

## Flow experiment with the ibidi pump system and $\mu$ -Slide I<sup>0.6</sup> Luer

### 1. General information

The following application note describes the performance of a perfusion assay combining the ibidi pump system with a  $\mu$ -Slide I<sup>0.6</sup> Luer. Furthermore some recommendations for the usage of human umbilical vein endothelial cells (HUVECs) are given. This protocol can be adapted for your special experimental demands.

For the setup you need the following material:

- $\mu$ -Slide I<sup>0.6</sup> Luer, ibiTreat
- ibidi pump system
  - air pressure pump
  - fluidic unit
  - perfusion set, 15 cm, ID 1.6 mm (red marking)
  - hose clip
- HUVECs
- Endothelial Cell Growth Medium

### 2. Cell Culture

Cultivate your cells according to your normal protocol. For HUVECs we recommend Endothelial Cell Growth Medium (PromoCell, Germany, C-22010) supplemented with 10% fetal calf serum (FCS). Always take care that the cells are just reaching confluence when starting a new experiment. Cells that are confluent over a longer period agglomerate very firmly and you might encounter difficulties by suspending the cells homogenously. Another important aspect is the fitness of the cells. Depleted cells may not endure shear stress and will be flushed from the surface. Do not use HUVEC at more than passage 4 (split ratio 1:2)!

### 3. Preparation of the material

Equilibrate all needed material like slides, medium and tubing (perfusion sets) **overnight** inside the incubator at 37°C and 5% CO<sub>2</sub>. This is essential to avoid air bubbles emerging over time.

Place the perfusion set on the fluidic unit as described in the ibidi pump system instructions and fill in about 12 ml of equilibrated medium (without slide). The level of both reservoirs should be equilibrated at 5 ml after removing all air from the tubes. To get out 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" and choose "Load demo setups" → "Remove air bubbles".

	<b>Pressure</b>	<b>Shear stress</b>	<b>Flow rate</b>	<b>Time span</b>
1)	50.0 mbar	None (no slide)	23.4 ml/min	infinite

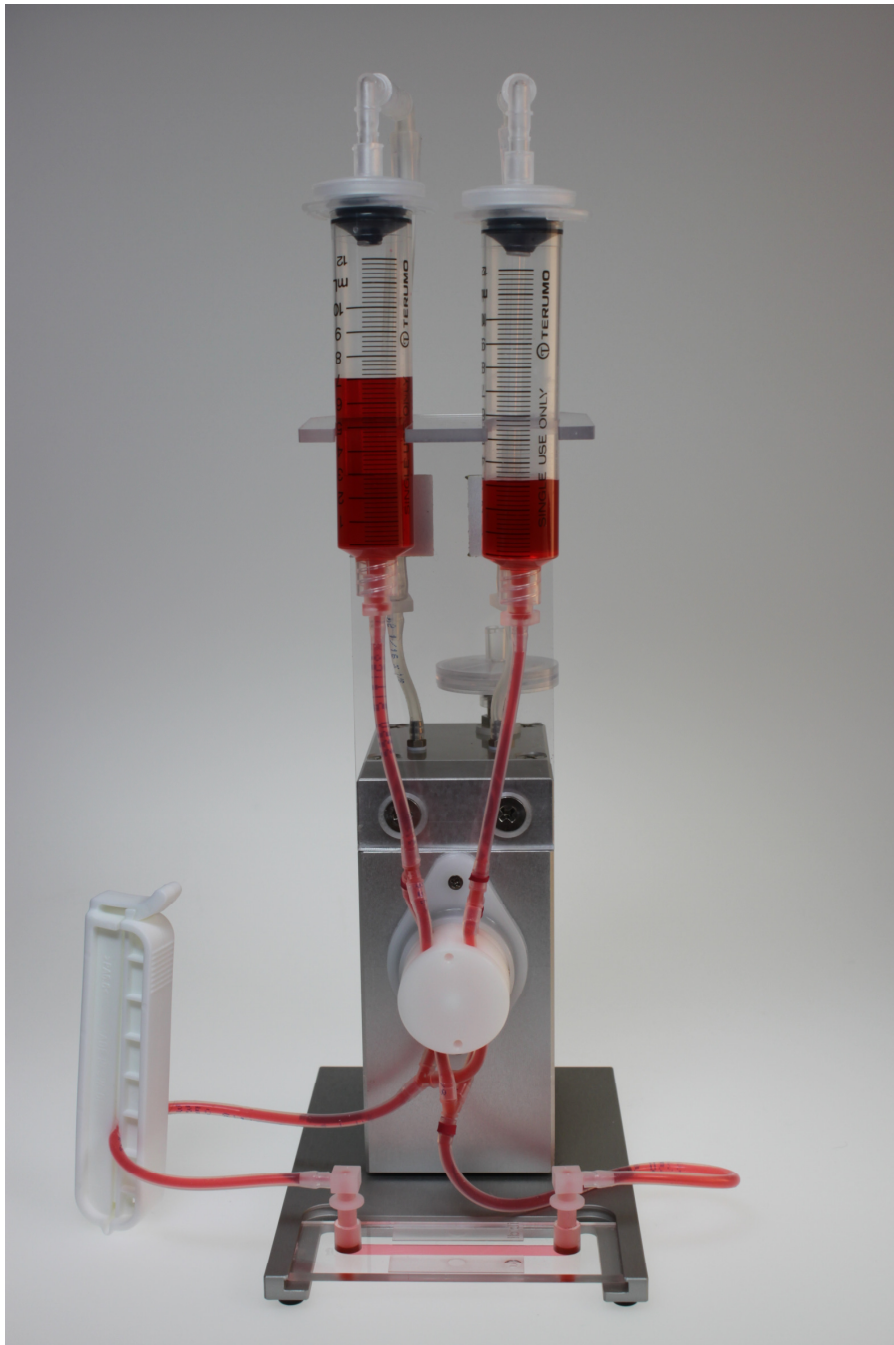
While running the program, gently flip on the tubes and on the adapters to remove the air bubbles.

It is crucial, that all bubbles are gone before adapting the slide to the fluidic unit! Gas remaining in the system can influence the flow rate and in the worst case stop the flow.

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

While the program is running check that the tubing is correctly put into the valves. Pinching off the tubing by hand, liquid movement in the reservoirs must stop **in both switching positions**.

The correct clamping position is shown in the picture below.



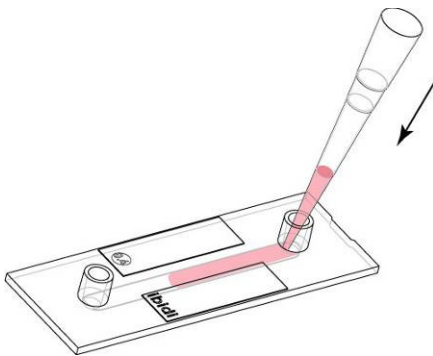
**Performing the test for correctly inserted tubing: When the tubing is blocked with a clamp, no liquid flow from one reservoir to the other should be observed.**

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

#### 4. Measuring the flow rate

To predict the correct shear stress or shear rate measure the flow rate of your experimental setup before you start working with cells. This may vary from the values the PumpControl calculations are based on, because of temperature or medium composition for example. Please find the detailed instructions for the recalibration in the PumpControl manual.

#### 5. Seeding the cells into the $\mu$ -Slide I<sup>0.6</sup> Luer

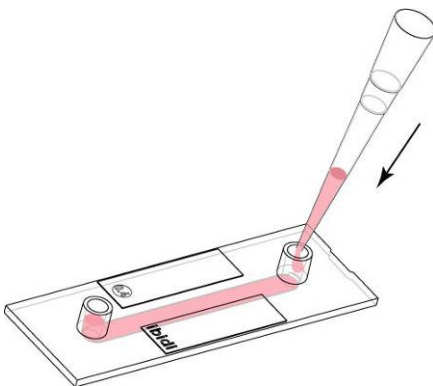


Seeding cells into the  $\mu$ -Slide

Unpack the  $\mu$ -Slide, place it on a  $\mu$ -Slide rack and put the caps on the Luer adapters while preparing the cell suspension. For a final cell number of  $1 \times 10^5$  cells/cm<sup>2</sup> ( $2.5 \times 10^5$  cells/ $\mu$ -Slide) prepare a concentration of  $1.6 \times 10^6$  cells/ml. Fill 150  $\mu$ l of the cell suspension into the channel by putting the pipet tip directly onto the channel's inlet. To help the suspension flow through the channel you may incline the slide slightly.

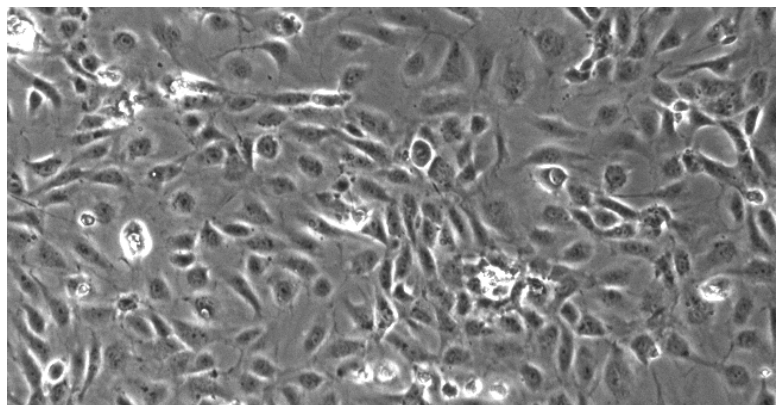
Put the caps on the Luer adapters and incubate at 37°C and 5% CO<sub>2</sub> for half an hour for cell attachment.

Fill the reservoirs with 60  $\mu$ l cell-free medium each. Avoid pointing the pipet tip directly onto the channel's inlet.



Filling the reservoirs with medium

After that, close the reservoirs again and incubate for two hours. After this time the cells should have formed a confluent cell layer like you can see in the figure below. This is important because the confluence of the layer is a crucial point in the ability of the cells to resist the shear stress.



Confluent cell layer of HUVECs, four hours after seeding them into  $\mu$ -Slide I Luer

If you want to cultivate the cells for more than one day under static conditions, a complete medium exchange has to be made every 24 hours!

For more detailed information about cell culture in the  $\mu$ -Slide I Luer please also refer to the instructions and to the Application Note 3 (Growing cells in  $\mu$ -channels).

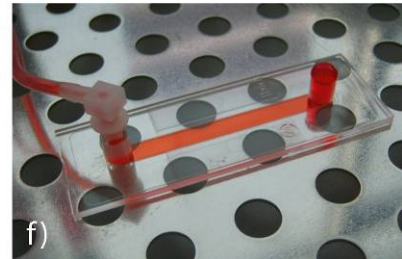
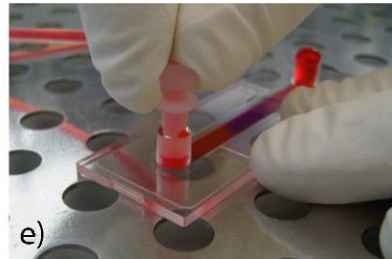
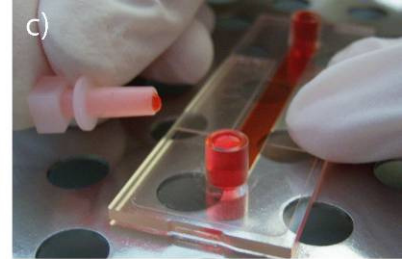
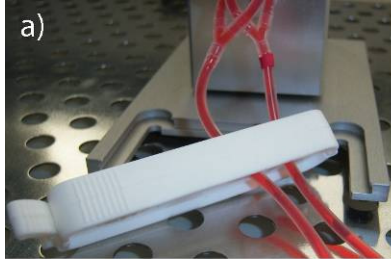
## 6. Connecting the Slide with the perfusion set:



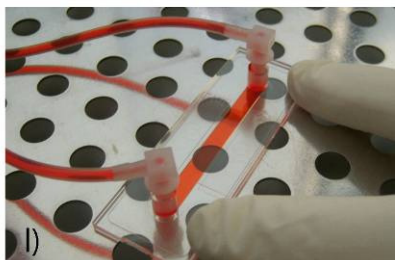
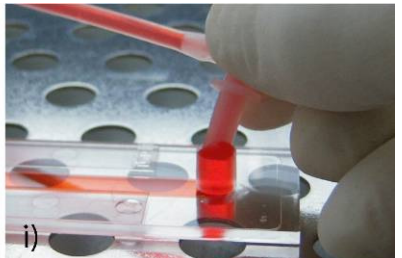
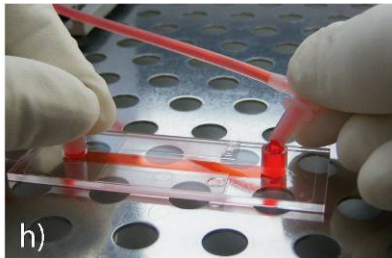
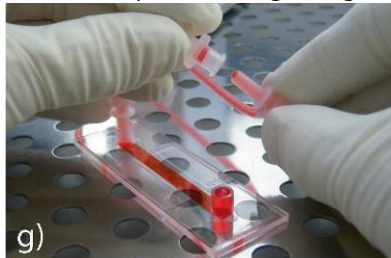
Place the fluidic unit with the mounted perfusion set under the sterile work bench and pinch off the tubes near the valve using the plastic clip (a). Put the  $\mu$ -Slide I Luer on the working surface, take off the caps and fill the reservoirs with medium until there is a small hump of liquid (see the small pictures).



Pull out the first male Luer adapter from the middle connector holding it upwards (b) to 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 the figures (c - f).



Repeat this procedure with the second male Luer adapter. Then remove the overspill with a wipe (see figure g - l).



Take care you work as fast and careful as possible. The cells are stressed at any disturbance and will detach if there is too much agitation.

Check your cells on the microscope after connection to the tubing!

## Application Note 13

### 7. Starting the pump

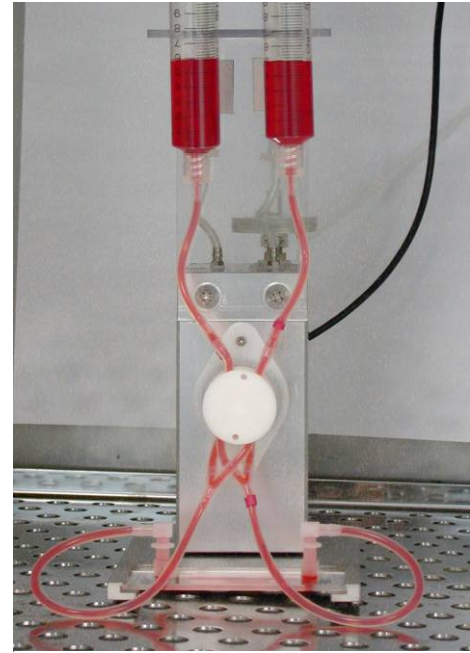
After adapting the slide to the tubing check the cells under the microscope. It is crucial that the cell layer is confluent and the cells outstretched when they are exposed to shear stress. If the cells are stressed it may be better to let them recover for a while and starting the flow later.

Put back the whole assembly in the incubator and connect the fluidic unit to the pump (air pressure tubing and electric cable).

Start the flow by switching on the air pressure pump with the ibidi Pump Control software.

For this special setup there exists a demo file in the Pump Control software (version 1.5). Go to the menu point "Tutorial" and choose "Load demo setups" → "Demo experiment".

The parameters for a flow experiment with moderate shear stress is loaded. The program consists of three cycles to habituate the cells with a stepwise increase to a final shear stress of 10 dyn/cm<sup>2</sup>.



**Fluidic unit with perfusion set and a  $\mu$ -Slide I Luer in the incubator.**

	<b>Pressure</b>	<b>Shear stress</b>	<b>Flow rate</b>	<b>Time span</b>
1)	6.1 mbar	2 dyn/cm <sup>2</sup>	3.3 ml/min	30 min
2)	15.8 mbar	5 dyn/cm <sup>2</sup>	8.3 ml/min	30 min
3)	33.9 mbar	10 dyn/cm <sup>2</sup>	16.6 ml/min	infinite

Unidirectional flow is maintained by the switching of the two valves of the fluidic unit. When applying positive pressure (as recommended above) the sink of flow is the tubing with color mark below the valve. Install the drying bottle between the pump back port and the tubing leading to the incubator for sucking in the gas atmosphere (see Pump Instruction Manual page 6).

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 pump instructions.

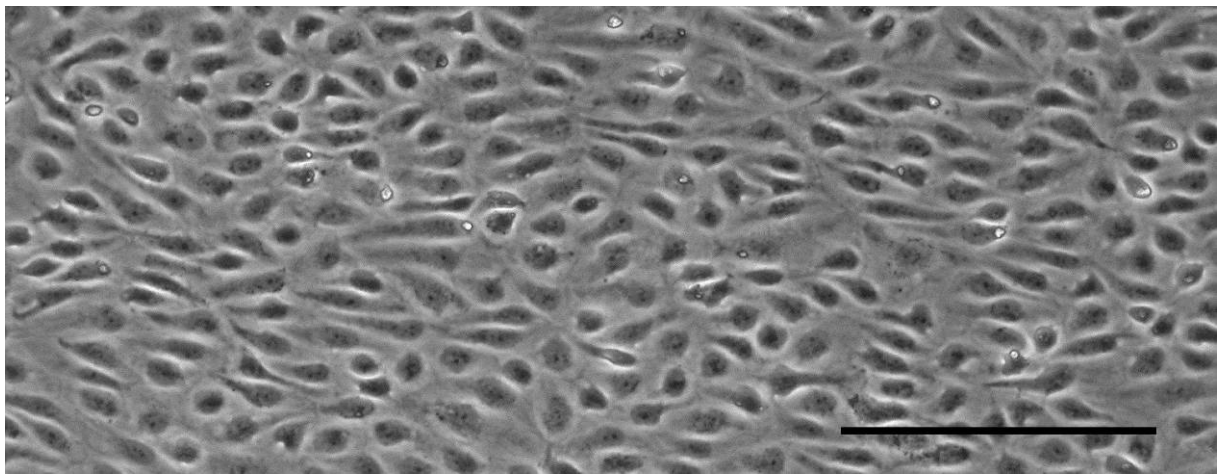
### 8. Observation of the cells on the microscope

To observe your cells on the microscope switch off the pump at that moment the levels in the reservoirs are equilibrated. Then detach the air pressure tubing and the electric cable of the fluidic unit. Take the  $\mu$ -Slide with the fluidic unit to the microscope and keep the adapters connected while watching the cells.

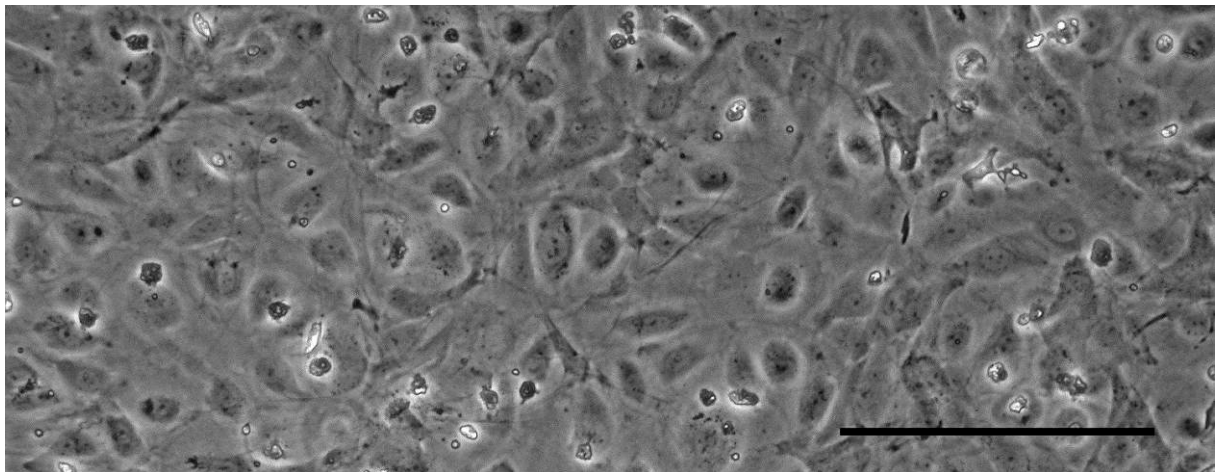
## 9. Cell Morphology

Endothelial cells are exposed to shear stress in their physiological environment. Thus the cultivation with a permanent circulating medium correlates more with the physiological conditions than a static cultivation.

With this experimental setup we observed cobblestone cell layer in the first two days after starting the flow experiment. After two days cells started elongating in the direction of the flow. In the figures below you can compare HUVECs cultivated under flow and static conditions respectively. All parameters except for the shear stress have been the same: Cells are of the same passage both cultivated for one week in the  $\mu$ -Slide I<sup>0.6</sup> Luer (ibiTreat). The medium of the static culture has been changed every day.



**HUVECs in  $\mu$ -Slide I<sup>0.6</sup> Luer cultivated for seven days at a flow rate of 22 ml/min (20 dyn/cm<sup>2</sup>). The cells show a good orientation in the direction of the flow. The scale bar indicates 200  $\mu$ m.**



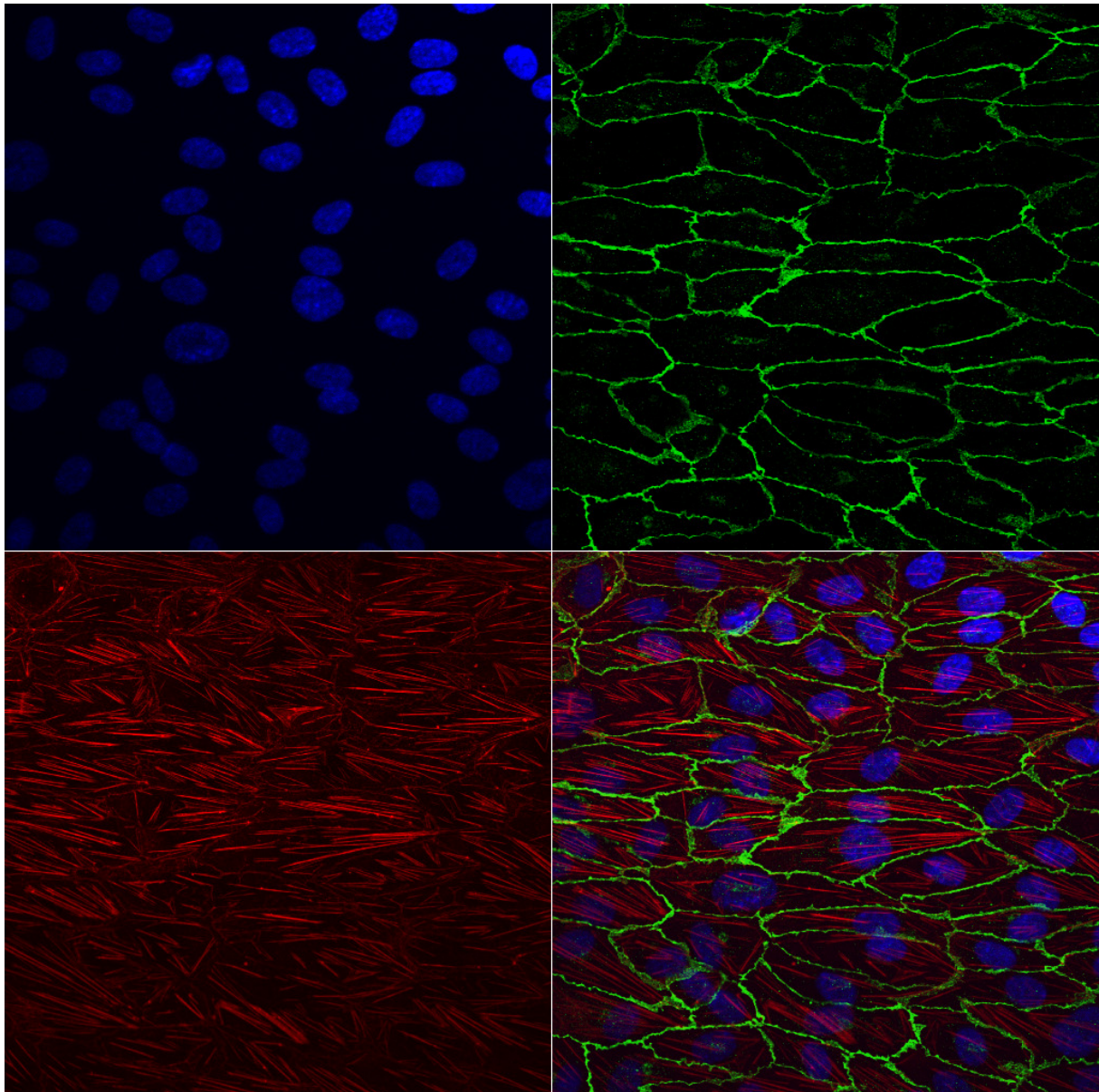
**HUVECs in  $\mu$ -Slide I<sup>0.6</sup> Luer cultivated for seven days under static conditions. The medium has been changed every day. The scale bar indicates 200  $\mu$ m.**

## 10. Immunofluorescence

Fix and stain your cells with the same procedure as usual. For changing the fluids first aspirate both reservoirs. Flush the channel two times with 140  $\mu$ l of the new solution. Always add the new solution from one side and aspirate it from the other. Take care that the channel is always filled with fluid!

An explicit protocol for immunofluorescence staining is given in Application Note 2 (Immunofluorescence in  $\mu$ -Slide I).

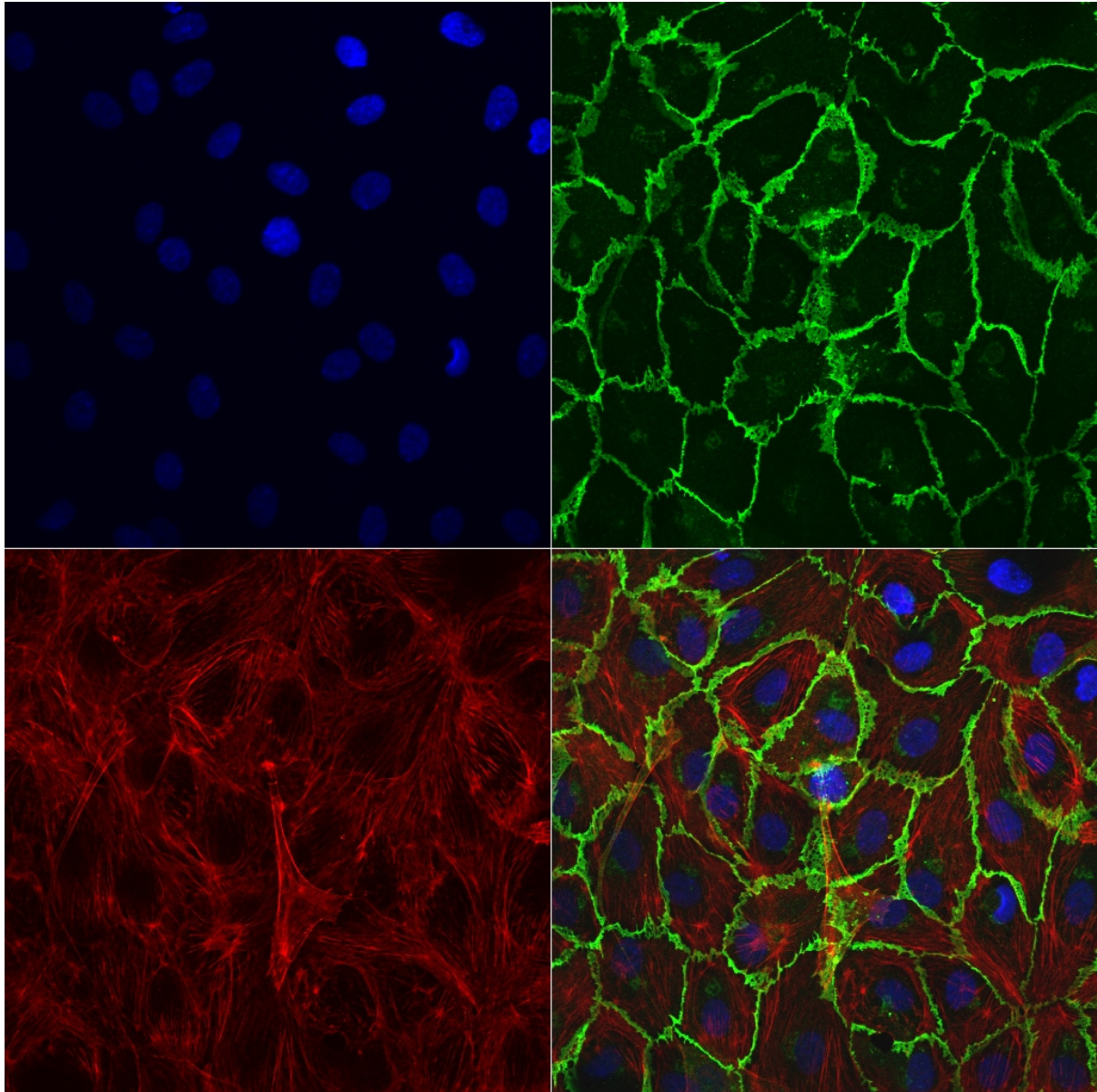
Perfusion experiment: The cells in the picture below are cultivated under flow conditions. The actin skeleton is aligned in the direction of the flow.



**HUVECs stained in the  $\mu$ -Slide I<sup>0.6</sup> Luer. HUVECs have been cultivated for seven days at a flow rate of 22 ml/min (20 dyn/cm<sup>2</sup>).**

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

Static control in  $\mu$ -Slide I<sup>0.6</sup> Luer: The cells shown on this page have been cultivated under static conditions over the same period (one week). The VE-cadherins are clearly visible, but the actin skeleton and the whole cell are not aligned in direction of the flow.



**HUVECs stained in the  $\mu$ -Slide I<sup>0.6</sup> 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.**