# Endothelial Cell Culture Under Perfusion with the ibidi Pump System and µ-Slide I $^{0.6}$ Luer

## 1. General Information

The following application note describes the protocol for a perfusion assay that combines the ibidi Pump System with a µ-Slide I $^{0.6}$ Luer. In addition, you will find some recommendations for using human umbilical vein endothelial cells (HUVECs). This protocol can be adapted for your specific experimental needs.

For the setup you need the following material:

- µ-Slide I $^{0.6}$ Luer, ibiTreat (ibidi 80186)
- ibidi Pump System (ibidi 10902, please see the ibidi Pump System Instructions for details)
- Perfusion Set RED, 15 cm, ID 1.6 mm (ibidi 10962)
- Hose clip
- HUVECs with Endothelial Cell Growth Medium, Fetal Calf Serum (FCS)
- Standard cell culture equipment (sterile working bench, cell detachment kit, culture flasks, etc.)
- µ-Slide Rack (ibidi, 80003)

## 2. Cell Culture

Cultivate your cells according to your standard protocol. For HUVECs, we recommend Endothelial Cell Growth Medium (PromoCell, Germany, C-22010) supplemented with 2% fetal calf serum (FCS).

Note: Endothelial cells should never grow to a density of 100%. A confluent cell layer enters into a growth inhibition state, which stops cell proliferation and changes the physiology of the cell.

Also important is that all cells must be healthy. Depleted cells may not endure shear stress and will be flushed from the surface. If you are working with primary cells, the cellular fitness could vary significantly from one batch to the other.

## 3. Preparation of the Material

### The Day Before Starting the Experiment

Place the channel slides and the Perfusion Set(s) within their packaging in the incubator. Fill the required amount of medium into a sterile tube and put it in the incubator as well. Leave the cap slightly unscrewed to allow the excessive pressure in the tube to escape.

Install the ibidi Pump next to the incubator and arrange the connection to the computer as well as the electric cable and the tubing that are led into the incubator. Install the drying bottle between the pump's back port and the tubing that leads to the incubator, in order to suck in the gas atmosphere.

**Important Note:** Equilibrate all required materials, such as slides, medium, and tubing (Perfusion Sets), **overnight** inside the incubator at 37°C and 5% $CO_2$. This is essential for keeping air bubbles from emerging over time.

The viscosity of medium at 37°C is approx. 0.007 dyn*s/cm² (see Application Note 11 "Shear Stress and Shear Rates").

### The Day of the Experiment

- Mount the Perfusion Set onto the Fluidic Unit as described in the ibidi Pump System Instructions.
- Add equilibrated medium to the reservoirs (up to the 5 ml mark on each side).
- Connect the Fluidic Unit to port 1 on the pump's back.
- To remove the air bubbles from the tubes, start a medium flow cycle. For this purpose, a predefined setup can be loaded in the Pump Control software. Go to the menu point "Tutorial", then choose "Load demo setups" → "Remove air bubbles".

| | Pressure | Shear stress | Flow rate | Time span |
|---|---|---|---|---|
| 1) | 50.0 mbar | None (no slide) | 35.6 ml/min | infinite |

- While running the program, gently tap on the tubes and the adapters with your finger to remove the air bubbles.
- The level of both reservoirs should be re-equilibrated to 5 ml after all air is removed from the tubes.

It is crucial that all bubbles are removed before adapting the slide to the Fluidic Unit! Any gas remaining in the system can influence the flow rate and, in the worst case, stop the flow or flush the cells away.

Please Note: The "Remove air bubble" setup is programmed for one Fluidic Unit connected to port 1.

If you want to use more than one Fluidic Unit, please make sure the corresponding boxes (P1, P2, P3, P4) are checked in the software (Figure 1).

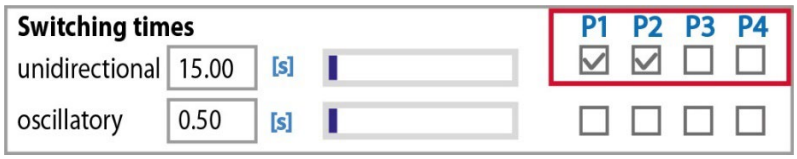


*Figure 1: Boxes to be checked in the PumpControl software when using more than one Fluidic Unit.*

### Pinch Test

This test must be done with every newly mounted Perfusion Set, to check for the correct insertion of the tubing!

Run the pump with a clearly visible flow (e.g., “Remove air bubbles”). Pinch off the tubing in the lower loop of the Perfusion Set (beneath the pinch valve). The correct clamping position is shown in Figure 2.

Make sure the flow fully stops when the tubing is pinched off. Be sure to make this test in both switching positions of the valve!

If the flow does not stop, check the insertion of the tubes in the pinch valve: adjust the insertion by stretching the tubing and moving it up and down. Then perform the pinch test again.

If you cannot solve the problem, please contact ibidi to check if the valve might be broken.

**Important!**
Test the correct insertion of the tubing with the Pinch Test!

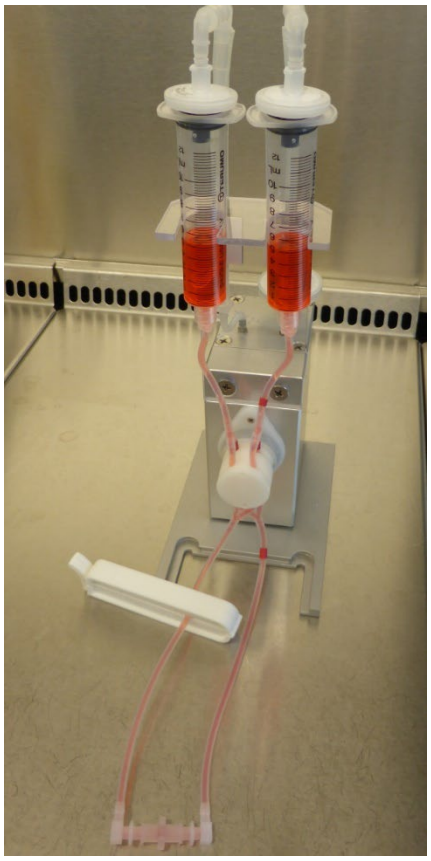

*Figure 2: Perform the pinch test to check if the tubing is inserted correctly: Once the tubing is blocked with a clamp, no liquid flow from one reservoir to the other should be observed.*

*It is crucial to do this test in both switching positions!*

## 4. Flow Rate Calibration

To predict the correct shear stress or shear rate, measure the flow rate of your experimental setup before you start working with cells. Due to factors such as temperature fluctuations or manufacture tolerances, the flow rate values may vary from those calculated by the software program. Please find detailed instructions for recalibration in the PumpControl Instructions.

After calibrating the flow rate, let the pump run at a medium pressure cycle (e.g., the “Remove air bubble” demo setup) while you are preparing the cells in the slide.

## 5. Seeding the Cells into the µ-Slide I $^{0.6}$ Luer

All of the following steps should be performed under sterile working conditions!

Unpack the µ-Slide and place it on a µ-Slide Rack, then put the caps on the Luer adapters while preparing the cell suspension. To achieve a final cell number of 1 x $10^5$ cells/cm², prepare a suspension of 1.6 x $10^6$ cells/ml. This will be a total of 2.5 x $10^5$ cells/µ-Slide.

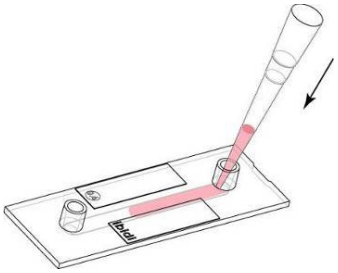

*Figure 3: Seeding cells into the µ-Slide.*

Remove the caps from the reservoirs.

Fill 150 µl of the cell suspension into the channel by putting the pipet tip directly onto the channel's inlet (Figure 3).

Put the caps on the Luer adapters and incubate at 37°C and 5% $CO_2$ for one to two hours to achieve cell attachment.

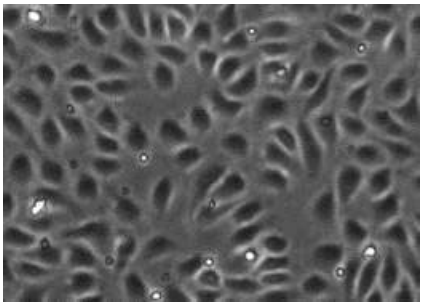

*Figure 4: Confluent cell layer of HUVECs, two hours after seeding.*

After this time, the cells should have formed a confluent cell layer as shown in Figure 4. A confluent cell layer is crucial for enabling the cells to resist the shear stress.

In principle, the cells are now ready for the connection to the Perfusion Set. If you are not connecting immediately, fill some surplus medium in the reservoirs, as shown in the next step.

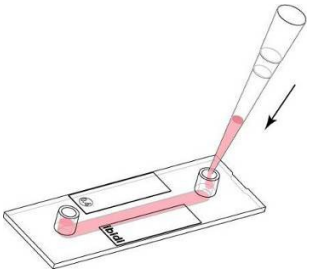

*Figure 5: Filling the reservoirs with medium.*

Fill each of the reservoirs with 60 µl of cell-free medium (Figure 5). Avoid pointing the pipet tip directly towards the channel's inlet, as this could lead to an undefined shear stress over the cell layer.

After filling the reservoirs, close them again, and then place the cells in the incubator while preparing the Fluidic Unit.

If you want to cultivate the cells for more than one day under static conditions, a complete medium exchange should be made at least once every 24 hours! For more detailed information about cell culture in the channel slides, please refer to the µ-Slide I Luer Instructions and Application Note 3 "Growing Cells in µ-Channels".

## 6. Connecting the µ-Slide I $^{0.6}$ Luer to the Perfusion Set

Once the cells have attached to the surface, you can connect the slide to the Perfusion Set. Take care not to make quick movements when inserting the Luer adapters, as this could lead to an abrupt impulse with a high shear stress for the cells.

- Stop the flow of the pump and place the Fluidic Unit with the mounted Perfusion Set in the laminar flow hood.
- Pinch off the tubes near the valve using the plastic clip (Figure 7-1, a). Place the µ-Slide I Luer next to the Fluidic Unit. Don't put it directly onto the cold metal surface. Use a µ-Slide Rack or a petri dish to prevent the slide from cooling down.
- Remove the caps and wash the channel with fresh pre-warmed medium. Apply the medium slowly, to avoid damage of the cells.
- Then fill the reservoirs with medium, until there is a small hump of liquid (Figure 6).
- Pull out the first male Luer adapter from the middle connector holding it upwards (Figure 7-1, b). Make sure there are no air bubbles remaining inside. Connect it to the female Luer on the slide, tipping it cautiously as you see in Figures 7-1, c–f.
- Press the Elbow Luer connector tightly into the Luer adapter on the slide by twisting the adapter (Figure 7-1, e).

*Figure 6: Luer port filled for connecting.*

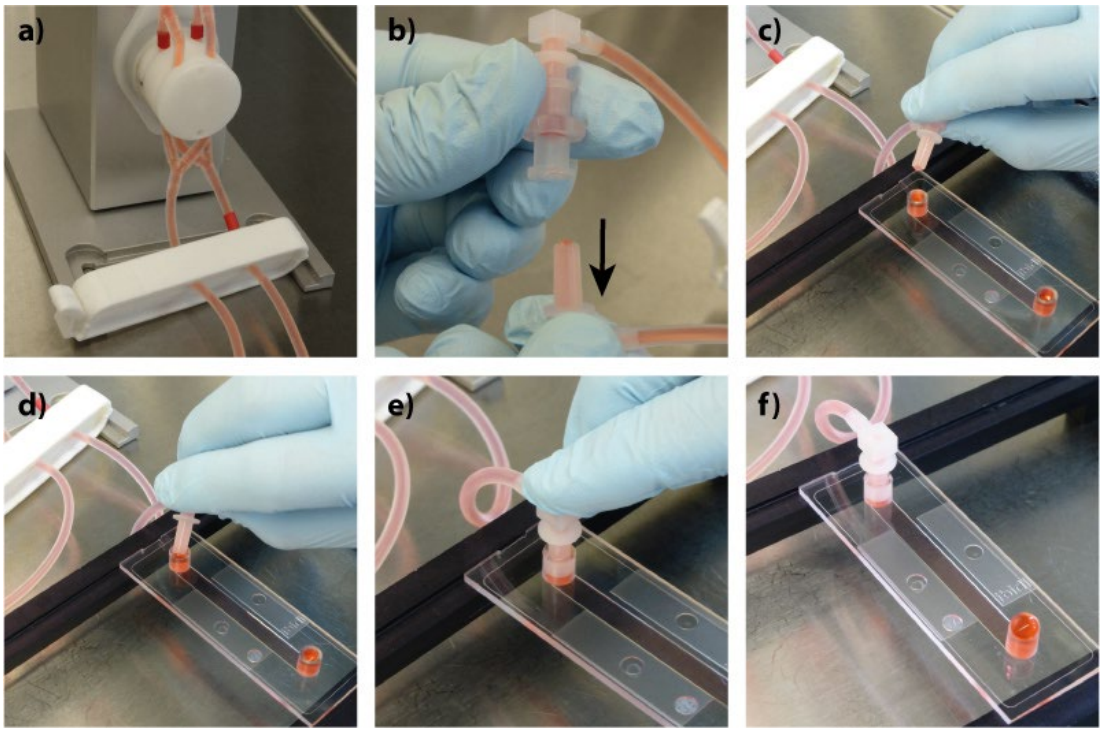


*Figure 7-1: Connecting the µ-Slide to the Perfusion Set (first adapter).*

Repeat this procedure with the second male Luer adapter, then remove the overspill with a wipe (see Figure 7-2, g–l).

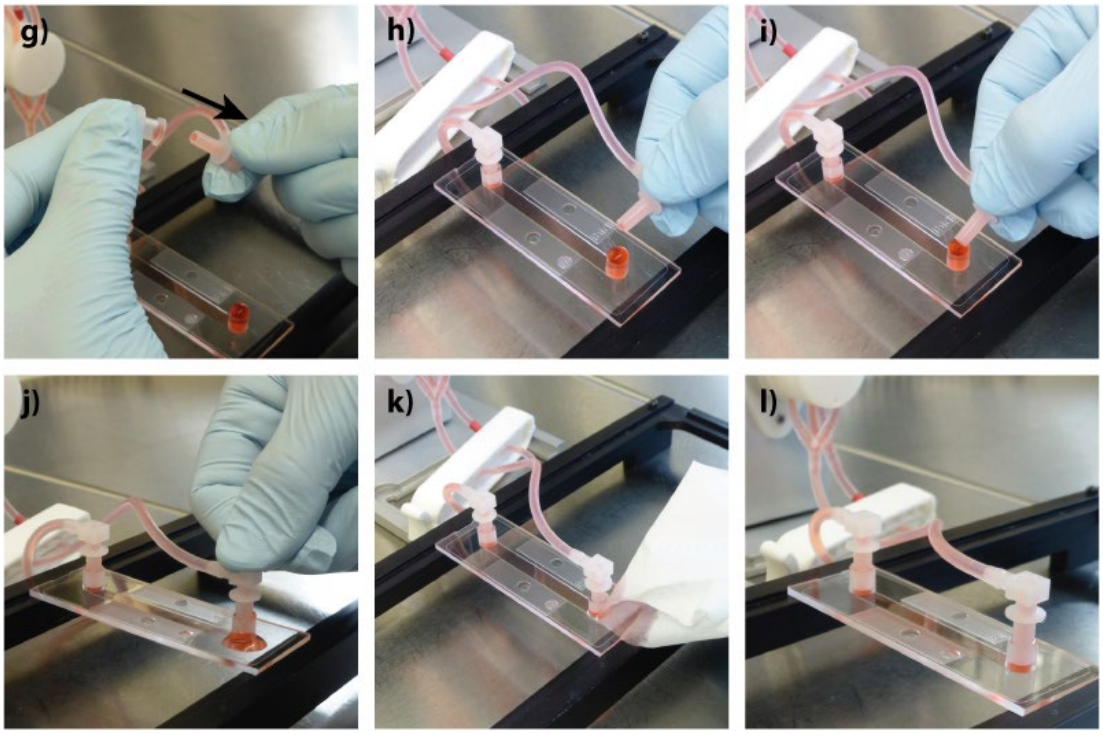


*Figure 7-2: Connecting the µ-Slide to the Perfusion Set (second adapter).*

Take care to work as fast and carefully as possible. The cells will become stressed with any disturbance and could detach if there is too much agitation.

- Check the cells under the microscope. It is crucial that the cell layer is confluent and the cells are well adherent when they are exposed to shear stress. If the cells become stressed from the connecting procedure, give them a few hours to recover before starting the flow.
- Replace the whole assembly into the incubator and connect the Fluidic Unit to the pump (air pressure tubing and electric cable).

## 7. Starting the Pump

If the cells look fine, then start the flow by switching on the air pressure pump with the ibidi Pump Control software.

For this special setup, a demo file is included in the Pump Control software. Go to the menu tab “Tutorial”, then choose “Load demo setups” → “Demo experiment”.

The parameters for a flow experiment with moderate shear stress will be loaded. The program consists of three cycles that will habituate the cells with a stepwise increase, until a final shear stress of 10 dyn/cm² is achieved (see the table below).

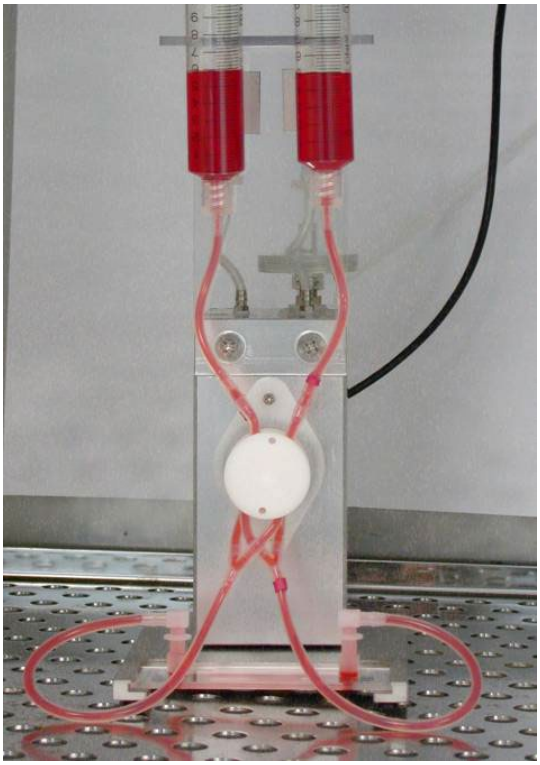

*Figure 8: Fluidic unit with Perfusion Set and a µ-Slide I Luer in the incubator.*

| | Pressure | Flow rate | Shear stress | Time span |
|---|---|---|---|---|
| 1) | 5.9 mbar | 4.8 ml/min | 2 dyn/cm² | 30 min |
| 2) | 15.4 mbar | 11.9 ml/min | 5 dyn/cm² | 30 min |
| 3) | 33.1 mbar | 23.8 ml/min | 10 dyn/cm² | infinite |

PumpControl 1.6.1

The unidirectional flow is maintained by the switching of the two valves of the Fluidic Unit. When applying positive pressure as recommended above, the source of flow will go from the unmarked tube to the marked tube (from left to right).

For a detailed description of shear stress and shear rates in the various slides, please refer to Application Note 11 “Shear Stress and Shear Rates”. For detailed information on the ibidi Pump System, please refer to the ibidi Pump System Instructions.

## 8. Observation of the Cells on the Microscope

To observe your cells on the microscope, switch off the pump at the moment the levels in the reservoirs are equilibrated. Next, detach the air pressure tubing and the electric cable from the Fluidic Unit. Take the Fluidic Unit with the connected µ-Slide to the microscope and watch your cells.

## 9. Cell Morphology

Endothelial cells are exposed to shear stress in their physiological environment. Therefore, cultivating cells with a permanent circulating medium will correlate more closely to their usual physiological conditions than cells in a static cultivation.

With this experimental setup ibidi observed a cobblestone cell layer building up in the first two days after starting the flow experiment. The cells oriented in the direction of the flow. In Figures 9 and 10, you can compare HUVECs cultivated under flow and static conditions, respectively. All of the parameters, except for the shear stress, have remained the same: the cells are in the same passage and have been cultivated for one week in the µ-Slide I $^{0.6}$ Luer (ibiTreat). The medium of the static culture was changed daily.

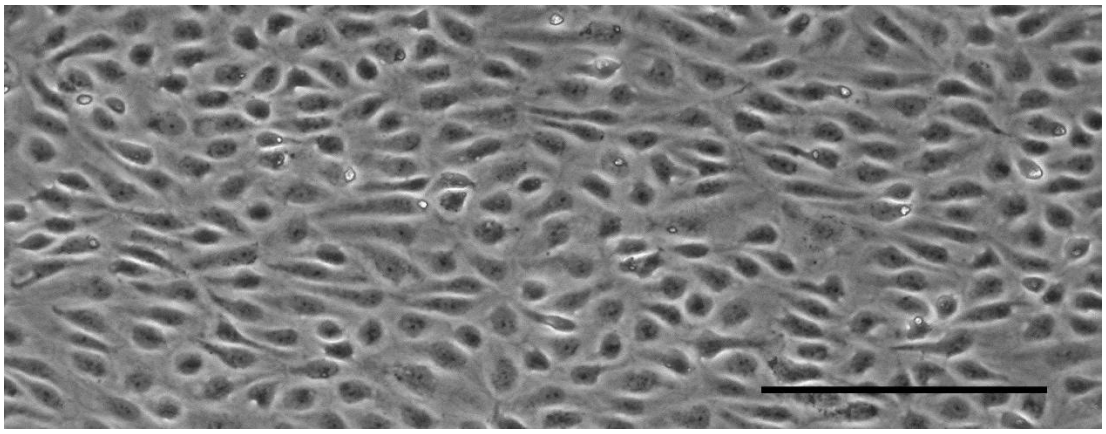

*Figure 9: HUVECs in µ-Slide I $^{0.6}$ Luer cultivated for seven days at 20 dyn/cm². The cells show a good orientation in the direction of the flow. The scale bar indicates 200 µm.*

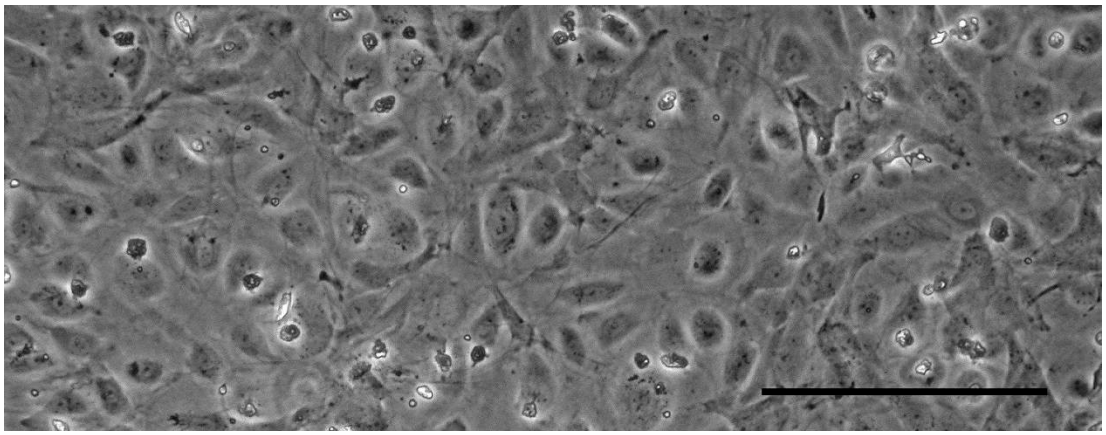

*Figure 10: HUVECs in µ-Slide I $^{0.6}$ Luer cultivated for seven days under static conditions. The medium was changed daily. The scale bar indicates 200 µm.*

## 10. Immunofluorescence

Fix and stain your cells with your standard procedure. When changing the solutions, first aspirate both reservoirs. Flush the channel two times with 140 µl of the new solution. Always add the new solution from one side and then aspirate it from the other side. Take care that the channel is always filled with liquid!

An explicit protocol for immunofluorescence staining is given in Application Note 2 “Fluorescence Staining using a µ-Slide I”.

### Perfusion Experiment

The cells in Figure 11 are cultivated under flow conditions. The actin skeleton is aligned with the direction of the flow. The cell shape is elongated.

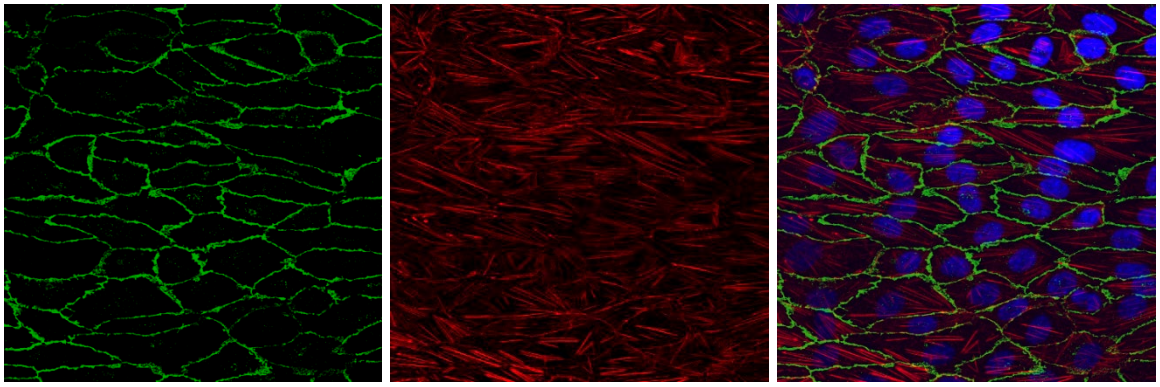

*Figure 11: HUVECs stained in the µ-Slide I $^{0.6}$ Luer. HUVECs have been cultivated for seven days at 20 dyn/cm².*

*Blue: cell nucleus; green: VE-cadherins; red: actin filaments.*

### Static Control in µ-Slide I $^{0.6}$ Luer

The cells in Figure 12 have been cultivated under static conditions over the same time period (one week). The VE-cadherins are clearly visible, but the actin skeleton is not arranged in stress fibers and the cells are not aligned in the direction of the flow.

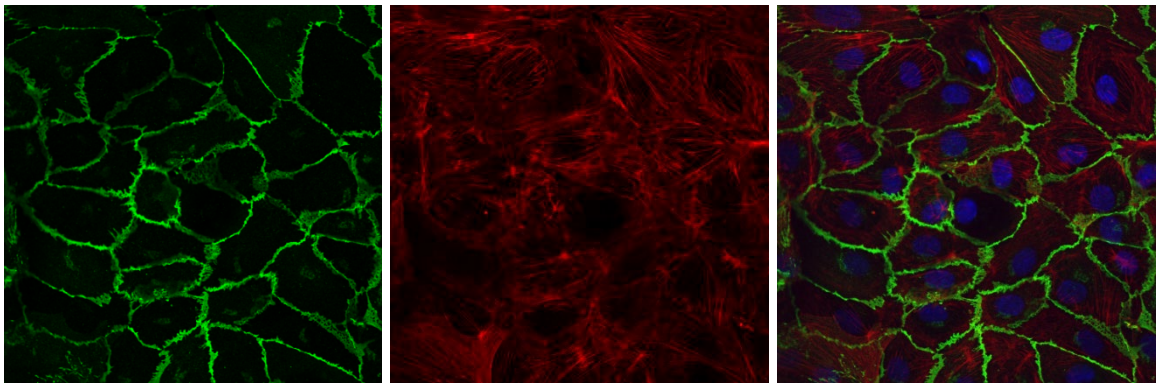

*Figure 12: HUVECs stained in the µ-Slide I $^{0.6}$ Luer. HUVECs have been cultivated for seven days at static conditions with a medium exchange every 24 hours.*

*Blue: cell nucleus; green: VE-cadherins; red: actin filaments.*