The effect of shear stress on circulating and vascular endothelial cells

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Introduction

This report describes the outcome of a study performed during my internship at the Department of Biomedical Engineering of Georgia Institute of Technology, from September 2001 till January 2002. To complete a part of my Master’s in Biomedical Engineering at Eindhoven University of Technology, I worked with Stephanie M. Kladakis and Prof. Dr. Robert M. Nerem on the effects of fluid flow (shear stress) on circulating and vascular endothelial cells.

An extensive amount of research has already been done to analyze the behavior of shear stress on vascular endothelial cells (VECs) in vitro. Vascular endothelial cells are endothelial cells that cover the luminal surface of a blood vessel. Various experiments were performed to determine how blood flow mechanically influences VECs in vivo in obtaining their functional, structural and mechanical characteristics. The outcome of these experiments can be useful in developing an endothelial cell monolayer on the luminal surface of a tissue engineered vascular graft. A complete endothelial cell monolayer does not form spontaneously on vascular grafts after implantation in humans, therefore graft endothelialization needs to be promoted. An endothelial cell monolayer is important, since it maintains the vascular tone, provides a permeability barrier and provides a nonthrombogenic surface in a native blood vessel.

One possible method of promoting graft endothelialization is to entice the VECs in vivo to migrate from the neighboring tissue onto the luminal surface of tissue engineered vascular grafts. Therefore, at Georgia Institute of Technology a lot of research has been done on the rate at which human artery endothelial cells (HAECs) migrate on the surface of vascular models in vitro [1]. Currently attention goes out to circulating endothelial cells (CECs), as another way of obtaining an endothelial cell monolayer on the luminal surface of tissue engineered vascular grafts. CECs are endothelial cells that are located in the peripheral blood of normal humans. One recent publication reports that the concentration of CECs in the peripheral blood of healthy humans is low (2.6±1.6 CECs per ml of blood), and increases substantially in patients with cancer, sickle cell anemia, or vascular injury [3]. Earlier experiments performed on baboon circulating endothelial cells (BCECs) showed that the proliferation rate of BCECs varies from that of bovine aortic endothelial cells (BAECs), whereas the morphology and F-actin organization respond similarly to BAECs when exposed to shear stress [4]. This behavior indicates that CECs may have some of the same functionalities as VECs, and provides motivation for a new avenue in cardiovascular research. If further experiments precisely indicate that functional similarities exist between CECs and VECs, then CECs could possible be recruited to the surface of tissue engineered vascular grafts in vivo to form an endothelial cell monolayer.

The overall goal of this study is to determine whether BCECs and BVEC, in attached status, show functional similarities under shear stress. Therefore BCECs and BVEC were seeded on collagen coated glass slides and exposed to various amounts of shear stress in flow systems (paragraph 3.2.2).

So far, a culture medium is determined to replace the Clonetics medium, which is commercially available to culture baboon circulating endothelial cells (BCECs) (chapter 2). A substitute for the Clonetics medium was desirable, since this medium is very expensive and the quantities of the components are unknown. Further, the effect of shear stress on the single cell BCEC migration is quantified (chapter 3). At the
moment, the migration characteristics of BCECs and BVECs cannot be compared, since the same migration experiments have not been performed on BVECs. In the overall discussion (chapter 5) the migration characteristics of BCECs are merely compared to earlier experiments performed on BAECs [7]. Last the effect of shear stress on the cell shape and the F-actin organization of baboon vascular endothelial cells (BVECs) is quantified (chapter 4). In earlier experiments the morphology and the F-actin organization of BCECs was determined using the same protocol (appendix VI) [4] and in chapter 4 both cell types are compared. In the overall discussion (chapter 5) the morphology and the F-actin organization of BVECs are also compared to results obtained from earlier flow experiments performed on BAECs [5, 6, 7]. And furthermore, in chapter 5 some characteristics of VECs in vivo are discussed. At this point no extrapolation can be made of the results obtained from the flow experiments performed on BCECs and BVECs to the situation in vivo. An extrapolation can only be made when the used vascular model corresponds to the situation in vivo with much more precision. As background to the study, chapter 1 provides and overview of the use of CECs in cardiovascular tissue engineering.
1. **The Use of CECs in Cardiovascular Tissue Engineering**

1.1 **Overview**
Cardiovascular disease is the leading cause of morbidity and mortality in the United States of America. One of the most severe forms of cardiovascular disease is associated with atherosclerosis, progressive narrowing of arteries. For coronary arteries this atherosclerosis leads to weakening of the myocardium, and ultimately to myocardial infarction. Each year over 12 million people are having coronary heart disease [1]. The most common form of treatment for coronary heart disease is coronary artery bypass graft surgery (CABG) [2]. Such surgeries frequently involve multiple bypasses, and commonly either the patient’s internal mammary artery or saphenous vein is used. Each year about a half million of these CABG surgeries are performed in the United States alone. Even so, there are many patients with coronary heart disease in need of surgery for whom a CABG is not possible because their native vessels are not available for use, either from being diseased themselves or because of previous surgery. Furthermore it is less than desirable to remove healthy native vessels from their original position in the vasculature. This all led to the development of vascular grafts. Some successes have already been made by using bypass grafts of synthetic materials, e.g. Dacron or expanded polytetrafluoroethylene (ePTFE), for replacing large blood vessels, i.e. 6-10 mm in diameter. When used in the coronary system, where the diameters are 3-4 mm, these synthetic grafts are all but successful; they rapidly occlude through thrombotic events.

To overcome the disadvantages of the current blood vessel substitutes, the principles of tissue engineering offer several potential advantages. Tissue engineering is a multidisciplinary science that combines engineering principles with those of life sciences to produce biological substitutes that resemble native tissue in a structural, functional and mechanical way.

The beginning of tissue engineering as applied to the cardiovascular system, was the development of an endothelial cell seeded synthetic graft [2]. The presence of a native endothelial cell layer is the proposed reason for the low incidence of thrombosis in autologous transplants for the CABG procedure [1]. The problem of thrombosis of the vascular grafts made of a synthetic material could perhaps be alleviated by providing the nonthrombogenic interface present in a native blood vessel, i.e. an endothelial cell monolayer. However only a few clinical successes have been reached by this technique. Currently the focus has shifted toward tissue engineering a complete vascular graft with all the functional characteristics of a normal blood vessel. This requires that the vascular graft not only be nonthrombogenic, it must also exhibit vasoactivity and possess mechanical properties that match a native blood vessel.

At Georgia Institute of Technology a lot of research has been done on creating a tissue engineered vascular model, of smooth muscle cells and collagen type I. And on promoting graft endothelialization by vascular endothelial cells (VECs). The overall goal of this study is, as noted earlier, to determine whether circulating endothelial cells (CECs) are suitable for graft endothelialization.

1.2 **Circulating endothelial cells**
Nonhematological cells were reported as early as 1934 in the blood of certain cancer patients. Since then, circulating endothelial cells (CECs) have been described in several pathological conditions that have in common the presence of vascular injury. One recent publication reports that there are $2.6 \pm 1.6$ CECs per milliliter of the peripheral blood in normal human adults [3]. The number of CECs is increased
The Effect of Shear Stress on CECs and VECs

substantially in the peripheral blood of patients with sickle cell anemia, cancer, or vascular injury. Experiments have revealed the possibility that not all CECs originate from the same source. It is believed that some of the CECs are mature endothelial cells that were shared of the vascular wall while others are marrow-derived endothelial cell precursors or angioblasts. This hypothesis is supported by accelerated growth rate of CECs in vitro compared to VECs of a large artery [3, 4]. Figure 1.2.1 shows baboon vascular endothelial cells (BVECs) and baboon circulating endothelial cells (BCECs) in culture.

![Figure 1.1](image1.png)

*Figure 1.1 Microscopic images of baboon vascular endothelial cells (BVEC) (a, magnification 40x) and baboon circulating endothelial cells (BCEC) (b, magnification 10x).*
2. Media Assay for BCECs

2.1 Introduction
A media assay was performed on baboon circulating endothelial cells (BCEC) to determine a medium to replace the Clonetics medium (table 2.2.2.1), which is commercially available to culture BCECs. A substitute for the Clonetics medium is desirable, because this medium is very expensive. Furthermore the quantities of the components of the Clonetics medium are unknown. In the media assay the proliferation of BCECs was analyzed in five different types of media (table 2.2.2.1) after consecutively two, three and four days of static culture. The BCECs were cultured in Clonetics medium, human coronary artery endothelial cell medium (HCAEC), HCAEC medium supplemented with Hepes (6mM), Flow medium (the medium used in flow experiments) and Flow medium supplemented with Hepes (6mM). Hepes was added to two types of media to determine whether it is preferred to add a buffer to maintain the pH of the medium.

2.2 Materials and methods

2.2.1 Cell culture
The baboon circulating endothelial cells (BCEC) were obtained from the laboratory of Prof. Stephen R. Hanson at Emory University (Atlanta, GA, USA). The BCECs were selectively cultured from the buffy coat layer of peripheral blood and were subcultured in the laboratory of Prof. Dr. Robert M. Nerem at Georgia Institute of Technology (Atlanta, GA, USA). The BCECs were grown in tissue culture flasks coated with 50 µg/ml type I rat tail collagen (0.5 ml per 25 cm², BD Bioscience) using Clonetics medium (table 2.2.2.1). The BCECs were fed every other day and were kept in an automatic CO₂ incubator at 37°C, with a 95 percent air, 5 percent CO₂ atmosphere. Confluent BCECs were subcultured using 0.025 percent trypsin and 0.27 mM EDTA in a Ca++-free Dulbecco’s Phosphate Buffer Saline (Gibco) and were replated in tissue culture flasks (1:3) for culture purposes (appendix I). To perform the media assay two nearly confluent T-75 flasks, approximately 4,500,000 cells, are needed on the first day of the assay. For the media assay cells were used at passage 7-9.

2.2.2 Media
The components of the five different types of media used in the media assay are shown in table 2.2.2.1. The product information of the components is given in appendix II.

2.2.3 Media assay
On the first day of the media assay fifteen T-25 flasks were coated with 50 µg/ml type I rat tail collagen (0.5 ml per 25 cm², BD Bioscience). After the collagen adhered for one hour and the remnants were removed, the BCECs were seeded in the T-25 flasks at a density of 10,000 cells/cm². On the second day of the media assay the Clonetics medium was removed from the T-25 flasks and replaced with the five different types of media summarized in table 2.2.2.1, in which each type of medium was added to three of the T-25 flasks. On the fourth day of the media assay the cells of each T-25 flask were counted, using the Coulter Counter, to determine the proliferation of the BCECs after two days of static culture.
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<table>
<thead>
<tr>
<th>Components</th>
<th>HCAEC</th>
<th>HCAEC+ Hepes</th>
<th>Flow</th>
<th>Flow+ Hepes</th>
<th>Clonetics</th>
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<td>MCDB 131</td>
<td>MCDB 131</td>
<td>MCDB 131</td>
<td>ECBM-2</td>
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<td>5%</td>
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<tr>
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<td>1%</td>
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</tr>
<tr>
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<td>0.05 mg/ml</td>
<td>-</td>
<td>-</td>
<td>*</td>
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<tr>
<td>hFGF-B</td>
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<td>0.002 µg/ml</td>
<td>0.002 µg/ml</td>
<td>0.002 µg/ml</td>
<td>*</td>
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<tr>
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<td>0.0005 µg/ml</td>
<td>*</td>
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<tr>
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<td>0.001 µg/ml</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>IGF-1</td>
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<td>0.002 µg/ml</td>
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<td>-</td>
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</tr>
<tr>
<td>Hydrocortisone</td>
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<td>0.001 mg/ml</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>R3-IGF-1</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
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<td>6 mM</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.2.2.1 The five different types of media, - indicates that the component is not included in the media and * indicates that the quantity of the used component is unknown.

After counting all fifteen T-25 flasks the BCECs were reseeded at a density of 10,000 cells/cm² into fifteen new T-25 flasks coated with 50 µg/ml type I rat tail collagen (0.5 ml per 25 cm²). The BCECs were reseeded in the tissue culture flasks to further determine the proliferation of BCECs after consecutively three and four days of static culture. The complete protocol for the media assay is given in appendix II. Endothelial cells form monolayers, they will not grow on top of one another. To prevent the results of the media assay from becoming unreliable, the assay should not be performed on cells that are nearly confluent; therefore a maximum is set at four days of static culture (~60-70% confluence).

2.2.4 Data analysis

Results data were expressed as mean number of cells ± standard deviation (SDEV). Statistical comparison of cell numbers was made using an ANOVA test. A significant result was assumed if the p value was less than 0.05.

2.3 Results

Figure 2.4.1 shows the number of BCECs after two days of static culture. It is clearly visible that there is no significant difference between the four types of media (HCAEC medium, HCAEC medium supplemented with Hepes, Flow medium and Flow medium supplemented with Hepes) and the Clonetics medium. Furthermore there is no significant difference between the HCEAC medium and the HCAEC medium supplemented with Hepes, as well as the Flow medium and the Flow medium supplemented with Hepes. So it can be concluded that Hepes does not have any effect on the total number of BCECs after two days of static culture.
Figure 2.4.2 shows the number of BCECs after three days of static culture. In comparison to figure 2.4.1 some changes have occurred. The Flow medium and the Flow medium supplemented with Hepes significantly differ from the Clonetics medium. Whereas the number of BCECs obtained from the HCAEC medium and the HCAEC medium supplemented with Hepes are comparable to those of the Clonetics medium. Furthermore it can be concluded that Hepes does not have any effect on the total number of BCECs after three days of static culture.
Figure 2.4.3 Media assay after 4 days of static culture

Figure 2.4.3 shows the number of BCECs after four days of static culture. Something inexplicable happened to the BCECs cultured in the Clonetics medium, which killed almost all of them. Therefore no comparison can be made between the four types of media and the Clonetics medium. What can be concluded is that Hepes still doesn’t have any effect on the total number of BCECs.

2.4 Discussion

From the obtained results can be concluded that the HCEAC medium can be used as a substitute for the Clonetics medium. Furthermore it can be concluded that the Hepes in the used concentration (6mM) does not have any effect on the total number of BCECs. An extra addition of 6 mM Hepes to the medium that can be used as a substitute for the Clonetics medium is therefore unnecessary. Last can be concluded that the Flow medium inhibits proliferation, as the number of BCECs after two, three and four days of static culture are almost the same.
3. **Single Cell BCEC Migration**

3.1 **Introduction**

Single cell baboon circulating endothelial cell (BCEC) migration experiments were performed to determine whether the migration characteristics of BCECs could be modified due to the effect of fluid flow (shear stress). The hypothesis states that shear stress stimulates cells to migrate in the direction of the imposed fluid flow. In this chapter it will be determined whether this hypothesis is correct regarding BCECs. The single cell BCEC migration was quantified after exposing the BCECs to approximately no shear stress (0.5 dynes/cm² = 0.05 Pa) and high shear stress (10 dynes/cm² = 1 Pa) for 24 hours.

3.2 **Materials and methods**

3.2.1 **Cell culture**

The BCECs were grown in tissue culture flasks coated with 50 µg/ml type I rat tail collagen (0.5 ml per 25 cm², BD Bioscience) using Clonetics media (table 2.2.2.1). The BCECs were fed every other day and were kept in an automatic CO₂ incubator at 37 °C, with a 95 percent air, 5 percent CO₂ atmosphere. Confluent BCECs were subcultured using 0.025 percent trypsin and 0.27 mM EDTA in a Ca⁺⁺-free Dulbecco’s Phosphate Buffer Saline (Gibco) and were replated in tissue culture flasks (1:3) for culture purposes (appendix I).

In order to expose the BCECs to shear stress the BCECs were labeled with 5 µM Vybrant CFDA (Molecular Probes), trypsinized and seeded onto the open area (10×15 mm) between a glass slide and a silicone gasket (figure 3.2.1.1) coated with 50 µg/ml type I rat tail collagen (appendix III). The BCECs were seeded at a density of 1000 cells/cm² and were statically cultured for 48 hours before exposing the BCECs to shear stress in a flow system. One hour after seeding the BCECs onto the glass slide the silicone gasket was removed. For migration experiments the BCECs were used at passage 7-9.

![Silicone gasket and Glass slide](image)

*Figure 3.2.1.1 Glass slide covered with a silicone gasket.*

3.2.2 **Flow system**

After statically culturing the BCECs on the glass slides for 48 hours, the BCECs were exposed to fluid flow using a parallel plate flow device. The flow system consists of the following four components looped in series: flow reservoir, pump, pulse dampener...
and a flow chamber. In figure 3.2.2.1 a flow system is shown. The glass slide with BCECs is placed inside the flow chamber, among the rubber gasket and the yellow incompressible spacer (figure 3.2.2.2 and 3.2.2.3). The pump circulates Flow medium (table 2.2.2.1) through the flow system, exposing the BCECs in the flow chamber to a constant shear stress of 10 dynes/cm$^2$ (= 1 Pa) for 24 hours. This level of shear stress is comparable to the normal physiological level of fluid shear stress in a blood vessel [7]. The control group was also placed in a flow system and exposed to approximately no shear stress (0.5 dynes/cm$^2$ = 0.05 Pa) for 24 hours. The sole purpose of the approximately no shear stress was to constantly refresh the media.

Figure 3.2.2.1  The flow system consists of a flow reservoir (a), a pump (b), a pulse dampener (c) and a flow chamber (d).

Figure 3.2.2  The flow chamber consists of an aluminum frame bottom with a rubber gasket (a), an incompressible spacer (b), a flow chamber with bypass tube (c) and an aluminum frame top (d). The components are placed on top of one another (a → d).
3. Single Cell BCEC Migration

To quantify the single cell BCEC migration at high shear stress (10 dynes/cm² = 1 Pa) and approximately no shear stress (0.5 dynes/cm² = 0.05 Pa), the flow chambers of two flow systems were placed on top of a motorized stage inside an automatic CO₂ incubator at 37° C for 24 hours. The incubator is built around a fluorescence microscope as can be seen in figure 3.2.2.4. By means of the fluorescence microscope, a CCD camera and the motorized stage, microscopic images were made of specific BCECs at a constant time interval. For the single cell BCEC migration experiments images were made of 32 fields, 16 fields per slide, at a 30 minute time interval for 24 hours.

Figure 3.2.2.3 Flow chamber with bypass tube (c, figure 3.2.2.2), the direction of the fluid flow is indicated.

Figure 3.2.2.4 Experimental equipment to monitor single cell BCEC migration. The experimental equipment consists of i.e. an incubator (a), a fluorescence microscope (b), and a motorized stage (d).
In figure 3.2.2.5 images are shown of one field after 0, 12 and 24 hours of exposure to fluid flow. The BCECs are visible as bright spots. By exposing the BCECs to fluorescent light, every time microscopic images are made, the Vybrant CFDA staining of the BCECs slightly diminishes. Therefore the exposure of BCECs to fluorescence light should be the least. The complete protocol to determine the single cell BCEC migration is given in appendix III.

Figure 3.2.2.5 Microscopic images of BCECs of one field after 0 (a), 12 (b) and 24 hours (c) of exposure to fluid flow (magnification 10x).

3.2.3 Data analyses
The obtained images were filtered to determine the centroid locations of the BCECs. The BCECs were tracked through the stack of filtered images (49 images in 24 hours), obtained form each field, using the nearest neighbor algorithm$^2$. After the BCECs were tracked in the fields, the distances that the BCECs migrated were determined. The results data of the single cell BCEC migration experiments were expressed as: (a) the normalized locations (x, y positions) of the BCECs through time, (b) the mean of the total (normalized) distances that the BCECs migrated from their initial position ± standard error of the mean (SEM) and (c) the mean of the (normalized) distances that the BCECs migrated in the direction of the fluid flow ± SEM.

The total distance that a BCEC migrated from their initial position was determined by the following equation.

\[
((x_{n,\text{last}}^2 + y_{n,\text{last}}^2)^{1/2})
\]

In this equation $x_{n,\text{last}}$ and $y_{n,\text{last}}$ indicate the final normalized location (x, y position) of a BCEC in one of the fields of a glass slide. The distance that a BCEC migrated in the direction of the fluid flow equals $y_{n,\text{last}}$, the final normalized y position.

Statistical comparison of the total distances that the BCECs migrated from their initial position, as well as in the direction of the fluid flow was made using a student t-test. A significant result was assumed if the p value was less than 0.05.

3.3 Results
Figure 3.3.1 shows the migration of BCECs of experiment 8 at approximately no shear stress (0.5 dynes/cm$^2$ = 0.05 Pa) and high shear stress (10 dynes/cm$^2$ = 1 Pa) for 24 hours. The hypothesis states that the BCECs migrate in the direction of the imposed fluid flow. However no preference direction can be found in which the BCECs tend to migrate. Furthermore no clear distinction can be found between the
3. Single Cell BCEC Migration

approximately no shear stress and high shear stress cases; the BCECs in both cases migrated in all directions equally. Figure 3.3.2 shows the migration of BCECs of experiment 9 at approximately no shear stress (0.5 dynes/cm$^2$ = 0.05 Pa) and high shear stress (10 dynes/cm$^2$ = 1 Pa) for 24 hours. Again no clear distinction can be found between the approximately no shear stress and high shear stress cases. However in comparison to experiment 8 the BCECs did not migrate in all directions equally, but migrated against the fluid flow. So a preference direction is found in which the cells tend to migrate, but this preference direction is opposite to the direction of the imposed fluid flow.

Figure 3.3.1 The 24 hour migration of BCECs of experiment 8 at 0.5 dynes/cm$^2$ (= 0.05 Pa) (a) and at 10 dynes/cm$^2$ (= 1 Pa) (b). The BCECs used are of passage 7.

Figure 3.3.2 The 24 hour migration of BCECs of experiment 9 at 0.5 dynes/cm$^2$ (= 0.05 Pa) (a) and at 10 dynes/cm$^2$ (= 1 Pa) (b). The BCECs used are of passage 8.
In appendix IV the migration of BCECs of i.e. experiment 9 is shown at approximately no shear stress and high shear stress for 12 hours. These graphs show a clear distinction between both shear stress cases. The BCECs exposed to approximately no shear stress migrated in the direction of the imposed fluid flow, whereas the BCECs exposed to high shear stress migrated against the fluid flow. After 12 hours some BCECs detached from the surface of the glass slides and are no longer visible in the graphs (figure 3.3.2). So with time BCECs disappear as well as the distinction between the approximately no shear stress and high shear stress cases.

Figure 3.3.3 shows the migration of BCECs of experiment 10 at approximately no shear stress (0.5 dynes/cm² = 0.05 Pa) and high shear stress (10 dynes/cm² = 1 Pa) for 24 hours. For the first time a distinction is found between both shear stress cases. The BCECs exposed to approximately no shear stress did not migrate in a specific direction, whereas the BCECs exposed to high shear stress migrated in the direction of the imposed fluid flow. The same pattern was seen after 12 hours of exposure to fluid flow (appendix IV).

So unfortunately the results of all three experiments are very different. What can be concluded though, is that in two of the three experiments preference directions exist in which the BCECs tend to migrate.

Figure 3.3.4 shows the total migration of BCECs from their initial position after 12 hours and 24 hours of exposure to approximately no shear stress and high shear stress. No significant difference can be found between both shear stress cases. What is found is an absolute increase in migration over time for both shear stress cases.
3. Single Cell BCEC Migration

Figure 3.3.4  The total distance the BCECs migrated at 0.5 dynes/cm² (= 0.05 Pa) and 10 dynes/cm² (= 1 Pa) after 12 and 24 hours.

Figure 3.3.5 shows the migration of the BCECs in the direction of the imposed fluid flow after 12 hours and 24 hours of exposure to approximately no shear stress and high shear stress.

Figure 3.3.5  The distance the BCECs migrated in the direction of the fluid flow at 0.5 dynes/cm² (= 0.05 Pa) and 10 dynes/cm² (= 1 Pa) after 12 and 24 hours. The numbers of BCECs (n) are the same as in figure 3.3.4.

Again no significant difference exists between both shear stress cases.
The reason for not finding any significant difference between the shear stress cases in figures 3.3.4 and 3.3.5 is probably the great variance in the results of the three experiments. This variance might have diminished the total effect. To determine whether this is true, the experiments in which a migration trend is visible are compared (figure 3.3.2.b and 3.3.3.b).

![Figure 3.3.6 Comparison of experiments 9 and 10 at 10 dynes/cm² (= 1 Pa).](image)

In figure 3.3.6 experiments 9 and 10 are compared for their high stress cases. The total displacements do not significantly differ, but there is a significant difference in the displacements of BCECs in the direction of the imposed fluid flow. Furthermore it can be concluded from figure 3.3.6 that two trends actually exist in which the BCECs tend to migrate and that these trends are opposite to one another. The existence of two opposite migration trends is therefore the most plausible explanation for not finding any difference in the total displacement, as well as in the displacement in the direction of the imposed fluid flow for the three experiments (figure 3.3.4 and 3.3.5).

### 3.4 Discussion

In the end it can be concluded that there is no significant difference in the total displacement of BCECs, as well as in the displacement of BCECs in the direction of the imposed fluid flow for the approximately no shear stress and high shear stress cases after 12 and 24 hours. Furthermore it can be concluded that there is an absolute increase in BCEC migration over time for both shear stress cases. And that two preference directions exist in which the BCECs tend to migrate under high shear stress. In experiment 8 the BCECs exposed to high shear stress migrate towards the fluid flow whereas in experiment 9 the BCECs migrate against the fluid flow.

The great variance in the data of the three experiments can be attributed to using BCECs of different passages for each experiment. To determine the actual migration trend of BCECs, either towards or against the fluid flow, it is recommended to repeat the experiment using BCECs of the same passage.
4. F-actin Organization and Cell Shape of BVECs

4.1 Introduction
The F-actin organization and cell shape experiments on baboon vascular endothelial cells (BVECs) were performed to complete a preliminary investigation focusing on the response of BCECs to the shear stress associated with fluid flow [4]. The same protocol was used for these experiments as the one that was used in the preliminary study (appendix VI), with the exception of the media that is used to culture the cells. The F-actin organization and cell shape of BVECs were quantified after 24 hours of static culture and exposure to shear stress (10 dynes/cm² = 1 Pa).

4.2 Materials and methods

4.2.1 Cell culture
The baboon vascular endothelial cells (BVEC) were obtained from the laboratory of Prof. Stephen R. Hanson at Emory University (Atlanta, GA, USA). The BVECs were selectively cultured from the intima of a blood vessel and were subcultured in the laboratory of Prof. Dr. Robert M. Nerem at Georgia Institute of Technology (Atlanta, GA, USA). The BVECs were grown in tissue culture flasks coated with 50 μg/ml type I rat tail collagen (BD Bioscience) using medium 199 (Gibco) supplemented with 20 percent Fetal Bovine Serum (FBS, Sigma), 1.2 percent L-Glutamine (Mediatech Cellgro), 1.2 percent Anitbiotic-Anitmycotic (Gibco), 0.8 ml Heparin solution (10000 u/ml, Sigma) and 1ml Endothelial Mitogen solution (25 mg/ml, Biomedical Technologies Inc.). The BVECs were fed every other day and were kept in an automatic CO2 incubator at 37 °C, with a 95 percent air, 5 percent CO₂ atmosphere. Confluent BVECs were subcultured using 0.025 percent trypsin and 0.27 mM EDTA in a Ca++-free Dulbecco’s Phosphate Buffer Saline (Gibco) and were replated in tissue culture flasks (1:3) for culture purposes (appendix V). In order to expose the BVECs to shear stress the BVECs were trypsinized and seeded onto the open area (20×40 mm) between a glass slide and a silicone gasket coated with 50 μg/ml type I rat tail collagen. The BVECs were seeded at a density of 25,000 cells/cm² and were statically cultured for 48 hours before exposing the BVECs to shear stress in a flow system. One hour after seeding the BVECs onto the glass slide, the silicone gasket was removed (appendix VI). For this experiment BVECs were used at passage 6.

4.2.2 Flow system
The BVECs grown to confluence on glass slides, were exposed to fluid flow using a parallel plate flow device (paragraph 3.2.2.). The flow system was placed in an automatic CO₂ incubator at 37 °C. And the pump of the flow system circulated Flow medium (table 2.2.2.1) through the system, exposing the BVECs in the flow chamber to a constant shear stress of 10 dynes/cm² (= 1 Pa) for 24 hours. The control group was given fresh Flow medium and left in static culture for 24 hours. The complete protocol to determine the F-actin organization and cell shape of BVECs is given in appendix VI.

4.2.3 Data analysis
Following the 24 hour experiment, the BVECs were fixed in 3.7 percent formaldehyde (Tousimis) solution in Phosphate Buffered Saline (PBS, Sigma) and incubated with 5 μl Oregon Green 488 Phalloidin (200 u/ml methanol, Molecular Probes) (appendix VI). This stain is a Phallotoxin and binds specifically to F-actin.
The BVECs were imaged using confocal microscopy. To reduce photobleaching before imaging the BVECs, the exposure of the BVECs to light should be minimized. The shape index of the BVECs was determined using the LSM 510 image software. The results data were expressed as: (a) confocal images of BCECs and BVECs stained for F-actin and (b) the mean of the shape index (SI) ± standard error of the mean (SEM). The shape index was obtained by the following equation.

\[
SI = \frac{4\pi A}{P^2}
\]

In this equation A is the area and P the perimeter of the cell. The SI is a dimensionless number that ranges from 0 for a straight line to 1 for a perfect circle. Statistical comparison of the shape indices was made using a student t-test. A significant result was assumed if the p value was less than 0.05.

4.3 Results

Figure 4.3.1 shows the confocal images of BCECs after 24 hours of static culture and exposure to shear stress (10 dynes/cm\(^2\) = 1 Pa), that were obtained in a preliminary investigation by C.L. Duvall at Georgia Institute of Technology [4]. These confocal images show that in statically cultured BCECs (figure 4.3.1.a), the F-actin filaments appear densely concentrated in the outer portion of the cytoplasm, with short filaments branching out in all directions. Whereas in BCECs exposed to shear stress (figure 4.3.1.b), the F-actin filaments are uniformly spread throughout the cell, with the long filaments aligned parallel to the direction of the imposed fluid flow.

In figure 4.3.2 confocal images are shown of BVECs after 24 hours of static culture and exposure to shear stress (10 dynes/cm\(^2\) = 1 Pa). These two images hardly differ with respect to their F-actin organization, in comparison to the earlier obtained results (figure 4.3.1). Some alignment of F-actin filaments parallel to the direction of flow is visible (figure 4.3.2b), but this alignment is not nearly as clear as in figure 4.3.1.b.
4. F-actin Organization and Cell Shape of BVECs

Figure 4.3.2  Confocal images of BVECs stained for f-actin after 24 hours of static culture (a) and exposure to shear stress (b). Magnification of 40x.

When exposed to shear stress, the BCEC monolayers shift from a random, confluent arrangement of rounded polygonal shapes to elongated shapes oriented lengthwise in the direction of fluid flow, causing a subsequent reduction in shape index (SI). A significant reduction (p<0.001) of the SI is shown in figure 4.3.3.

Some lengthwise elongation of BVECs in the direction of fluid flow is visible (figure 4.3.2b), but this elongation is not nearly as enormous as in figure 4.3.1.b. Furthermore, no significant reduction in SI is found (p>0.05), when exposing the BVECs to shear stress for 24 hours (figure 4.3.4).
4.4 Discussion

What can be concluded from the obtained results is that some alignment of F-actin filaments and lengthwise elongation of BVECs occurs in the direction of the imposed fluid flow. But this alignment and elongation is small compared to the alignment and the elongation obtained by exposing BCECs to shear stress in previous experiments. And furthermore no significant reduction in the shape index of BVECs is found \((p>0.05)\), whereas a distinct reduction in the shape index of BCECs was found in previous experiments \((p<0.001)\). The obtained results are everything but expected. Since vascular endothelial cells show alignment of their F-actin filaments and lengthwise elongation in the direction of the blood flow in vivo [7].

The reason for these strange results is perhaps that the BVECs were not completely confluent prior to the experiment. Furthermore the outcome of the experiment could also be affected by the fact that the BVECs may not have been very healthy since they were not proliferating properly prior to the experiment. Therefore it is recommended to use a new batch of BVECs for future experiments and to determine the optimal seeding density to obtain confluence. To obtain more likely results it is further recommended to increase the time of the experiments from 24 hours to 48 hours of exposure to shear stress.
5. Discussion

The overall goal of this study was to determine whether BCECs and BVECs, in attached status, show functional similarities under shear stress. Therefore BCECs and BVECs were seeded on collagen coated glass slides and exposed to various amounts of shear stress in flowsystems. If the experiments precisely indicate that functional similarities exist between CECs and VECs, then CECs could possibly be recruited to the surface of tissue engineered vascular grafts in vivo to form an endothelial cell monolayer. Graft endothelialization needs to be promoted, since a complete endothelial cell monolayer does not form spontaneously on the luminal surface of a vascular graft after implantation in humans.

In Chapter 2 a medium is determined to replace the Clonetics medium, which is commercially available to culture BCECs. Based upon the proliferation of BCECs in five different types of media after consecutively two, three and four days of static culture, the human coronary artery endothelial cell (HCAEC) medium turned out to be the most suitable substitute for the Clonetics medium. This medium will be used to culture BCECs in the near future. Furthermore, the media assay showed that Flow medium, the medium used in flow experiments (chapter 3 and 4), inhibits the proliferation of BCECs. Earlier flow experiments performed on bovine aortic endothelial cells (BAECs) showed that the rate of cell proliferation in subconfluent monolayers is also decreased by fluid flow [5, 6]. These facts should be kept in mind while performing the cell shape and F-actin organization experiments on BVECs (chapter 4), since confluent monolayers are used for these experiments. For the single cell BCEC migration experiments (chapter 3) only few cells are needed and whether these cells proliferate or not, is of no importance. The single cell BCEC migration is determined by making images of BCECs at a constant time interval. What is important for these migration experiments is that the cells do not detach from the surface of the collagen coated glass slides, otherwise the migration of these cells cannot be tracked through time.

In chapter 3 the single cell BCEC migration is quantified after exposing the BCECs to approximately no shear stress (0.5 dynes/cm$^2$ = 0.05 Pa) and high shear stress (10 dynes/cm$^2$ = 1 Pa) for 24 hours. No significant difference was found in the total displacement of BCECs, as well as in the displacement of BCECs in the direction of the imposed fluid flow for the approximately no shear stress and the high shear stress cases. However it can be concluded that there is an absolute increase in migration over time for both shear stress cases. Furthermore two preference directions were found in which the BCECs tend to migrate under high shear stress. In one of the three experiments the BCECs migrated in the flow direction, while in another the BCECs migrated against the flow. Almost all of the findings above are contradictory to the hypothesis stated in paragraph 3.1 and to earlier in vitro experiments performed on bovine aortic endothelial cells (BAECs) [7]. The experiments showed that the cells exposed to high shear stress (10 dynes/cm$^2$) migrated in the direction of the imposed fluid flow. Whereas the BAECs exposed to approximately no shear stress (0.6 dynes/cm$^2$ = 0.06 Pa) did not show a preference direction and migrated in all directions equally. Furthermore, it appeared that no BAEC was able to migrate against the fluid flow for a prolonged period of time. At this point no conclusions can be made regarding the migration characteristics of BCECs. To determine the actual migration trend of BCECs, the single cell BCEC migration experiments have to be repeated using cells of the same passage. Furthermore, the same experiments have to be performed on BVECs as well; otherwise no real comparison can be made between the two cell types.
In chapter 4 the cell shape and F-actin organization of BVECs is quantified after 24 hours of static culture and exposure to shear stress (10 dynes/cm² = 1 Pa). Some alignment of F-actin filaments and lengthwise elongation of BVECs occurs in the direction of the imposed fluid flow, but no significant reduction is found in the shape index (SI). Furthermore this alignment and elongation is small compared to earlier experiments performed on BCECs (figure 4.3.1 and 4.3.3) [4]. In these experiments a clear distinction is found between the statically cultured BCECs and the BCECs exposed to shear stress. In the statically cultured BCECs, the F-actin filaments appear densely concentrated in the outer portion of the cytoplasm, with short filaments branching out in all directions. Whereas in BCECs exposed to shear stress, the F-actin filaments are uniformly spread throughout the cell, with the long filaments aligned parallel to the direction of the imposed fluid flow. Furthermore the BCEC monolayers shift from a random, confluent arrangement of rounded polygonal shapes to elongated shapes oriented lengthwise in the direction of fluid flow, causing a subsequent reduction in shape index (SI). The results obtained from the flow experiments performed on BVECs are also contradictory to the to the results obtained from earlier flow experiments performed on BAECs in vitro [7]. These experiments also showed a clear elongation of the cells in the flow direction after 48 hours of exposure to 9.4 dynes/cm² (= 0.94 Pa). The F-actin organization was not determined in this specific experiment. However, other experiments showed that alignment of F-actin filaments in the direction of imposed fluid flow occurs when exposing BAECs to various amounts of shear for 24 hours [5, 6]. Furthermore it is well known that VECs exhibit spindle shaped overall morphology in vivo and that the long axis of these spindle shaped cells is generally parallel to the long axis of the blood vessel or the direction of the blood flow [7]. The SI of VECs in vivo seems to depend on the level of shear stress as well, since the SI of VECs in areas of high shear stress is smaller compared to areas of low shear stress. Also, the F-actin filaments of VECs in a blood vessel are aligned parallel to the blood flow direction. At this point again no conclusion can be made regarding the cell shape and F-actin organization of BVECs. To determine whether similarities exist between BVECs and BCECs some changes have to be made to the current protocol (appendix IV). First the optimal seeding density to obtain confluence has to be determined. Second it’s recommended to increase the exposure time to shear stress form 24 hours to 48 hours. Since the experiments performed on BAECs showed significant elongation of the cells after 48 hours of exposure to shear stress [7]. Based upon the obtained results no comparisons can be made between BCECs and BVECs. So unfortunately, no similarities are found at the moment between both cell types to determine whether BCECs are suitable for graft endothelialization. However, after performing the recommended adjustments to the single cell migration experiments, as well as to the F-actin organization and cell shape experiments some good results will hopefully be obtained in the near future. Furthermore it has to be noted that together the experiments will give a good indication of the characteristics of BCECs and BVECs, since not only the F-actin organization and cell shape is studied at a given moment in time but also the migration is studied at a constant time interval. Whenever the experiments succeed it is further recommended to decrease the constraints of the current vascular model, so that it better corresponds to the situation in vivo. First of all this can be done by changing the celsubstrate from a collagen coated glass slide to a flexible layer of smooth muscle cells (co-culture). And second, by changing the laminar fluid flow into a cyclic flow that corresponds to the
5. Discussion

blood flow *in vivo*. Last the geometry of blood vessel is of great importance and should also be included into the vascular model.
References


Appendices

I  Culture Processes for BCECs
II Media Assay for BCECs
III Single Cell BCEC Migration Assay
IV Single Cell BCEC Migration after 12 Hours
V  Culture Processes for BVECs
VI F-actin Organization Cell Shape Assay for BVECs
I Culture Processes for BCECs

Reagents:

Collagen Type I Rat Tail (3.48 mg/ml acetic acid)  
BD Biosciences, Cat. No. 35-4236 (100 mg)  
Tissue Culture Rm → Fridge

Dimethyl Sulfoxide (DMSO)  
J.T.Baker, Cat. No. 922401 (500 ml)  
Dry Chemical Storage

Dulbecco’s Phosphate-Buffered Saline (DPBS) (w/o Calcium)  
Gibco, Cat. No. 14190-144 (500 ml)  
Tissue Culture Rm → Fridge

Trypsin-EDTA  
Gibco, Cat. No. 25300-054  
0.05% Trypsin, 0.53 mM EDTA (100 ml)  
Tissue Culture Rm → Fridge

Water, Double Processed Tissue Culture Water  
Sigma, Cat No. W3500 (500 ml)  
Tissue Culture Rm → Fridge

Medium:

Medium ECBM-2  
Clonetics, Cat. No. CC-3156 (500 mL)  
Tissue Culture Rm → Fridge

Ascorbic Acid  
Clonetics, Cat. No. CC-4116 (0.5 ml)  
Tissue Culture Rm → Fridge

Fetal Bovine Serum (FBS)  
Clonetics, Cat. No. CC-4101 (10 mL)  
Tissue Culture Rm → Fridge

hEGF  
Clonetics, Cat. No. CC-4317 (0.5 ml)  
Tissue Culture Rm → Fridge

Heparin  
Clonetics, Cat. No. CC-4396 (0.5 ml)  
Tissue Culture Rm → Fridge

Hydrocortisone  
Clonetics, Cat. No. CC-4112 (0.2 mL)  
Tissue Culture Rm → Fridge

hFGF-B  
Clonetics, Cat. No. CC 4113 (2 mL)  
Tissue Culture Rm → Fridge

GA-1000  
Clonetics, Cat. No. CC-4381 (0.5 ml)  
Tissue Culture Rm → Fridge

R3-IGF-1  
Clonetics, Cat. No. CC-4115 (0.5 ml)  
Tissue Culture Rm → Fridge

VEGF  
Clonetics, Cat. No. CC-4114 (0.5 ml)  
Tissue Culture Rm → Fridge
Appendix I

Solutions:

Collagen Coating Solution
Add 1.437 ml rat tail collagen type I in an autoclaved bottle and fill it up with tissue culture water until a 100 ml solution (50 µg/ml) is obtained. Filter the solution.

Trypsin/DPBS
Dilute trypsin with DPBS in a ratio of 1:1.

A. Preparation of the Clonetics media:

1. Take the bottle of Medium ECBM-2 and all singlequots with a sterile pipette:
   - 10 ml FBS
   - 0.2 ml Hydrocortison
   - 2 ml hFGF-B
   - 0.5 ml VEGF
   - 0.5 ml R3–IGF-1
   - 0.5 ml Ascorbic Acid
   - 0.5 ml hEGF
   - 0.5 ml GA-1000
   - 0.5 ml Heparin
   Optional: Filter the obtained Clonetics media.

B. Procedure for plating BCECs:

1. Coat the tissue culture flask(s):
   a) Coat the new culture flask(s) with collagen coating (0.5 ml per 25 cm²).
      (Normally one vial of cells is plated in a T-75 flask.)
   b) The collagen coating has to adhere for one hour.
   c) Remove the remnants of the collagen coating from the new flask(s).

2. Place reagents and solutions into a warm water bath until they have warmed.

3. Plate the cells:
   a) Obtain a vial of cells from the cryo-conservation container.
   b) Thaw the vial under a warm water tab.
   c) Place the cells into the coated flask(s) with a sterile pipette.
   d) Add 5 ml/25 cm² media to the flask(s).
   e) Replace the media with fresh media after 24 hours to remove the remnants of the freezing media.
   f) After the first media change, the media of the cells is refreshed every other day.
   g) The cells are split in a ratio of 1:3, when they reach 80-90 % confluence.

C. Procedure for trypsinizing BCECs:

1. Place reagents and solutions into a warm water bath until they have warmed and coat the tissue culture flasks.

2. Trypsinize and reseed the cells:
   a) Remove the old media from flasks containing the cells.
   b) Add 5 mL/25 cm² of DPBS to the flask.
   c) Place cells in the incubator for 10 minutes.
   d) Remove the DPBS.
   e) Add 1 mL/25 cm² of the diluted trypsin.
   f) Wait 3-5 minutes with flask in the incubator.
g) After 3 minutes, view the cells under a microscope to ensure they have released from the flask wall. If the cells have not released from the wall, lightly tap on the side of the flask with your hand to remove the extras. If the cells still have not released from the wall, place them back into the incubator for another 2 minutes.

h) Add 5 mL/ 25 cm² of media to neutralize the trypsin.

i) Rinse the cells off the side of the flask using the cell suspension.

j) Place cells into new coated culture flasks.

D. Procedure for freezing the BCECs:

1. Prepare a freezing solution:
   - 90% BCEC media
   - 10% DMSO

2. Label vials:
   - cell type
   - passage
   - freezing date
   - initials

3. Freezing the cells:
   a) Trypsinize the cells of a T-75 flask(s) using the above procedure.
   b) Transfer the cell solution to a 50 ml tube.
   c) Centrifuge the cell solution in the tube for 5 min. at 1000 rpm.
   d) Remove the supernatant of the tube.
   e) Add 3 ml of freezing solution to the pellet for each T-75 flask from which the cells have spun down.
   f) Resuspend the cells in the freezing solution.
   g) Add 1 ml of cell solution into each vial.
   h) Freeze at -70° C for one day in ETOH cold tray in freezer.
   i) Place the vials in the liquid nitrogen cryo-conservation container.

Notes:

- The BCECs are only used for experiments at passage 6 to 10.
II Media Assay for BCECs

Reagents:

Collagen Type I Rat Tail (3.48 mg/ml acetic acid)  
BD Biosciences, Cat. No. 35-4236 (100 mg)  
Tissue Culture Rm → Fridge

Dulbecco’s Phosphate-Buffered Saline (DPBS) (w/o Calcium)  
Gibco, Cat. No. 14190-144 (500 ml)  
Tissue Culture Rm → Fridge

Hepes Buffer (1M)  
Fisher Scientific, Cat. No.BP299-100 (100 ml)  
Tissue Culture Rm → Fridge

Water, Double Processed Tissue Culture Water  
Sigma, Cat No. W3500 (500 ml)  
Tissue Culture Rm → Fridge

Medium MCDB 131  
Mediatech Cellgro, Cat. No. 99-555-CV (2 bottles of 500mL)  
Tissue Culture Rm → Fridge

Ascorbic Acid  
Sigma, Cat. No. A-4034 (500g/bottle)  
Dry Chemical Storage

Fetal Bovine Serum (FBS)  
Mediatech Cellgro, Cat. No. 35-011-CV (500 ml)  
Tissue Culture Rm → Freezer

hEGF  
Gibco, Cat. No. 13247-051 (100 µg)  
Tissue Culture Rm → Freezer

hFGF-Basic  
Pepro Tech, Cat. No. 100-18B (50 µg)  
Tissue Culture Rm → Freezer

HyQ Penicillin-Streptomycin  
Mediatech Cellgro, Cat. SV30010 (100 ml)  
Tissue Culture Rm → Freezer

IGF-1  
Gibco, Cat. No. 13245-014 (10 µg at 100 µg/mL)  
Tissue Culture Rm → Freezer

Hydrocortisone  
Sigma, Cat. No. H-4001 (5g/bottle)  
Dry Chemical Storage

L-Glutamine  
Mediatech Cellgro, Cat. No. 25-005-C1 (100 ml)  
Tissue Culture Rm → Freezer

Vascular Endothelial Growth Factor (VEGF)  
Sigma, Cat. No. V-7259 (10 µg)  
Tissue Culture Rm → Freezer

Medium ECBM-2  
Clonetics, Cat. No. CC-3156 (500 mL)  
Tissue Culture Rm → Freezer

Ascorbic Acid  
Clonetics, Cat. No. CC-4116 (0.5 ml)  
Tissue Culture Rm → Freezer

Fetal Bovine Serum (FBS)  
Clonetics, Cat. No. CC-4101 (10 mL)  
Tissue Culture Rm → Freezer

GA-1000  
Clonetics, Cat. No. CC-4381 (0.5 ml)  
Tissue Culture Rm → Freezer
The Effect of Shear Stress on CECs and VECs

Heparin
Clonetics, Cat. No. CC-4396 (0.5 ml)  Tissue Culture Rm → Freezer

Hydrocortisone
Clonetics, Cat. No. CC-4112 (0.2 mL)  Tissue Culture Rm → Freezer

hEGF
Clonetics, Cat. No. CC-4317 (0.5 ml)  Tissue Culture Rm → Freezer

hFGF-B
Clonetics, Cat. No. CC 4113 (2 mL)  Tissue Culture Rm → Freezer

R3-IGF-1
Clonetics, Cat. No. CC-4115 (0.5 ml)  Tissue Culture Rm → Freezer

VEGF
Clonetics, Cat. No. CC-4114 (0.5 ml)  Tissue Culture Rm → Freezer

Solutions:
Sterile Phosphate Buffered Saline (PBS)
Sigma P-3813 (Made according to package)  Tissue Culture Rm → Fridge

Collagen Solution Type I Rat Tail (50 µg/ml)
Add 1.437 ml rat tail collagen type I in an autoclaved bottle and fill it up with tissue culture water until a 100 ml solution (50 µg/ml) is obtained. Filter the solution.  Tissue Culture Rm → Fridge

Aliquots
hFGF-B (1µg/ml tissue culture water).  Tissue Culture Rm → Freezer
hEGF (5 µg/ml tissue culture water).  Tissue Culture Rm → Freezer
VEGF (1 µg/ml tissue culture water).  Tissue Culture Rm → Freezer
IGF-1 (1 µg/ml tissue culture water).  Tissue Culture Rm → Freezer
Hydrocortisone (2 mg/ml tissue culture water).  Tissue Culture Rm → Freezer
Ascorbic Acid (25 mg/ml tissue culture water).  Tissue Culture Rm → Freezer
hEGF (0.25 µg/mL tissue culture water)  Tissue Culture Rm → Freezer

A. Procedure:

One to two weeks prior to the actual experiment a small vial of BCECs has to be taken out of the cryoconservation container and cultured to obtain two nearly confluent T-75 flasks on day 2.

Day 0:
1. Place all Reagents in a warm water bath until they have completely thawed and/or warmed.

2. Preparation of HCAEC medium:
   a) Take one of the bottles of MCDB 131 medium and add the following components with a sterile pipette:
      • 5 mL L-Glutamine (1%).
Appendix II

- 5 mL Penicillin (1%).
- 25 mL FBS (5%).
- 1 ml hFGF-B (0.002 µg/ml).
- 1 ml hEGF (0.01 µg/ml).
- 0.5 ml VEGF (0.001 µg/ml).
- 1 ml IGF-1 (0.002 µg/ml).
- 0.25 ml Hydrocortisone (0.001 mg/ml).
- 1 ml Ascorbic Acid (0.05 mg/ml).
Optional: Filter the obtained HCAEC medium.

3. Preparation of HCAEC medium supplemented with Hepes (6mM):
   a) Take half a bottle of the unfiltered HCAEC medium (~ 267.5 ml) and put it into a 500 ml filter unit.
   b) Add 1.605 ml Hepes buffer (6mM).
Optional: Filter the obtained HCAEC medium supplemented with Hepes.

4. Preparation of Clonetics medium:
   a) Take the bottle of Medium ECBM-2 and add the following components with a sterile pipette:
      - 10 ml FBS
      - 0.2 ml Hydrocortison
      - 2 ml hFGF-B
      - 0.5 ml VEGF
      - 0.5 ml R3–IGF-1
      - 0.5 ml Ascorbic Acid
      - 0.5 ml hEGF
      - 0.5 ml GA-1000
      - 0.5 ml Heparin
Optional: Filter the obtained Clonetics medium.

5. Preparation of Flow medium:
   a) Take the other bottle of MCDB 131 medium and add the following components with a sterile pipette.
      - 5 ml L-Glutamine (1%)
      - 5 ml Penicilline-Streptomycin (1%)
      - 25 ml FBS (5%)
      - 1 ml hFGF-B (0.002 µg/mL)
      - 1 ml hEGF (0.0005 µg/mL)
Optional: Filter the obtained Flow medium.

6. Preparation of Flow medium supplemented with Hepes (6mM):
   a) Take half a bottle of the unfiltered Flow medium (~267.5 ml) and put it into a 500 ml filter unit.
   b) Add 1.605 ml Hepes buffer (6mM)
Optional: Filter the obtained Flow medium supplemented with Hepes.

Day 1:
Two nearly confluent T-75 flaks should be obtained (~ 6,000,000 cells) on day 1.

1. Coat tissue culture flasks:
   a) Coat 15 T-25 flasks with 0.5 ml 25 cm² collagen solution (50 µl/ml).
   b) The collagen coating has to adhere for one hour.
   c) Remove the remnants of the collagen coating from the flasks after one hour.

2. Trypsinize the cells:
   a) Remove the old medium from the two T-75 flasks.
   b) Add 15 ml DPBS to each flask.
   c) Incubate for 10 minutes.
   d) Remove DPBS.
e) Add 3 ml trypsin to each flask.
f) Wait 5 min. with the T-75 flasks in the incubator.
g) Check if cells released from walls.
h) If so, add ~ 12 ml regular medium (Clonetics medium) in each flask to neutralize the trypsin.
i) Transfer the entire volume of each flask to one 50 ml tube.

3. Count the cells:
   a) Centrifuge the cell solutions in the tube for 5 min. at 1000 rpm.
   b) Remove the supernatant of the tube.
   c) Add 10 ml regular media to the tube and resuspend the cells.
   d) Put 20 ml (sterile) PBS in a cuvet.
   e) Add 200 µl of the cell solution to the specific cuvet.
   f) Use the Coulter counter according to directions on the bench.

4. Seeding the cells:
   a) Place 2 ml cell suspension, containing approximately 250,000 cells, in each T-25 flask.
   b) Add another 3 ml of regular medium in each flask.

**Day 2:**
1. Change the media of the flasks
   a) Remove the old media from the flask.
   b) Add 5 ml of the specific media into each flask.

**Day 4:**
1. Trypsinize the cells:
   This procedure has to be carried out in groups of five T-25 flasks, in which each flask contains a different kind of media.
   a) Remove the old media from the flasks.
   b) Add 5 ml DPBS to each flask.
   c) Incubate for 10 minutes.
   d) Remove DPBS.
   e) Add 1 ml trypsin to each flask.
   f) Wait 5 min. with the T-25 flasks in the incubator.
   g) Check if cells released from walls.
   h) If so, add 5 ml media to neutralize trypsin.
   i) Transfer the entire volume of each flask to 50 ml tubes.

2. Count the cells of each tube using a coulter counter:
   a) Centrifuge the cell solutions in the tubes for 5 min. at 1000 rpm.
   b) Remove the supernatant of each tube.
   c) Add 1 ml of the specific media to according tube and resuspend the cells.
   d) Put 20 ml PBS in five cuvets.
   e) Add 200 µl of each cell solution to the specific cuvet.
   f) Use the Coulter counter according to directions on the bench (each cell solution has to be counted three times).

After counting all fifteen T-25 flasks the cells have to be reseeded into 15 new collagen-coated T-25 flasks. The response of the BCECs to the five different types of media can be determined in time, by counting the cells after respectively 2, 3 and 4 days of static culture, in which the media is changed every other day. The media assay should not be performed on cells that are nearly confluent, otherwise the results will be unreliable because endothelial cells do not grow on top of each other, they only form monolayers. Therefore a maximum is to set a cell confluence of 60-70% for use in the media assay.
### III Single Cell Migration Assay for BCECs

**Reagents:**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
<th>Catalog Number</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen Type I Rat Tail (3.48 mg/ml acetic acid)</td>
<td>BD Biosciences</td>
<td>35-4236</td>
<td>Tissue Culture Rm → Fridge</td>
</tr>
<tr>
<td>Dimethyl Sulfoxide (DMSO)</td>
<td>J.T. Baker</td>
<td>922401</td>
<td>Dry Chemical Storage</td>
</tr>
<tr>
<td>Dulbecco’s Phosphate-Buffered Saline (w/o Calcium)</td>
<td>Gibco, Cat. No. 14190-144</td>
<td></td>
<td>Tissue Culture Rm → Fridge</td>
</tr>
<tr>
<td>Trypsin-EDTA</td>
<td>Gibco, Cat. No. 25300-054</td>
<td></td>
<td>Tissue Culture Rm → Fridge</td>
</tr>
<tr>
<td>0.05% Trypsin, 0.53 mM EDTA (100 ml)</td>
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<td></td>
<td>Tissue Culture Rm → Fridge</td>
</tr>
<tr>
<td>Vybrant™ CFDA SE Cell Tracer Kit</td>
<td>Molecular Probes, Cat. No. V 12883</td>
<td></td>
<td>Tissue Culture Rm → Freezer</td>
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<tr>
<td>Water, Double Processed Tissue Culture Water</td>
<td>Sigma, Cat No. W3500 500 ml</td>
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<td>Tissue Culture Rm → Fridge</td>
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<tr>
<td><strong>Medium ECBM-2</strong></td>
<td>Clonetics, Cat. No. CC-3156 (500 mL)</td>
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<td>Tissue Culture Rm → Fridge</td>
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<td>Ascorbic Acid</td>
<td>Clonetics, Cat. No. CC-4116 (0.5 ml)</td>
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<td>Tissue Culture Rm → Fridge</td>
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<td>Fetal Bovine Serum (FBS)</td>
<td>Clonetics, Cat. No. CC-4101 (10 mL)</td>
<td></td>
<td>Tissue Culture Rm → Fridge</td>
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<tr>
<td>hEGF</td>
<td>Clonetics, Cat. No. CC-4317 (0.5 ml)</td>
<td></td>
<td>Tissue Culture Rm → Fridge</td>
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<td>Heparin</td>
<td>Clonetics, Cat. No. CC-4396 (0.5 ml)</td>
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<td>Tissue Culture Rm → Fridge</td>
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<tr>
<td>Hydrocortisone</td>
<td>Clonetics, Cat. No. CC-4112 (0.2 mL)</td>
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<td>Tissue Culture Rm → Fridge</td>
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<tr>
<td>hFGF-B</td>
<td>Clonetics, Cat. No. CC 4113 (2 mL)</td>
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<td>Tissue Culture Rm → Fridge</td>
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<td>GA-1000</td>
<td>Clonetics, Cat. No. CC-4381 (0.5 ml)</td>
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<td>Tissue Culture Rm → Fridge</td>
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<td>R²-IGF-1</td>
<td>Clonetics, Cat. No. CC-4115 (0.5 ml)</td>
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<td>Tissue Culture Rm → Fridge</td>
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<td>VEGF</td>
<td>Clonetics, Cat. No. CC-4114 (0.5 ml)</td>
<td></td>
<td>Tissue Culture Rm → Fridge</td>
</tr>
<tr>
<td><strong>Medium MCDB 131</strong></td>
<td>Mediatech Cellgro, Cat. No. 99-555-CV</td>
<td></td>
<td>Tissue Culture Rm → Fridge</td>
</tr>
</tbody>
</table>
Fetal Bovine Serum (FBS)  
Mediatech Cellgro, Cat. No. 35-011-CV (500 ml)  
Tissue Culture Rm → Freezer

hEGF  
Gibco, Cat. No. 13247-051 (100 µg)  
Tissue Culture Rm → Freezer

hFGF-Basic  
Pepro Tech, Cat. No. 100-18B (50 µg)  
Tissue Culture Rm → Freezer

HyQ Penicillin-Streptomycin  
Mediatech Cellgro, Cat. SV30010 (100 ml)  
Tissue Culture Rm → Freezer

L-Glutamine  
Mediatech Cellgro, Cat. No. 25-005-C1 (100 ml)  
Tissue Culture Rm → Freezer

Solutions:

Clonetics medium (see appendix I, II for medium preparation)  
Tissue Culture Rm → Fridge

Collagen Coating Solution  
Add 1.437 ml rat tail collagen type I in an autoclaved bottle and fill it up with tissue culture water until a 100 ml solution (50 µg/ml) is obtained. Filter the solution.

Flow medium (see appendix II for medium preparation)  
Tissue Culture Rm → Fridge

Phosphate Buffered Saline (PBS)  
Sigma P-3813 (Made according to package)  
Filter the solution

Stock Vybrant  
Add 90 µl DMSO to the vial of vybrant to obtain a 10 mM stock solution.

Trypsin/DPBS  
Dilute Trypsin with DPBS in a ratio of 1:1.  
Tissue Culture Rm → Fridge

Cells:

Baboon Circulating Endothelial Cells (BCEC)  
Incubator

A. Autoclave:

1. Instruments:
   - 4 flat tweezers
   - 2 specimen spatulas

2. For each sample:
   - Glass slide with clean silicone gasket adhered
   - Flow chamber with bypass tube
   - One complete and connected flow loop apparatus including: reservoir cup and top, pressure vessel, necessary tubing
3. Per experiment:
   - Microscope mountable aluminum frame bottom with rubber gasket on stainless steel tray
   - Microscope mountable aluminum frame top

B. UV sterilize flow spacers:

1. UV sterilize one yellow spacer per flow loop.
   a) Put yellow spacer in a plastic petri dish underneath the UV lamp with lid off, inside up.
   b) Turn on UV light and sterilize for 1½ hour.
   c) Turn off UV light.
   d) Place top on petri dish, and flip yellow spacer over, remove bottom of petri dish (which should now be on top).
   e) Turn on UV light and sterilize for another 1½ hour.

C. Procedure:

Day 1:
1. Coat tissue glass slides:
   a) Place clean slide with adhered collagen coating silicone gasket in a square petri dish.
   b) Add 400 \( \mu l \) of collagen solution.
   c) Allow to sit in incubator overnight for passive absorption for 24 hours.

Day 2:
1. Label the cells with 5\( \mu M \) Vybrant CFDA:
   a) Prepare 5 \( \mu M \) Vybrant by adding 10 \( \mu l \) of stock Vybrant (10mM) to 20 ml of sterile PBS.
   b) Label the cells in a T-25 with 5 ml of 5 \( \mu M \) Vybrant.
   c) Wait 15 minutes in incubator.
   d) Replace labeling media with fresh complete media and incubate for 2 hours.

2. Preparing slides for cell seeding:
   a) Remove collagen solution from slide.
   b) Replace it with 400 \( \mu l \) of sterile PBS.
   c) Incubate for 1 hour.

3. Trypsinize cells:
   a) Add 5 ml DPBS to cells.
   b) Incubate for 10 minutes.
   c) Remove DPBS.
   d) Add 1 ml trypsin.
   e) Wait 3 min. with the T-25 in the incubator.
   f) Check if cells released from walls.
   g) If so, add 5 ml media to neutralize trypsin.
   h) Transfer entire volume to a 50 ml tube.

4. Count cells:
   a) Centrifuge cell solution in 50 ml tube for 5 min. at 1000 rpm.
   b) Remove the supernatant.
   c) Add 5 ml media and resuspend the cells.
   d) Put 20 ml PBS in a cuvet.
   e) Add 200 \( \mu l \) of the cell solution to the cuvet.
   f) Use the Coulter counter according to directions on the bench.
   g) Resuspend the cells to a final concentration of 3,750 cells/ml (1000 cells/cm\(^2\)).

5. Seed cells on slides:
a) Put 400 µl (~1,500 cells) of concentrated cell solution into center of gasket.
b) Allow cells to adhere for 1 hour in incubator.
c) Remove the silicone gasket from the slide with a tweezers.
d) Cover the slide with 25 ml flow media, and place it in incubator.

**Day 4:**

1. Prepare microscope for imaging:
   a) Turn on fluorescent lamp.
   b) Turn on power strip
   c) Turn on heater
   d) Turn on CO2 controller

2. Place slides into flow chamber in laminar flow hood:
   a) Add 125 ml of flow media to reservoir.
   b) Allow media to flow through tubing in incubator for 1 hour.
   c) Remove aluminum frame bottom from autoclave bag and keep the rubber gasket inside the frame bottom.
   d) Place slides with cells on top of rubber gasket.
   e) Place yellow spacer(s) on top.
   f) Put flow chamber with bypass tube on top of that structure.
   g) Cover with the aluminum frame top, and screw top and bottom plates together evenly.
   h) Connect to flow loop on the side portals of the flow chamber (Be sure not to drip media.)
   i) Attach to pump ensuring that the flow will be pulled in the right direction.
   j) Tilt pressure vessel so that media will fill it up beyond the outlet to prevent air bubbles from passing through flow chamber.
   k) Once there is no air in the system, clamp off bubble bypass loops (the pressure is then increased).
   l) Be sure that there are no leaks.
   m) Release bubble bypass loops.
   n) Move flow chamber onto microscope.
   o) Reclamp bubble bypass loops (the air bubbles will remove from the glass slide).
   p) Start image acquisition software.
   q) Program 16 fields for imaging for each sample.
   r) Image fields every 30 minutes for 24 hours.

**Notes:**

**Day 1:** Autoclave, collagen coat slides, UV sterilize (about 3 hours)

**Day 2:** Label and seed cells (about 3 hours)

**Day 4:** Start flow loop
IV Single Cell BCEC Migration after 12 Hours

The 12 hour migration of BCECs of experiment 8 at 0.5 dynes/cm² (0.05 Pa) (a) and at 10 dynes/cm² (= 1 Pa) (b). The BCECs used are of passage 7.

The 12 hour migration of BCECs of experiment 9 at 0.5 dynes/cm² (0.05 Pa) (a) and at 10 dynes/cm² (= 1 Pa) (b). The BCECs used are of passage 8.
The 12 hour migration of BCECs of experiment 10 at 0.5 dynes/cm² (= 0.05 Pa) (a) and at 10 dynes/cm² (= 1 Pa) (b). The BCECs used are of passage 9.
V Culture Processes for BVECs

Reagents:

Collagen Type I Rat Tail (3.48 mg/ml acetic acid)
BD Biosciences, Cat. No. 35-4236 (100 mg)

Dimethyl Sulfoxide (DMSO)
J.T.Baker, Cat. No. 922401 (500 ml)

Dulbecco’s Phosphate-Buffered Saline (DPBS) (w/o Calcium)
Gibco, Cat. No. 14190-144 (500 ml)

Trypsin-EDTA
Gibco, Cat. No. 25300-054
0.05% Trypsin, 0.53 mM EDTA (100 ml)

Water, Double Processed Tissue Culture Water
Sigma, Cat No. W3500 (500 ml)

Medium:

Medium 199 (1X), liquid
Gibco, Cat. No. 12350-039 (500 ml)

Anitbiotic-Antimycotic
Gibco, Cat. No. 15240-062 (100 ml)

Endothelial Mitogen
Biomedical Technologies Inc., Cat. No. BT-203 (50 mg)

Fetal Bovine Serum (FBS)
Mediatech Cellgro, Cat. No. 35-011-CV (500 ml)

Heparin Sodium Salt
Grade I-A: From Porcine Intestinal (187 USP units/mg)
Sigma, Cat. No. H-3149 (100,000 units)

L-Glutamine
Mediatech Cellgro, Cat. No. 25-005-C1 (100 ml)

Solutions:

Collagen Coating Solution
Add 1.437 ml rat tail collagen type I in an autoclaved bottle and fill it up with tissue culture water until a 100 ml solution (50 µg/ml) is obtained.
Filter the solution.

Endothelial Mitogen Solution
Add 2 ml of medium 199 to the bottle.

Heparin solution
Add 106.95 mg of heparin to 2 ml of tissue culture water.
Filter the solution.

Trypsin/DPBS
Dilute trypsin with DPBS in a ratio of 1:1.
A. Preparation of the BVEC Media:

1. Take the bottle of medium 199 and add the following components with a sterile pipette:
   - 100 ml FBS.
   - 6 ml L-Glutamine.
   - 6 ml Antibiotics-Antimycotics.
   - 0.8 ml Heparin solution.
   - 1 ml Endothelial Mitogen solution.
   Optional: Filter the obtained BVEC Media.

B. Procedure for plating BVECs:

1. Coat the tissue culture flask(s):
   a) Coat the new culture flask(s) with collagen coating (0.5 ml per 25 cm²).
      ( Normally one vial of cells is plated in a T-75 flask.)
   b) The collagen coating has to adhere for one hour.
   c) Remove the remnants of the collagen coating from the new flask(s).

2. Place reagents and solutions into a warm water bath until they have warmed.

3. Plate the cells:
   a) Obtain a vial of cells from the cryo-conservation container
   b) Thaw the vial under a warm water tab.
   c) Place the cells into the coated flask(s) with a sterile pipette.
   d) Add 5 ml/ 25 cm² media to the flask(s).
   e) Replace the media with fresh media after 24 hours to remove the remnants of the freezing media.
   f) After the first media change, the media of the cells is refreshed every other day.
   g) The cells are split in a ratio of 1:3, when they reach 80-90 % confluence.

C. Procedure for trypsinizing BVECs:

1. Place reagents and solutions into a warm water bath until they have warmed and coat the tissue culture flasks.

2. Trypsinize and reseed the cells:
   a) Remove the old media from flasks containing the cells.
   b) Add 5 mL/ 25 cm² of DPBS to the flask.
   c) Place cells in the incubator for 10 minutes.
   d) Remove the DPBS.
   e) Add 1 mL/25 cm² of the diluted trypsin.
   f) Wait 3-5 minutes with flask in the incubator.
   k) After 3 minutes, view the cells under a microscope to ensure they have released from the flask wall. If the cells have not released from the wall, lightly tap on the side of the flask with your hand to remove the extras. If the cells still have not release from the wall, place them back into the incubator for another 2 minutes.
   g) Add 5 mL/ 25 cm² of Media to neutralize the trypsin.
   h) Rinse the cells off the side of the flask using the cell suspension.
   i) Place cells into new coated culture flasks.

D. Procedure for freezing the BVECs:

1. Prepare a freezing solution:
   - 90% BVEC media
   - 10% DMSO
2. Label vials:
   - cell type
   - passage
   - freezing date
   - initials

3. Freezing the cells:
   a) Trypsinize the cells of a T-75 flask (s) using the above procedure.
   b) Transfer the cell solution to a 50 ml tube.
   c) Centrifuge the cell solution in the tube for 5 min. at 1000 rpm.
   d) Remove the supernatant of the tube.
   e) Add 3 ml of freezing solution to the pellet for each T-75 flask from which the cells have spun down.
   f) Resuspend the cells in the freezing solution.
   g) Add 1 ml of cell solution into each vial.
   h) Freeze at -70°C for one day in ETOH cold tray in freezer.
   i) Place the vials in the liquid nitrogen cryo-conservation container.

Notes:

- The BVEC are only used for experiments at passage 6 to 10.
VI  F-actin Organization and Cell Shape Assay for BVECs

Reagents:

Albumin Bovine (BSA)  
Sigma, Cat. No. A7030  
Gel img. Rm → Refrigerator

Anitbiotic-Antimycotic  
Gibco, Cat. No. 15240-062 (100 ml)  
Tissue Culture Rm → Freezer

Collagen Type I Rat Tail (3.48 mg/ml acetic acid)  
BD Biosciences, Cat. No. 35-4236 (100 mg)  
Tissue Culture Rm → Fridge

Dulbecco’s Phosphate-Buffered Saline (DPBS) (w/o Calcium)  
Gibco, Cat. No. 14190-144  
Tissue Culture Rm → Fridge

Formaldehyde, 20%  
Tousimis, Cat. No. #1008A  
Gel img. Rm → Refrigerator

Oregon Green 488 Phalloidin  
Molecular Probes , Cat. No. O-7466  
Freezer

Prolong Antifade Kit  
Molecular Probes, Cat. No. P-7481  
Freezer

Triton-X 100  
Sigma, Cat. No. 12H0766  
Dry Chemical Storage

Trypsin-EDTA  
Gibco, Cat. No. 25300-054. 0.05% Trypsin, 0.53 mM EDTA (100 ml)  
Tissue Culture Rm → Fridge

Water, Double Processed Tissue Culture Water  
Sigma, Cat No. W3500 (500 ml)  
Tissue Culture Rm → Fridge

Medium 199 (1X)  
Gibco, Cat. No. 12350-039 (500 ml)  
Tissue Culture Rm → Fridge

Endothelial Mitogen  
Biomedical Technologies Inc. , Cat. No. BT-203 (50 mg)  
Dry Chemical Storage

Fetal Bovine Serum (FBS)  
Mediatech Cellgro, Cat. No. 35-011-CV (500 ml)  
Tissue Culture Rm → Freezer

Heparin Sodium Salt  
Sigma, Cat. No. H-3149 (100,000 units) Grade I-A: From Porcine Intestinal (187 USP units/mg)  
Dry Chemical Storage

L-Glutamine  
Mediatech Cellgro, Cat. No. 25-005-C1 (100 ml)  
Tissue Culture Rm → Freezer

Medium MCDB 131  
Mediatech Cellgro, Cat. No. 99-555-CV  
Tissue Culture Rm → Fridge

Fetal Bovine Serum (FBS)  
Mediatech Cellgro, Cat. No. 35-011-CV (500 ml)  
Tissue Culture Rm → Freezer
Appendix VI

hEGF
  Gibco, Cat. No. 13247-051 (100 µg)  Tissue Culture Rm → Freezer

hFGF-Basic
  Pepro Tech, Cat. No. 100-18B (50 µg)  Tissue Culture Rm → Freezer

HyQ Penicillin-Streptomycin
  Mediatech Cellgro, Cat. SV30010 (100 ml)  Tissue Culture Rm → Freezer

L-Glutamine
  Mediatech Cellgro, Cat. No. 25-005-C1 (100 ml)  Tissue Culture Rm → Freezer

Solutions:

BVEC medium (see appendix V for medium preparation)  Tissue Culture Rm → Fridge

Collagen Coating Solution
  Add 1.437 ml rat tail collagen type I in an autoclaved bottle and fill it up with tissue culture water until a 100 ml solution (50 µg/ml) is obtained. Filter the solution.

Flow medium (see appendix II for medium preparation)  Tissue Culture Rm → Fridge

Phosphate Buffered Saline (PBS)
  Sigma P-3813 (Made according to package)  Tissue Culture Rm → Fridge
  Filter the solution

Trypsin/DPBS
  Dilute trypsin with DPBS in a ratio of 1:1.  Tissue Culture Rm → Fridge

Cells:

Baboon Vascular Endothelial Cells (BVEC)  Incubator

A.  Autoclave:

1. Instruments:
   • 4 flat tweezers
   • 2 specimen spatulas

2. For each sample:
   • Glass slide with clean silicone gasket adhered.
   • Flow chamber with bypass tube.
   • One complete and connected flow loop apparatus including: reservoir cup and top, pressure vessel, necessary tubing.

3. Per experiment:
   • Microscope mountable aluminum frame bottom with rubber gasket on stainless steel tray.
   • Microscope mountable aluminum frame top.

B.  UV sterilize flow spacers:

1. UV sterilize one yellow spacer per flow loop.
   a)  Put yellow spacer in a plastic petri dish underneath the UV lamp with lid off, inside up.
b) Turn on UV light and sterilize for 1½ hour.
c) Turn off UV light.
d) Place top on petri dish, and flip yellow spacer over, remove bottom of petri dish (which should now be on top).
e) Turn on UV light and sterilize for another 1½ hour.

C. Procedure:

Day 1:
1. Coat tissue glass slides:
   a) Place clean slide with adhered collagen coating silicone gasket in a square petri dish.
   b) Add 2 ml of collagen solution.
   c) Allow to sit in incubator overnight for passive absorption for 24 hours.

Day 2:
1. Preparing slides for cell seeding:
   a) Remove collagen solution from slide.
   b) Replace it with 2 ml of sterile PBS.
   c) Incubate for 1 hour.

2. Trypsinize cells:
   a) Add 5 ml DPBS to cells.
   b) Incubate for 10 minutes.
   c) Remove DPBS.
   d) Add 1 ml trypsin.
   e) Wait 3-5 min. with the T-25 in the incubator.
   f) Check if cells released from walls.
   g) If so, add 5 ml media to neutralize trypsin.
   h) Transfer entire volume to a 50 ml tube.

3. Count cells:
   a) Centrifuge cell solution in 50 ml tube for 5 min. at 1000 rpm.
   b) Remove the supernatant.
   c) Add 5 ml media and resuspend the cells.
   d) Put 20 ml PBS in a cuvet.
   e) Add 200 μl of the cell solution to the cuvet.
   f) Use the Coulter counter according to directions on the bench.
   g) Resuspend the cells to a final concentration of 200,000 cells/ml (25,000 cells/ cm²)

4. Seed cells on slides:
   a) Put 1ml (~200,000 cells) of concentrated cell solution into center of gasket.
   b) Allow cells to adhere for 1 hour in incubator.
   c) Remove the silicone gasket from the slide with a tweezers.
   d) Cover the slide with 25 ml flow media, and place it in incubator.

Day 4:
1. Place slides into flow chamber in laminar flow hood:
   a) Add 125 ml of flow media to reservoir.
   b) Allow media to flow through tubing in incubator for 1 hour.
   c) Remove aluminum frame bottom from autoclave bag and keep the rubber gasket inside the frame bottom.
   d) Place slides with cells on top of rubber gasket.
   e) Place yellow spacer(s) on top.
   f) Put flow chamber with bypass tube on top of that structure.
   g) Cover with the aluminum frame top, and screw top and bottom plates together evenly.
   h) Connect to flow loop on the side portals of the flow chamber (Be sure not to drip media).
i) Attach the flow loop to the pump in the incubator ensuring that the flow will be pulled in the right direction.  

j) Tilt pressure vessel so that media will fill it up beyond the outlet to prevent air bubbles from passing through flow chamber. 

k) Once there is no air in the system, clamp off bubble bypass loops (the pressure is then increased). 

l) Be sure that there are no leaks. 

m) Release bubble bypass loops. 

n) Allow media to flow through the flow loop for 24 hours. 

**Day 5:**

1. **Staining of the samples:**
   a) Place the slides in a glass petri dish. 
   b) Wash the sample twice with 10 ml prewarmed PBS. 
   c) Fix the sample in 10 ml formaldehyde solution for 10 minutes at room temperature. 
   d) Wash the sample twice with 10 ml PBS. 
   e) Extract each sample with 10 ml Triton solution for 3-5 minutes. 
   f) Wash the sample twice with 10 ml PBS. 
   g) Incubate the fixed cells with 10 ml BSA solution for 20-30 minutes. 
   h) Dilute 5 µl Oregon Green methanolic stock solution into 200 µl BSA solution for each slide to be stained. 
   i) Place the staining solution on the sample and put a cover on the petri dish. Allow sample to incubate at room temperature for 20 minutes. 
   j) Wash the samples twice with 10 ml PBS. 

2. **Use the Prolong Antifade Kit**
   a) Add approximately 1 ml of ProLong mounting medium (component B) to one of the brown vials containing the ProLong antifade reagent (Component A). 
   b) Mix by gently pipetting until antifade no longer adheres to the sides of the vial. Be careful not to generate any bubbles. 
   c) Apply 100 µl of the mixture to the sample and gently put a cover slip on the sample with no trapped air. 
   d) Apply transparent nail polish at border of the cover slip to prevent the sample from drying out. 

**Day 6:**

1. Image the samples using confocal microscopy. 

**Notes:**

Day 1: Autoclave, collagen coat slides, UV sterilize (about 3 hours)
Day 2: Label and seed cells (about 3 hours)
Day 4: Start flow loop
Day 5: Stain the samples
Day 6: Confocal microscopy