Characterization and Analysis Techniques of a Dynamic *in vitro* Blood-Brain Barrier Model

A Senior Project

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Contents

Abstract/Executive Summary

The blood-brain barrier (BBB) is responsible for maintaining the sensitive environment required by the brain. Although the BBB is necessary for proper functioning of the brain, it acts as an obstacle for doctors attempting to treat neurological disease. For a drug to act upon the brain, it must first pass through the discriminating BBB. For this reason, much research has been performed in recent years in order to create an *in vitro* model of the BBB on which drugs targeted for the central nervous system may be tested. The main goal of this project is to create an *in vitro* BBB model using both endothelial and glial cells, while my specific goals are to decide which techniques for characterization and analysis are best suited to our needs and resources as well as establish effective protocols for these techniques. To achieve these goals, several analysis techniques were chosen and protocols for three fluorescent assays were established. Although not a comprehensive method for characterization, these fluorescent assays allow for basic analysis of the system and act as a stepping-stone for future work.

I. Introduction

1.1 *Anatomy & Physiology of Blood-Brain Barrier*

Because proper functioning of the central nervous system (CNS) is critical to survival, its extracellular environment is highly regulated in the body (a). The extracellular environment of the CNS has many requirements, such as precise ion concentrations and protection from substances circulating throughout the rest of the body (a). In order to preserve optimal functioning of the CNS, it is protected by the blood-brain barrier (BBB). The many constituents of the BBB have been grouped together into what are known as the neurovascular unit and the junctional complex (a).

1.1.1 Neurovascular Unit

A neurovascular unit is composed of: cerebral microvascular endothelium, neurons, astrocytes, pericytes, and the extracellular matrix (ECM) (a, b).

Cerebral Microvascular Endothelium

Endothelial cells comprising the BBB are distinguishable from periphery endothelium due to their increased mitochondrial content, lack of fenestrations, minimal pinocytotic activity, and the presence of tight junctions (a). Cerebral endothelium also enhance the growth and differentiation of associated astrocytes (f).

Neurons

As neurons are the main communicators of the CNS, a central function of the BBB is to insure that the composition of the interstitial fluid allows for optimal functioning of neurons (f).

Astrocytes

Although astrocytes do not make a significant contribution to the physical barrier of the BBB (a), the tightness of the endothelial junctions *in vitro* are dependent upon the presence of astrocytes, or astrocyte-conditioned medium (c). *In vitro* studies show that astrocytes can up-regulate several BBB phenotypic features, such as narrower tight junctions. These interactions also lead to the correct association of endothelial cells and pericytes in tube-like structures *in vitro* (f).

Pericytes

In peripheral tissues, pericytes are involved in a range of endothelial-specific functions. Although little is known with respect to the role of cerebral pericytes, pericytes are considered an essential component of blood vessel assembly within the BBB(e).

ECM

The ECM, specifically that of the basal lamina, interacts with the cerebral microvascular endothelium, influencing the expression of endothelial tight junction proteins. Disruption of the ECM is associated with an increase in BBB permeability, which is attributed to the likely fact that proteins of the ECM are involved in the maintenance of tight junctions (a).

1.1.2 Junctional Complex

The cerebral microvasculature can be characterized by its junctional complex, which consists of adherens junctions (AJs), tight junctions (TJs), and gap junctions (b).

Adherens Junctions (AJs)

The constituents of the AJs work to mediate adhesion of endothelial cells to each other. AJs also restrict permeability across the endothelium and supplement the TJs in the regulation of paracellular permeability (b).

Tight Junctions (TJs)

TJs are the main components that confer low paracellular permeability and high electrical resistance (b). Specific proteins, such as the occludins and ZO-1, exist in relatively high concentrations in the TJs of the BBB. Although it is not certain, it is most likely due to the existence of these proteins that TJs exhibit selective permeability (b). The molecular organization of TJs is shown below in Figure 1.

Gap Junctions

The main purpose of the gap junctions is to mediate intercellular communication (b).

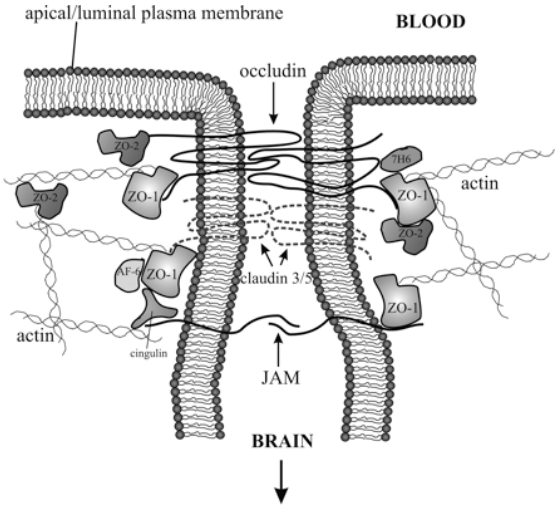


Figure 1: tight Junction Organization (a)

1.2 Overall Project Purpose

This project was initiated with the goal of developing an *in vitro* blood-brain barrier model consisting of a dual culture of bovine aortic endothelial cells and C6 glioma cells. To achieve this, a hollow-fiber pulsatile-flow bioreactor and pump were purchased and co-culture and cell sodding protocols were established.

Due to the cost of the single-use commercial bioreactors, it became clear that it would be necessary to manufacture a reactor in-house.

1.3 Individual Project Purpose

My individual goals for this project focused on the characterization and analysis of the system. Initially, my goal was to research methods of characterizing the blood-brain barrier and sift out the methods that were feasible for our lab to perform. Once a list of analytical methods was constructed, experiments were to be designed and performed in order to establish protocols for these techniques and ensure efficacy when applied to the model. The analytical approaches researched allow the determination of the quality of the BBB phenotype.

II. Methods

2.1 Literature Search

Initially, a literature search was carried out in order to determine the current state of research in the field. The information gleaned from the literature search was also used to determine which experiments would be most beneficial and feasible for the lab to perform.

2.2 Live/Dead Fluorescent Stain

Using the Live/Dead Viability/Cytotoxicity Kit from Invitrogen, 3T3 fibroblasts were stained with two fluorescent probes.

The working solution was prepared in a 15 mL conical, consisting of 20 µL Ethidium homodimer-1 (EthD-1), 10 mL Dulbecco's cation-free phosphate buffered saline (DCF-PBS), and 5 µL Calcein AM. The working solution was then vortexed.

The 3T3s, which were previously passed into a six-well plate as described in Appendix B, were allowed the previous two days to adhere to the plate. The media was aspirated from each well and the cells were subsequently rinsed with DCF-PBS. The DCF-PBS was then aspirated and 500 mL of the Live/Dead working solution was pipetted into each well, a sufficient amount to cover the bottom of each well. The six-well plate was then placed in an incubator for 40 minutes prior to visualizing using a fluorescent microscope.

2.3 CellTracker Green CMFDA Fluorescent Molecular Probe Test #1

Invitrogen's CellTracker Green CMFDA fluorescent probe was first tested on 3T3 fibroblasts in order to determine the optimum concentration of the working solution. Six concentrations of working solution were tested.

Six stock solutions were first prepared by dissolving 50 µg CMFDA in 10.8 µL anhydrous DMSO (Sigma-Aldrich), yielding a 10 mM solution of CMFDA. Approximately 150 µL anhydrous DMSO was extracted from the bottle using a sterile syringe and placed in a 15 mL conical. Using a micropipette, 10.8 µL DMSO was transferred into each CMFDA vial and the vials were manually shaken. Dulbecco's Modified Eagle Medium (DMEM) and 10 mM stock solution were combined in six 15 mL conicals at volumes described below in Table 1. Each conical of working solution was then vortexed.

Conical/ Well #	Concentration CMFDA (μM)	Volume Stock Solution (μL)	Volume DMEM (μL)	Total Volume (μL)
1	0.5	0.05	999.95	1000.0
2	1.0	0.10	999.9	1000.0
3	5.0	0.50	999.5	1000.0
4	10.0	1.0	999.0	1000.0
5	15.0	1.5	998.5	1000.0
6	20.0	2.0	998.0	1000.0

Table 1: CellTracker Green Test #1 Solution Volumes and Concentrations

The 3T3s, which had been passed into a six-well plate according to Appendix B, were given the previous 24 hours to adhere to the plate. The fibroblast media was aspirated, the T75 was rinsed with DCF-PBS, and working solution was transferred to each well as described above in Table 1. After the six-well plate was placed in an incubator for 30 minutes, the working solution was aspirated, and 1 mL fibroblast media was transferred to each well. The cells went through another 30-minute incubation, after which the media was aspirated, the wells were rinsed with DCF-PBS, and fresh fibroblast media was pipetted into each well (1 mL/well).

The cells were visualized using a fluorescent microscope at 24 hours, 5 days, and 7 days. Characteristic images can be seen below in Figure 2.

2.4 Dual Staining using CellTracker Green CMFDA and CellTracker Red (3T3s)

Invitrogen's CellTracker Green CMFDA and CellTracker Red CMTPX (Invitrogen) fluorescent probes were first tested on 3T3 fibroblasts in order to determine if the stains could be used to differentiate BAECs and C6s.

A green stock solution was first prepared by dissolving 50 µg CMFDA in 10.8 µL anhydrous DMSO, yielding a 10 mM solution of CMFDA. Approximately 100 µL anhydrous DMSO was extracted from the bottle using a sterile syringe and placed in a 15 mL conical. Using a micropipette, 10.8 µL DMSO was transferred into the vial containing 50 µg CMFDA and the vial was manually shaken. This was then diluted to create a 10 µM working solution by combining 10 mL DMEM and 10 µL of 10 mM stock solution in a 25 mL conical, which was subsequently vortexed.

This procedure was carried out a second time, but 5 mL DMEM was combined with 10 µL of 10 mM stock solution in order to achieve a working concentration of 20µM.

Two red working solutions were created in the same manner as the green working solution, except the CMFDA was replaced by CMTPX.

The 3T3s, which had been passed into four T75s according to Appendix A, were given the previous 24 hours to adhere to the plate. The fibroblast media was aspirated and each T75 was rinsed with DCF-PBS. One of the four working solutions (10 µM green; 10 µM red; 20 µM green; 20 µM red) was then transferred to each flask. After the four T75s were placed in an incubator for 30 minutes, the working solution was aspirated, and 3 mL fibroblast media was transferred to each flask. The cells went through another 30-minute incubation, after which the media was aspirated, the flasks were rinsed with DCF-PBS, and 3 mL trypsin was pipetted into each T75. Once the cells had detached from the surface of the flasks, the trypsin was deactivated with 3 mL fibroblast media. The cells, now tagged with either red or green fluorescent probes, were transferred to a six-well plate.

The cells were visualized using a fluorescent microscope. Characteristic images can be seen below in Figure 3.

2.5 Dual Staining using CellTracker Green CMFDA and CellTracker Red (BAECs and C6s)

The CellTracker Green CMFDA and CellTracker Red CMTPX fluorescent probes were then tested for efficacy in differentiating BAECs and C6s.

A green stock solution was first prepared by dissolving 50 µg CMFDA in 10.8 µL anhydrous DMSO, yielding a 10 mM solution of CMFDA. Approximately 100 µL anhydrous DMSO was extracted from the bottle using a sterile syringe and placed in a 15 mL conical. Using a micropipette, 10.8 µL DMSO was transferred into the vial containing 50 µg CMFDA and the vial was manually shaken. This was then diluted to create a 10 µM working solution by combining 10 mL DMEM and 10 µL of 10 mM stock solution in a 25 mL conical, which was subsequently vortexed.

A red working solutions was created in the same manner as the green working solution, except that the CMFDA was replaced by CMTPX.

The C6s, which had been passed into a T225 culture flask according to Appendix E, were given the previous 24 hours to adhere to the plate. The C6 media was aspirated and the culture flask was rinsed with DCF-PBS. 9 mL of the 10 µM green working solution was then transferred to the T225 and allowed to incubate for 30 minutes. The working solution was then aspirated, and 9 mL C6 media was transferred to the flask. The cells went through another 30-minute incubation, after which the media was aspirated, the flask was rinsed with DCF-PBS, and 3.5 mL trypsin was pipetted into the flask. Once the cells had detached from the surface of the flask, the trypsin was deactivated with 12.5 mL C6 media. The glial cells, now tagged with green fluorescent probes, were transferred to a six-well plate.

Similarly, the BAECs had been passed into a T75 culture flask according to Appendix D and were given 24 hours to adhere to the plate. The endothelial cell (EC) media was aspirated and the culture flask was rinsed with DCF-PBS. 3mL of the 10 µM CellTracker red working solution was then pipetted

into the T75 and the cells were given 30 minutes to incubate. The working solution was then aspirated and 3 mL EC media was transferred to the flask. The cells went through another 30-minute incubation period, the media aspirated, the flask was rinsed with DCF-PBS, and 2 mL trypsin was transferred to the T75. After the cells had detached from the surface of the flaks, the trypsin was deactivated with 2 mL EC media. The endothelial cells, which were now marked with red fluorescent probes, were transferred to a six-well plate.

2.6 Glucose Consumption

The rate of glucose consumed by the BAECs in the experimental setup was measured using a glucose meter. The baseline glucose concentration was measured during the experimental setup of the system as well as 4 and 5 days later, allowing the calculation of glucose consumption rate.

III. Results

3.1 Live/Dead Fluorescent Stain

Using Invitrogen's recommended concentrations of calcein AM and EthD-1, the 3T3's were successfully tagged. This assay imparted live cells with a green fluorescence (calcein AM) and dead cells with a red fluorescence (EthD-1), allowing differentiation between the two.

3.2 CellTracker Green CMFDA Fluorescent Molecular Probe Test #1

At 24 hours, each concentration of CellTracker working solution proved successful in allowing visualization of the fibroblasts. Higher concentrations of CellTracker working solution correlated with brighter fluorescence of the cells.

Five days later, although the fluorescence was not as bright as at the 24-hour mark, the results were similar. The cells tagged with the lowest concentration, 0.5 µM CMFDA solution, exhibited sufficient fluorescence to visualize the 3T3's, as shown in Figure 2.

A larger contrast between CMFDA concentrations was observed at seven days. Each concentration exhibited fluorescence, but this fluorescence was faint in wells one, 2, and 3

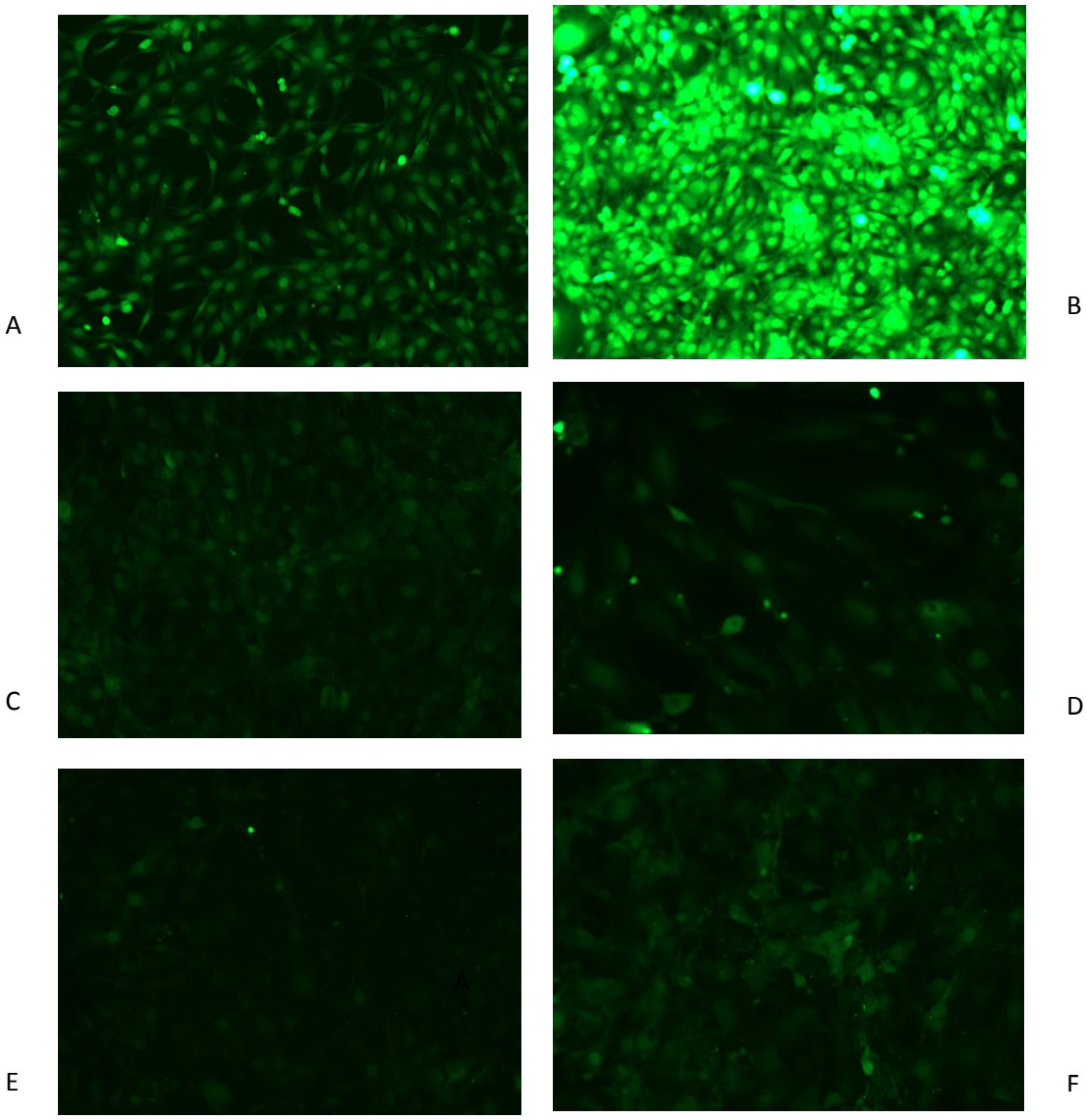


Figure 2: CellTracker Green CMFDA Characteristic Images (10x)

A: 24 hours; 5.0 µM CMFDA **B**: 24 hours; 20.0 µM CMFDA
C: 5 days; 5.0 µM CMFDA **D:** 5 days; 20 µM CMFDA
E: 7 days; 5.0 µM CMFDA **F**: 7 days; 20.0 µM CMFDA

3.3 Dual Staining using CellTracker Green CMFDA and CellTracker Red CMPTX (3T3s)

The 3T3's were successfully tagged at both 10 µM and 20 µM. The green-stained and red-stained fibroblasts were differentiable, but the red CMPTX gave off a much brighter fluorescence than the green CMFDA. A characteristic image of each can be seen below in Figure 3.

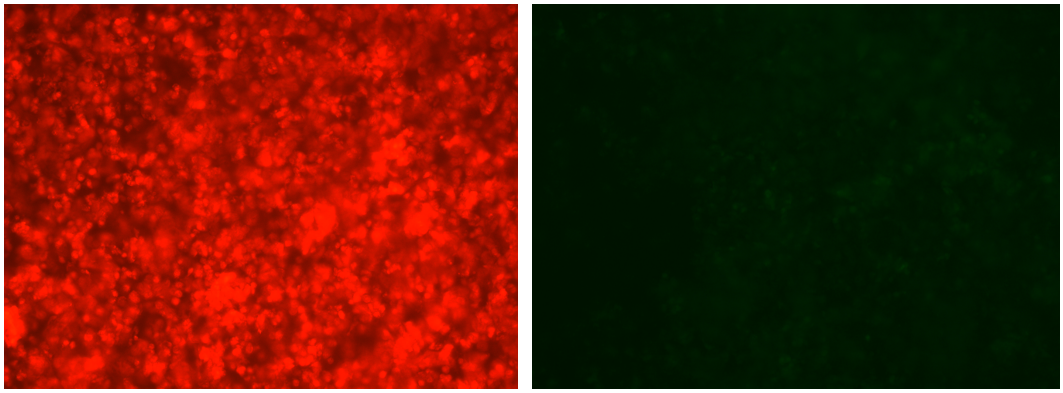
A  B

Figure 3: Dual Staining using CellTracker Green CMFDA and CellTracker Red CMPTX (10x)

A: 10 µM CMPTX B: 10 µM CMFDA

3.4 Dual Staining using CellTracker Green CMFDA and CellTracker Red (BAECs and C6s)

After the C6's were incubated with the CellTracker Green working solution, many of the cells had detached from the surface of the culture flask, suggesting that the CMFDA killed many of the C6's. The red-stained BAEC's were successfully tagged with red CMPTX, as shown in Figure 4.

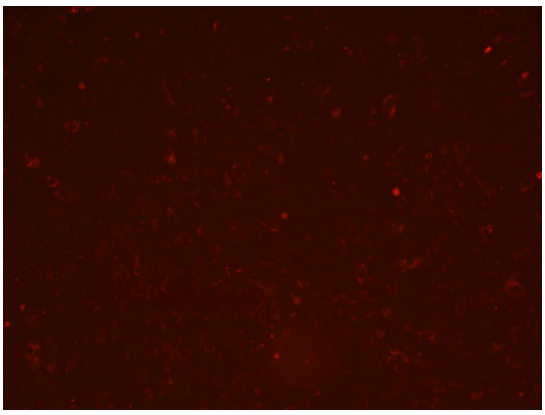


Figure 4: BAECs visualized at 10x (10 µM CMPTX)

3.5 Glucose Consumption

The baseline glucose level was measured at 348 mg/dL. Four days later, the glucose concentration in the system was 422 mg/dL. Five days post-inoculation, the glucose concentration was 423 mg/dL.

IV. Discussion/Conclusion

The results of the dual staining using CellTracker green CMFDA and CellTracker red CMPTX suggest that clearer images may be produced by using a lower concentration of red CMPTX and a higher concentration of green CMFDA.

The glucose concentration in the bioreactor setup was higher on the fourth and fifth day than it was on the first. Because there were a large number of BAECs alive, it is known that they had to be consuming some amount of glucose. One possible reason for the increase in glucose concentration is that the meter was designed to measure blood-glucose levels in humans, which is usually between 65 and 130 mg/dL. At concentrations as high as those in our system, the meter may not be sensitive to small changes as the meter may be overloaded with glucose.

Future Work

As this project evolves, more in-depth analysis techniques may be used. One such method is measuring the trans-epithelial electrical resistance (TEER), a measure of tightness of the junctions. Another possible analysis technique would be immunocytochemistry to further verify the BBB phenotype is being expressed. Some TJ proteins to be targeted are von Willebrand Factor (vWF), zonula Occluden-1 (ZO-1), and claudin-5.

Furthermore, once a reactor is developed that allows the sampling of fibers without taking down the entire system, the model can be assessed at several time points rather than merely post-experiment.

Further down the line, it would be ideal to test the permeability of substances across the barrier. These substances can range from a simple water-soluble dye to more complex chemicals, such as drugs that target the brain and therefore must cross the BBB.

V. Appendices

Appendix A: Protocol for Thawing Cells

Supplies:

Vial of cryopreserved cells

20 mL Growth Medium

T75 culture flask

1. Bring BAEC media to 37 ˚C in water bath
2. Remove vial of cells from liquid nitrogen dewer and thaw in water bath, making sure not to submergethe cap or threading
3. Label flask with cell type, passage number, date, and initials.
4. When media is warm, spray container with 70% Isopropyl alcohol (IPA) and place in laminar flow hood
5. Transfer the 20 mL of medium into the labeled flask, using the single-hand method
6. Once the cells have thawed, spray the vial with 70% IPA and place in laminar flow hood
7. Transfer the cells into the T75 culture flask
8. Place flask in incubator

Appendix B: Protocol for Freezing Down Cells

Supplies

~80% confluent T225 flask

Growth Medium

Vial of Trypsin

DMSO

DCF-PBS

4 freezing vials

1 Sterile 10mL conical

1 Sterile 50mL conical

Protocol

1. Warm growth medium and trypsin in 37°C water bath
2. Label freezing vials with cell type, initials, and date
3. When medium is warm, spray with 70% IPA and bring into laminar flow hood
4. Spray DMSO bottle with 70% IPA and bring into laminar flow hood
5. Prepare 5 mL freezing solution in 10 mL conical: 10% DMSO in growth medium
6. 0.5 mL DMSO
7. 4.5 mL growth medium
8. Transfer T225 flask from incubator to laminar flow hood
9. Aspirate medium from the culture flask
10. Rinse with sufficient DCF-PBS to cover the bottom of the T225
11. Aspirate DCF-PBS
12. Vortex the warmed trypsin, spray with 70% IPA and bring into laminar flow hood

13. Pipette 9 mL trypsin into culture flask and leave for several minutes until all cells have detached from the flask
14. If necessary, the flask may be knocked against the palm of your hand to agitate the cells
15. Pipette 9 mL growth medium into flask to deactivate the trypsin
16. Aspirate up and down several times to break up cell clumps
17. Transfer cell suspension to 50 mL conical and centrifuge at setting 4 for 4 minutes
18. Aspirate supernatant
19. Resuspend cell pellet in 4 mL freezing solution
20. Place 1 mL of cell suspension in each freezing vial
21. Place freezing vials in -80˚C freezer overnight
22. Transfer vials to liquid nitrogen dewer for long term storage

Appendix C: 3T3 Passage Schedule (1:4)

Supplies

2 T75 culture flasks

Fibroblast growth medium

1 vial trypsin

DCF-PBS

~75% confluent T75 flask of 3T3s

Protocol

1. Warm fibroblast growth medium and trypsin in 37˚C water bath
2. Bring new T75s into laminar flow hood using aseptic technique
3. Label T75s with cell type, passage number, initials, and date
4. When fibroblast media is warm, spray with 70% IPA and bring into laminar flow hood
5. Using the single-hand method, transfer 12 mL fibroblast media into each T75
6. Bring ~75% confluent T75 culture flask into laminar flow hood
7. Aspirate the medium from the culture flask
8. Rinse with DCF-PBS and aspirate
9. Vortex the warmed trypsin, spray with 70% IPA, and bring into laminar flow hood
10. Pipette 3 mL trypsin into culture flask and leave for several minutes until all cells have detached from the flask
11. If necessary, the flask may be knocked against the palm of your hand to agitate the cells
12. Pipette 5 mL fibroblast media into the flask to deactivate the trypsin
13. Aspirate up and down to break up cell clumps
14. Transfer 2 mL cell suspension to each of the labeled T75 flasks

15. Place each T75 in incubator

Appendix D: BAEC Passage Schedule (1:3)

Supplies

3 T75 culture flasks

BAEC growth medium

1 vial trypsin

DCF-PBS

~75% confluent T75 flask of BAECs

Protocol

1. Warm BAEC growth medium and trypsin in 37˚C water bath
2. Bring new T75s into laminar flow hood using aseptic technique
3. Label T75s with cell type, passage number, initials, and date
4. When BAEC media is warm, spray with 70% IPA and bring into laminar flow hood
5. Using the single-hand method, transfer 12 mL BAEC media into each T75
6. Bring ~75% confluent T75 culture flask into laminar flow hood
7. Aspirate the medium from the culture flask
8. Rinse with DCF-PBS and aspirate
9. Vortex the warmed trypsin, spray with 70% IPA, and bring into laminar flow hood
10. Pipette 3 mL trypsin into culture flask and leave for several minutes until all cells have detached from the flask
11. If necessary, the flask may be knocked against the palm of your hand to agitate the cells
12. Pipette 3 mL BAEC media into the flask to deactivate the trypsin
13. Aspirate up and down to break up cell clumps
14. Transfer 2 mL cell suspension to each of the labeled T75 flasks

15. Place each T75 in incubator

Appendix E: C6 Passage Schedule (1:4)

Supplies

2 T75 culture flasks

C6 complete growth medium

1 vial trypsin

DCF-PBS

~75% confluent T75 flask of C6s

Protocol

1. Warm C6 growth medium and trypsin in 37˚C water bath
2. Bring new T75s into laminar flow hood using aseptic technique
3. Label T75s with cell type, passage number, initials, and date
4. When C6 media is warm, spray with 70% IPA and bring into laminar flow hood
5. Using the single-hand method, transfer 12 mL C6 media into each T75
6. Bring ~75% confluent T75 culture flask into laminar flow hood
7. Aspirate the medium from the culture flask
8. Rinse with DCF-PBS and aspirate
9. Vortex the warmed trypsin, spray with 70% IPA, and bring into laminar flow hood
10. Pipette 3 mL trypsin into culture flask and leave for several minutes until all cells have detached from the flask
11. If necessary, the flask may be knocked against the palm of your hand to agitate the cells
12. Pipette 5 mL C6 media into the flask to deactivate the trypsin
13. Aspirate up and down to break up cell clumps
14. Transfer 2 mL cell suspension to each of the labeled T75 flasks

15. Place each T75 in incubator

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