequently, the cells were introduced into the lower microchannels and incubated at 37^0C for 1 hr. After the HUVECs were adhered, the harvested BeWo cells were resuspended in DMEM/F12 medium at a density of 4×10^6 cells ml^{-1} and seeded in the upper microchannels. Thereafter, the device was incubated at 37^0C for 2 hr at an inverted position. Following 2 hr of incubation, a 1:1 mixture of ECM and DMEM/F-12K medium was prepared and used in replace of the current medium in the microsystem. After the first day, a syringe pump injected ECM medium and DMEM/F-12k medium to the lower and upper channels, respectively. The volumetric flow rate for the perfusion of the channels respective mediums was 10 μl h^{-1}. Then, differentiated THP-1 cells were resuspended in RPMI 1640 medium at a density of 1×10^5 cells ml^{-1} and then perfused them into the upper microchannel for 30 min at 40 μl h^{-1}. The same cell seeding process was done similarly by *Yin et al*. [8], with the absence of THP-1 cell perfusion.

Pemathilaka et al. [9] cell seeding process began by inserting PEEK tubes to the inlets and outlets of the microfluidic device (**Figure S1**). Then, the microchip was UV-sterilized for 20 min. Following UV-sterilization, an Entactin collagen IV-laminin (E-C-L) solution was prepared. The solution was processed from a diluted solution of E-C-L with a sterile serum-free

medium until a 10 mg ml^{-1} concentration was reached. Afterward, both sides of the membrane were coated with the E-C-L solution, and then refrigerated at 4^0C overnight. The following day, the excess E-C-L from the chip was washed away with PBS, and then prepared for infusion. Both the BeWo cells and HUVECs densities were adjusted to 5×10^6 cells ml^{-1}. Next, the HUVECs were resuspended with EGM medium and then seeded into the lower channel. Subsequently, the chip was incubated at an inverted position at 37^0C with 5% CO_2 for 1 hr. The same process was carried out for the BeWo cells, but were resuspended in F-12K medium and were introduced into the upper channel. After the microfluidic device was incubated, 3 ml syringes were attached to the channels and perfused their respective mediums at a constant volumetric flow rate of 50 μl hr^{-1}.

S.4. Verification of Placental Barrier

Miura et al. [1] began the verification of their placental barrier by analyzing the RNA interference in their cultured and seeded BeWo cells to determine the presence of receptor potential, TRPV2. Next, immunofluorescence was carried out to acquire images from the BeWo cells and own channel tissue. Afterward, reverse transcription polymerase chain reaction (RT-PCR) and TaqMan gene expression were performed. Following RT-PCR and TaqMan gene expression evaluations, *Miura et al*. perfused 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) a-mino]-2-deoxy-D-glucose（2-NBDG) across the fetal channel to analyze for glucose uptake. Shortly after, they scanned the maternal chamber with electron microscopy. After electron microscopy was performed, *Miura et al*. measured the length of their microengineered microvilli and conducted Ca^{2+} imaging. Finally, they examined immunoblotting in their microfluidic device.

Through the following evaluations, they discovered that FSS is an extracellular sign that promotes microvilli formation influx; intracellular Ca^{2+} increased levels play a crucial role in the development of the microvilli formation; and the activation of TRPV6 is mostly dependent on FSS triggering Ca^{2+} within their chip. In other words, microvilli formation is primarily depended on the direct relationship between FSS and Ca^{2+} and TRPV6.

Lee et al. [2] placental barrier analysis began with the monitoring of cell growth. Confocal microscopy was used to examine the structural integrity of their placental barrier based on the confluent monolayers of JEG-3 cells and HUVECs on the opposite sides of the membrane. After the confocal microscopy finished the analysis, fluorescent imaging took place, which included the imaging of GFP-stained HUVECs and CellTracker™ Red CMTPX stained JEG-3 cells. When both studies were completed, the cultured-channels were envaulted with an image-processing MATLAB algorithm. The following examinations provided evidence that *Lee et al.* placenta-on-a-chip reached a confluency between 99.3% and 99.4%, which reveals exceedingly confluent monolayers of HUVECs and JEG-3 cells on the chip's membranes. The following percentages supported the continuation of *Lee et al.* study when they began perfusing and evaluating glucose transfer across their microchip's barrier.

Preceding after the cell seeding in *Blundell et al*. [5] placenta-on-a-chip, they began the analysis of their microengineered barrier. The investigation of the intercellular junction started in a room temperature environment and fixing the BeWo cells and HPVECs in 4% paraformaldehyde (PFA) for 15 min and permeabilized in 0.25% Triton X-100 for 10 min. Afterward, the cells were incubated in 2% bovine serum albumin (BSA) for 1 hr. Subsequently, the primary antibodies (incubation of the BeWo cells and HPVECs was done in E-cadherin and VE-cadherin antibodies, respectively) were diluted in 2% BSA and incubated within the

microfluidic device for 1 hr. Next, the samples were washed with PBS, and preparation for the secondary antibodies was carried out. Similarly, the secondary antibodies were diluted in 2% BSA, but incubated for 45 min. After both sides of the membrane were washed with PBS, the membrane was removed from the microfluidic device and mounted on a coverslip. Finally, images were acquired from an inverted microscope and confocal laser-scanning microscope.

Following the analysis of the intercellular junction, *Blundell et al*. analyzed for microvilli formation and trophoblast syncytialization in their placenta-on-a-chip. They began the microvilli formation examination by staining the BeWo cell lines from their chip with Alexa 488-conjugated phalloidin as described by the manufacturer. Next, the cell line was washed to visualize the F-actin in the microvilli. Afterward, images were acquired through the utilization of a confocal laser scanning microscope. Then, the images underwent isolation to capture fluorescent pixels. After the microvilli formation was analyzed, trophoblast syncytialization began. *Blundell et al*. started by activating the protein kinase A pathway through treating the apical side of the epithelium with forskolin. Subsequently, the perfusion of a forskolin solution (at 50 μM in F-12K medium) was carried out in the upper channel for 72 hr. The cells were then isolated and fixed in 4% PFA, permeabilized in Triton X-100, and incubated in with 2% BSA for immunofluorescence staining. [5]

Once the explained procedures were carried out efficiently, *Blundell et al*. evaluated for barrier permeability after syncytialization and visualization of cell membrane transporters. The barrier permeability of the syncytialized epithelium was examined by measuring the 3 kDa fluorescein isothiocyanate (FITC)-dextran between the fetal and maternal channels. This process included the perfusion of FITC-dextran in DMEM/F-12K media at a concentration of 0.1 mg $ml^{-1}$to the upper channel. After 3 hr of perfusion, the media perfusate was collected from both

microchannels, and then processed for analysis. After *Blundell et al.* assessed the barrier permeability of their microchip, they examined the visualization of cell membrane transporters. This study began by isolating the seeded cells from the placental barrier and placing them in 4% PFA, permeabilized in Triton-X 100, and then incubated in 2% BSA in PBS. Subsequently, the samples were then incubated with mouse anti-glucose transporter 1 antibody. Images from the sample were acquired from a confocal laser-scanning microscope, while Velocity software was used to process the images. Finally, FIJI was used to assess transporter membrane localization across the obtained samples.

After the images and immunofluorescence staining were processed from the above procedures, *Blundell et al.* were able to conclude their microengineered barrier effectively replicated the microarchitecture of an *in vivo* placenta. The model produced confluent layers of endothelial and trophoblast cells that demonstrated barrier integrity. Not to mention, the microvilli formation exceeded expectations by revealing a distributed number of microvilli per cell. Additionally, trophoblast epithelium showed successful syncytialization through the production of the placental hormone, human chorionic gonadotropin. Thus, the following successful outcomes of *Blundell et al.*[5] study have provided *Blundell et al.* [6] the opportunity to carry out the evaluation of glyburide and heparin on their placenta-on-a-chip accordingly.

Zhu et al. [7] performed live cell staining, immunohistochemistry, live/dead cell assay, microvilli staining for their placental barrier verification. The process of live cell staining was carried out through CellTracker^{TM} Green or Red. The HUVECs and BeWo cells were incubated for 30 min at 37^0C with dye in basal medium. Shortly after, the cells were washed with PBS before imaging. Following live cell staining, *Zhu et al.* began mRNA extraction and RT-PCR. After mNRA extraction and RT-PCR was performed, immunohistochemistry was carried out.

Briefly, OCCLUDIN and VE-CADHERIN antibodies were used to conduct the assessment, and stained images from the study were acquired from a confocal microscope. Succeeding after the immunohistochemistry examination, *Zhu et al.* stained trophoblast cells cultured in microfluidic device to investigate microvilli formation. This included fixing the trophoblast cells with 4% paraformaldehyde for 10 min, and then permeabilized them with 0.25% Triton X-100 for 5 min. Next, the samples were stained with Alexa 488-conjugated phalloidin for 30 min, while the cell nuclei were counterstained with DAPI. Inspection of microvilli formation was performed under a confocal microscope. Following the staining for microvilli, the assay of live and dead cells in *Zhu et al.* microchip was performed using a Live/Dead viability assay kit. Once the following steps were finished, *Zhu et al.* concluded that their chip had efficiently formed the characteristics of an *in vivo* microvilli, along with confluent and tight monolayers of trophoblast and endothelial cells. After placental barrier verification was complete, *Zhu et al.* began preparation for bacterial transformation and infection within their microfluidic device.[7] Likewise, *Yin et al*. [8] performed the same placental barrier verification analyses for their nanoparticle study, with the addition of a molecular permeability assay. Initially, the dilution of FITIC-dextran with a molecular weight of 10,000 Da in cell culture medium was executed until a final concentration of 10 μM was reached. Lastly, the fluorescein was injected into the maternal channel, while the fluorescence intensity in the fetal side was observed for 24 hr.

The observation of live cells, barrier permeability, and intercellular junctions were all included in the initial stages of placental barrier analyses in the study by *Pemathilaka et al*. [9] The live cell study began with the staining of the HUVECs and BeWo cells with CellTracker^{TM} Green and CellTracker^{TM} Red, respectively. The dissociated cells were diluted with serum-free medium and incubated at 37^0C with 5% CO_2 in the air for 45 min. After the live cells were

observed, the investigation of barrier permeability was executed. The study began by measuring the transport between the maternal and fetal channels using a 3000 MW fluorescein-dextran anionic probes. Beforehand, the fluorescein-dextran anionic was diluted in PBS to 0.1 mg ml^{-1} in F-12K medium from a stock solution (100 mg ml^{-1} in PBS). Next, the dextran-mixed F-12K was introduced into the maternal channel, replacing the F-12K supplement. Finally, the flow was collected for 4 hr with 1 hr intervals from both channels and then analyzed using a microplate reader.

Succeeding after the barrier permeability analysis, *Pemathilaka et al*. [9] began the cell characterization for analyzing intercellular junctions. The following process began when *Pemathilaka et al*. cleansed the microchannels with PBS followed by a cell fixation with 4% PFA. Next, the channels were rinsed with PBS before incubated in a blocking solution (5% normal donkey serum, 0.4% BSA, and 0.2% Triton X-100 in PBS) for 60 min at room temperature. After incubation, the primary antibodies, E-cadherin and VE-cadherin, were diluted in the previously stated blocking solution and then incubated in the channels overnight at 4^0C. Then, the channels were washed with PBS and then incubated for 90 min with DAPI solution diluted in secondary antibodies with the blocking solution. Following a PBS wash, the membrane was separated from the microchip, and then imaged with an inverted microscope. After the analysis of intercellular junctions, *Pemathilaka et al*. confirmed that their chip produced confluent layers of trophoblast and endothelial cells. Once *Pemathilaka et al*. verified their placental barrier was successfully fabricated and seeded, they perfused and examined the transportation of caffeine across their microengineered barrier.

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