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TITLE:

Pneumococcus Infection of Primary Human Endothelial Cells in Constant Flow

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KEYWORDS:

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SUMMARY:

This study describes the microscopic monitoring of pneumococcus adherence to von Willebrand factor strings produced on the surface of differentiated human primary endothelial cells under shear stress in defined flow conditions. This protocol can be extended to detailed visualization of specific cell structures and quantification of bacteria by applying differential immunostaining procedures.

ABSTRACT:

Interaction of *Streptococcus pneumoniae* with the surface of endothelial cells is mediated in blood flow via mechanosensitive proteins such as the Von Willebrand Factor (VWF). This glycoprotein changes its molecular conformation in response to shear stress, thereby exposing binding sites for a broad spectrum of host-ligand interactions. In general, culturing of primary endothelial cells under a defined shear flow is known to promote the specific cellular differentiation and the formation of a stable and tightly linked endothelial layer resembling the physiology of the inner lining of a blood vessel. Thus, the functional analysis of interactions between bacterial pathogens and the host vasculature involving mechanosensitive proteins requires the establishment of pump systems that can simulate the physiological flow forces known to affect the surface of vascular cells.

The microfluidic device used in this study enables a continuous and pulseless recirculation of fluids with a defined flow rate. The computer-controlled air-pressure pump system applies a defined shear stress on endothelial cell surfaces by generating a continuous, unidirectional,

and controlled medium flow. Morphological changes of the cells and bacterial attachment can be microscopically monitored and quantified in the flow by using special channel slides that are designed for microscopic visualization. In contrast to static cell culture infection, which in general requires a sample fixation prior to immune labeling and microscopic analyses, the microfluidic slides enable both the fluorescence-based detection of proteins, bacteria, and cellular components after sample fixation; serial immunofluorescence staining; and direct fluorescence-based detection in real time. In combination with fluorescent bacteria and specific fluorescence-labeled antibodies, this infection procedure provides an efficient multiple component visualization system for a huge spectrum of scientific applications related to vascular processes.

INTRODUCTION:

The pathogenesis of pneumococcus infections is characterized by a multifaceted interaction with a diversity of extracellular matrix compounds and components of the human hemostasis, such as plasminogen and VWF[1-8]. The multidomain glycoprotein VWF serves as key regulator of a balanced hemostasis by mediating thrombocyte recruitment and fibrin incorporation at the site of vascular thrombus formation[9]. The importance of functional, active VWF for bleeding control and wound healing is demonstrated by von Willebrand's disease, a common inherited bleeding disorder[10].

Globular VWF circulates in the human blood system at a concentration of up to 14.0 µg/mL[11,10]. In response to vascular injury, the local release of VWF by endothelial Weibel Palade Bodies (WBP) is markedly increased[11,12]. Previous studies show that pneumococcus adherence to human endothelial cells and its production of the pore-forming toxin pneumolysin significantly stimulates luminal VWF secretion[13]. The hydrodynamic forces of the blood flow induce a structural opening of the mechanoresponsive VWF domains. At flow rates of 10 dyn/cm^2 the VWF multimerizes to long protein strings of up to several hundred micrometers in length that remain attached to the subendothelium[10,12].

To understand the function of multimerized VWF strings generated under shear stress in the interaction of pneumococcus with the endothelial surface, a microfluidic-based cell culture infection approach was established. A microfluidic device with a software-controlled air-pressure pump system was used. This enabled a continuous, unidirectional recirculation of cell culture medium with a defined flow rate. Thereby, the system applied a defined shear stress on the surface of endothelial cells, which remained attached inside specialized channel slides. This approach enabled the simulation of the shear force within the blood stream of the human vascular system, in which VWF strings are generated on differentiated endothelial cells under defined constant flow conditions. For this purpose, the endothelial cells were cultivated in specific channel slides (see **Table of Materials**), which were adapted for microscopic analyses during flow. The microfluidic pump system provided the highly defined and controlled shear stress situation required for the formation of extended VWF strings on the confluent endothelial cell layer. After the stimulation of VWF-secretion of confluently grown human umbilical vein endothelial cells (HUVEC) by histamine supplementation, the string formation was induced by applying a shear stress (τ) of 10 dyn/cm^2. The shear stress is defined as the force acting on the cell layer. It is calculated approximately according to Cornish et. al.[14] with equation 1:

$$\tau = \frac{6 \times \eta}{h^2 \times w} \times \Phi \quad (1)$$

Where τ = shear stress in dyn/cm^2, η = viscosity in (dyn·s)/cm^2, h =half of channel height, w = half of channel width, and Φ = flowrate in mL/min.

The result of equation 1 depends on the different heights and widths of the different slides used (see **Table of Materials**). In this study a Luer channel slide of 0.4 µm resulting in a chamber slide factor of 131.6 was used (see formula 2).

$$\tau = 131.6 \times \eta \times \Phi \quad (2)$$

Viscosity of the medium at 37 °C is 0.0072 dyn·s/cm² and a shear stress of 10 dyn/cm² was used. This resulted in a flow rate of 10.5 mL/min (see formula 3).

$$\Phi = \frac{10}{0.0072 \times 131.6} = 10.5 \quad (3)$$

Here, the adaptation and advancement of a microfluidic cell culturing procedure using a unidirectional laminar flow system for the investigation and visualization of bacterial infection mechanisms in the host vasculature is described in detail. The generation of VWF strings on endothelial layers can also be stimulated by using other pump systems that are able to apply a continuous and steady shear stress[15].

After cultivation of primary endothelial cells to confluence in flow and stimulation of VWF string formation, pneumococci expressing red fluorescence protein (RFP)[16] were added to the endothelial cell layer under constant microscopic control. The attachment of bacteria to VWF strings on the surface of endothelial cells was microscopically visualized and monitored for up to three hours in real time by using VWF-specific fluorescent-labelled antibodies. With this approach, the role of VWF as an adhesion cofactor promoting bacterial attachment to the vascular endothelium was determined[8].

In addition to the microscopic visualization of protein secretion and conformational changes, this method could be used to monitor single steps of bacterial infection processes in real time and to quantify the amount of attached bacteria at different time points of infection. The specific software-controlled pump system also provides the possibility to culture the endothelial cells in defined constant flow conditions for up to several days and enables a defined pulsed medium flow incubation. Moreover, this method can be applied using different cell types. Adapting the staining protocol also enables the detection and visualization of bacteria internalized into eukaryotic cells.

This manuscript describes this advanced experimental protocol that can be used as a defined, reliable, and reproducible approach for an efficient and versatile characterization of pathophysiological processes.

PROTOCOL:

The microfluidic cell cultivation was performed with commercial primary human umbilical vein endothelial cells (HUVEC). The company isolated the cells with informed consent of the donor.

This study was approved by the Ethics Committee of Doctors Chamber of the Federal State Baden-Wuerttemberg with the reference number 219-04.

NOTE: See **Table of Materials** for protocol supplies.

1. Precultivation of primary endothelial cells

1.1. Thaw a frozen glycerol vial containing 1 x 10^5 primary HUVEC from three different donors gently at 37 °C and seed the cells in 7 mL of prewarmed endothelial cell growth medium (ECGM, ready to use with supplements) in a 25 cm^2 cell flask.

NOTE: The primary endothelial cells lose differentiation capacity after more than 5 proliferation cycles. Therefore, only cells with less than 5 passages can be used if high grades of cell differentiation are required.

1.2. Cultivate the cells at 37 °C in 5% CO_2 atmosphere for 60 min to allow surface attachment and exchange the ECGM cell culture medium to get rid of residue from cryoconservation.

1.3. Continue culturing the cells at 37 °C in 5% CO_2 atmosphere until they form a subconfluent cell layer.

NOTE: The HUVEC must not grow to a confluent layer since the tight cell-cell contacts prevent formation of a stable cell layer later in flow.

2. Precultivation of *Streptococcus pneumoniae*

CAUTION: *Streptococcus pneumoniae* is a biosafety level 2 agent and is only allowed to be cultured in biosafety level 2 laboratories. Use a clean bench classified for safety level 2 for all bacterial treatments, strictly avoid aerosol formation, and use a centrifuge with aerosol protection for sedimentation of bacteria.

2.1. Inoculate a Columbia blood agar plate with *Streptococcus pneumoniae* clinical isolate ATCC11733 derived from a glycerol stock constantly stored at -80 °C and cultivate the agar plate overnight at 37 °C and 5% CO_2.

2.2. Prepare 40 mL of Todd Hewitt liquid broth supplemented with 1% yeast extract (THY) and 15 mL of sterile phosphate-buffered saline (PBS) pH 7.4, for bacterial cultivation and washing steps.

2.3. Use a sterile tube for bacterial cultivation and inoculate the liquid culture broth with bacterial mass. Control the amount of inoculation by photometric measurement of 1 mL aliquots at 600 nm against non-inoculated liquid broth as reference. Fill in bacterial mass into the liquid broth until it reaches an optical density at 600 nm (OD_{600}) of 0.15.

2.4. Incubate the inoculated liquid broth without shaking at 37 °C and 5% CO_2 and determine the OD_{600} every 30 min by measuring 1 mL aliquots using plastic cuvettes.

2.5. As soon as the bacterial culture has reached an OD_{600} of 0.4, which corresponds to the exponential growth phase, centrifuge the bacterial culture suspension for 10 min at 1,000 x *g* at room temperature (RT).

NOTE: Do not allow a pneumococcus culture to reach an OD_{600} of more than 1.0, because a high pneumococcus culture density is known to trigger bacterial autolysis, which might affect overall bacterial fitness.

2.6. Resuspend the bacterial sediment gently with 10 mL PBS and sediment again for 10 min at 1,000 x *g* at RT.

2.7. Resuspend the washed bacterial sediment gently in 1 mL PBS and determine the OD_{600} of 10 µL of the bacterial suspension using 1 mL of PBS as a reference.

2.8. Adjust the amount of bacteria in PBS to an OD_{600} of 2.0. According to formerly determined bacterial counting, an OD_{600} of 2.0 corresponds to 2 x 10^9 colony forming units (CFU). Immediately proceed with the infection procedure to prevent bacterial autolysis.

3. Endothelial cell cultivation of HUVEC under microfluidic conditions

3.1. Detach the primary endothelial cells from the cell culture flask by controlled proteolysis. Perform the following steps in a sterile environment using a clean bench. Prepare a volume of 15 mL of sterile PBS for the washing steps.

3.1.1. Remove the ECGM from a subconfluently-grown HUVEC layer and wash the cell layer with 10 mL PBS using a serological pipette to get rid of the cell culture medium.

3.1.2. Incubate the washed HUVEC with 3 mL of 37 °C prewarmed cell dissociation solution for cell detachment for 5 min at 37 °C. Observe the proteolytic cell detachment by microscopic monitoring each minute.

3.1.3. Pipette the detached cell suspension into a tube containing 7 mL of ECGM supplemented with 2% fetal calf serum (FCS) for stopping proteolysis and sediment the cells for 3 min at 220 x *g* at RT.

3.1.4. Remove the supernatant and resuspend the HUVEC in 250 µL of ECGM supplemented with 5% FCS and 1 mM $MgSO_4$. Use 10 µL of the cell suspension for cell counting using a Neubauer cell counting chamber and adjust the cell count to 4 x 10^6 cells/mL ECGM supplemented with 5% FCS and 1 mM $MgSO_4$.

NOTE: For each flow experiment 30 mL of ECGM medium supplemented with 5% FCS and with 1 mM $MgSO_4$ will be required. From here on this medium composition is named ECGMS-medium. The increase of FCS concentration in the culture medium from 2% to 5% supports cell attachment and cell viability of HUVEC seeded in the channel slide. The medium supplements FCS and $MgSO_4$ substantially stabilize the cell attachment of HUVEC under shear stress conditions.

3.2. Seed and cultivate the HUVEC in a channel slide. Work in a sterile environment using a clean bench. The cells will be cultivated under shear stress for 2 days followed by infection with bacteria and microscopic monitoring for another 2 h.

3.2.1 Equilibrate a channel slide, a perfusion set 1.6 mm in diameter and 50 cm in length, an aliquot of the ECGMS-medium, and a Luer channel slide 0.4 µm in height, for 24 h in an incubator with 5% CO_2 atmosphere at 37 °C to reduce the number of air bubbles.

NOTE: This procedure is recommended to degas the plastic equipment and to prewarm the medium, the perfusion tubes, and the reservoirs. If materials or liquids have been stored at RT or in the refrigerator, gases dissolved in the plastic and liquids will be released when heated up in the incubator during the experiment. Gas bubbles will then appear. Degassing all plastic components before the experiment will eliminate this effect. Each time the system is taken out of the incubator, the process of gas absorption begins again. Therefore, work quickly at RT and never leave the fluidic unit outside the incubator for longer time periods.

3.1.2. Use a Luer syringe to inject 100 µL of a 2% sterile-filtered porcine gelatin solution in PBS solution into one of the reservoirs of a temperature-equilibrated channel slide. Incubate the gelatin-solution for 1 h at 37 °C and rinse the channel of the slide with 1 mL PBS under sterile conditions using a 1 mL Luer syringe.

3.1.3. Place the gelatin-coated channel slide on a thin polystyrene or styrofoam plate to prevent a drop in the slide temperature. Add 100 µL of the 4 x 10^6/mL HUVEC suspension with a 1 mL Luer syringe into the slide.

NOTE: Placing the channel slide on the cold metal surface of the clean bench could decrease the temperature of the slide bottom, thereby generating cold stress to the endothelial cells. During cell pipetting hold the slide a bit upwards to let air bubbles rise and disappear from inside the slide.

3.1.4. Incubate the channel slide with the HUVEC for 60 min at 37 °C and 5% CO_2 and fill up the medium reservoirs at both ends of the channel slide with 60 µL ECGMS-medium each. Incubate for 1 h at 37 °C and 5% CO2.

3.2. Adjust the microfluidic pump and the software settings.

3.3.1. Connect the equilibrated perfusion set to the pump unit, fill with 13.6 mL of ECGMS-medium, and start the pump control software. Select the adequate perfusion set and type of chamber slide using the scroll down windows in the menu of the fluidic unit set up. Choose 0.007 (dyn·s/cm^2) in the software for medium viscosity. (Refer to the pressure pump software settings marked with red arrows in **Supplementary Figure 1**).

3.2.2. Outside of the incubator, connect a glass bottle filled with drying silica beads to the air pressure tubing (refer to **Figure 1**, **Inset 3**). The air of the pressure pump circulates between the perfusion reservoirs and the pump and must be dry before reentering the pump device. Select **Flow Parameters** in the software menu, set the pressure to 40 mbar, and flush the pump tubes with the liquid medium by starting the continuous medium flow. (These settings are also indicated by red arrows in **Supplementary Figure 1**).

3.2.3. Program the desired shear stress cycles of flow cultivation. Start with 5 dyn/cm^2, control balanced reservoir pumping and assure that no air bubbles are circulating in the pump system.

NOTE: The wall shear stress in a channel slide depends on the flow rate and the viscosity of the perfusion medium. If using another pump system, please refer to the equations described in the introduction to set a flow rate generating the desired shear stress level. The described settings correspond to a flow rate of 5.42 mL/min. (An exemplary screen shot showing the adequate flow parameter settings in the pressure pump software is shown in **Supplementary Figure 2**).

3.2.4. Stop the flow circulation in the pump control software and hold the medium flow in the perfusion tubing by clamping the tubes near the Luer connections. Connect the channel slide, thereby avoiding air bubbles, and place the fluidic unit with the connected channel slide in a CO_2 incubator at 37° C and 5% CO_2. Start the shear stress at 5 dyn/cm^2 for 30 min to smoothly adapt the cells to the forces generated by the shear stress before accelerating the shear stress level (see **Supplementary Figure 3**).

NOTE: Take care that no air bubbles remain in the tube system or in the slide after connecting to the tube system because the movement of air bubbles in the flow might lead to cell detachment.

3.2.5. Accelerate the shear stress to 10 dyn/cm^2 (which corresponds to 10.86 mL/min in this flow setting) and incubate the channel slide in continuous shear stress for 48 h in a small CO_2 incubator at 37 °C and 5% CO_2 to allow cell differentiation (the respective software settings are indicated with red arrows in **Supplementary Figure 4**).

NOTE: HUVEC cells tend to detach from the channel surface if flow cultivation is directly started at 10 dyn/cm^2. The cells remain attached to the chamber surface if flow cultivation is started with less shear stress using 5 dyn/cm^2 for a minimum of 30 min followed by increasing the shear stress slowly up to the desired 10 dyn/cm^2. A shear stress of 10 dyn/cm^2 is the minimum value in this perfusion setting required for VWF string formation.

3.2.6. After 24 h of microfluidic cell cultivation, stop the medium flow with the pump control software exactly when a balanced medium level is reached in both medium reservoirs. Place the fluidic unit in a clean bench and remove 10 mL of the circulated cultivation medium of the perfusion reservoirs using a 10 mL serological pipette. Add 10 mL of ECGMS-medium into the reservoirs to renew the medium, place the fluidic unit back into the CO_2 incubator at 37 °C and 5% CO_2, and restart the fluidic cultivation using the pump control software.

NOTE: The function of the pressure pump can suddenly be disrupted by laboratory machines such as large centrifuges, which might create a strong magnetic field disturbance. This sudden disruption might lead to cell detachment. Take care that such machines are not active near the pressure pump during the experiment.

3.2.7. Start the prewarming of the temperature incubation chamber covering the stage of the fluorescence microscope to 37 °C for temperature equilibration 24 h before the

microscopic visualization. After the microscope is prewarmed, start the microscope software control and adjust the principal settings for the fluorescence microscopic monitoring by selecting the appropriate filter settings (540 nm/590 nm for detection of the RFP-expressing bacteria and 470 nm/515 nm for detection of fluorescein emission of the FITC-conjugated VWF-specific antibodies). Prewarm an additional heating chamber for incubation of the fluidic unit at 37 °C.

NOTE: During the infection analyses and microscopic monitoring the temperature of the channel slide and the circulating medium should not decrease substantially, because this would generate cold stress on the cells. In general, the size of the temperature chambers covering the microscope stage is not enough to cover the whole fluidic unit. Therefore, the use of an additional heating chamber prewarmed to 37 °C is recommended.

3.2.8. For microscopic visualization, place the fluidic unit into the 37 °C prewarmed heating chamber and place the channel slide on a stage of the 37 °C prewarmed microscope.

NOTE: For microscopic visualization, the fluidic unit and the channel slide needed to be removed from the CO_2 incubator due to the limited length of the perfusion tubing. If infection times and microscopic monitoring longer than 180 min are required outside the 5% CO_2 atmosphere for pH buffering, a pH-buffered medium should be used for microfluidic cultivation.

3.2.9. Control the cell morphology and the integrity of the HUVEC layer prior to injection of histamine and bacteria to the flow circulation throughout the time of the flow experiment and after finishing the flow experiment using the bright field mode of the microscope.

4. Induction of VWF-release and visualization of multimerized VWF strings

4.1. Maintain the flow setting, because a shear stress of 10 dyn/cm^2 is required to trigger the multimerization of VWF to long strings of up to 200 MDa. Induce the release of VWF from endothelial WPB by injecting 136 µL of a 100 mM histamine stock solution into the ECGMS-medium circulating in the perfusion tubing using an injection port. The final concentration of histamine in the flow medium will be 1 mM. If no injection port is available, the histamine can be added alternatively by pipetting into the medium of the pump reservoirs.

4.2. For immunofluorescence detection of multimerized VWF strings, stop the flow when a balanced medium level in the reservoirs is reached, and inject 20 µg of a VWF-specific FITC-conjugated antibody in a volume of 200 µL PBS (pH 7.4) into the circulating 13.6 mL of ECGMS-medium using an injection port. If no injection port is available, the antibody can be added alternatively by pipetting into the medium of the pump reservoirs. This results in a final antibody concentration of 1.3 µg/mL.

4.3. For microscopic scanning of several fields of view in a short time, use the fluorescence unit of the microscope with a Xenon fluorescence device at 30% power and an epifluorescence camera. Monitor the shape and morphology of the HUVEC layer with the bright field mode to select representative cells suitable for the VWF-strings visualization.

4.4. For visualization of green fluorescent VWF strings, select a 63x/1.40 oil objective and a 470 nm detection filter in the fluorescence unit menu of the microscope software (LasX). Create snapshots of Z-stacks of at least 50 representative field views, each containing approximately 10 morphologically intact HUVEC. For quantification of the green fluorescent VWF strings at different time points, scan several fields of view.

5. Microscopic evaluation of bacterial attachment to VWF-strings in flow in real time

5.1. Quantify pneumococcal attachment to the VWF-strings generated on HUVEC cell surfaces via immunofluorescence detection.

5.1.2. Hold the flow and inject 1.35 x 10^8 CFU/mL RFP-expressing pneumococci in a maximum volume of 1 mL into the ECGMS-medium using the injection port. Alternatively, pipette the bacteria into the medium in the pump reservoir. Restart the shear stress at 10 dyn/cm^2 to let the bacteria circulate within the pump system.

5.1.3. Select a 63x oil-immersion objective for microscope magnification and adjust the fluorescence filter settings in the microscope software to the RFP-channel (540 nm detection filter) for detection of RFP-expressing pneumococci.

5.1.4. For quantification of bacterial attachment to the VWF strings, stop the flow and create snapshots of Z-stacks of at least 30 representative field views, each containing approximately 10 morphologically intact HUVEC, and count the amount of pneumococci.

5.1.5. Use the ANOVA one-factorial statistics algorithm in order to evaluate the data, followed by a post hoc two-tailed unpaired sample test for detailed statistical comparison. P values of <0.05 were considered statistically significant.

6. Microscopic evaluation of bacterial attachment to VWF-strings after sample fixation

6.1. Sample the fixation prior to immunofluorescence staining.

6.1.2. Stop the flow, remove 10 mL of ECGMS-medium from the pump reservoirs, and add 10 mL of PBS supplemented with 5% paraformaldehyde (PFA). Let the PFA solution circulate for 10 min at a shear stress of 10 dyn/cm^2.

6.1.3. Disconnect the channel slide from the pump unit.

6.2. Block unspecific binding sites on the cell surface and perform immunodetection of VWF strings and attached bacteria.

6.2.2. Prepare 4 mL of a washing solution containing 100 mM Na_2CO_3 (pH 9.2) supplemented with 4% sucrose for all washing steps. Prepare 1 mL of a blocking solution containing 100 mM Na_2CO_3 (pH 9.2) supplemented with 4% sucrose and 2% bovine serum albumin (BSA) for blocking of unspecific binding sites.

6.2.3. Wash the PFA-incubated channel slide 3x using a 1 mL Luer syringe to inject 200 µL of the washing solution and incubate the slide for 120 min at RT with 200 µL blocking solution.

6.2.4. Prepare 4 mL of another blocking solution containing 100 mM Na_2CO_3, (pH 9.2) supplemented with 4% sucrose and 0.5% BSA for the dilution of the antibodies. Use 200 µL of this blocking solution to dilute the pneumococcus-specific rabbit antiserum 1:100. Use 200 µL of this blocking solution to dilute the VWF-specific mouse antibody 1:50 to make a VWF-specific antibody concentration of 4 µg/mL. Dilute the AlexaFluor488-conjugated secondary antibody from a 2 mg/mL stock solution 1:100 in 200 µL of PBS (pH 7.4) to generate a final concentration of 20 µg/mL.

NOTE: In the described immunofluorescence settings, the antibody detection delivered optimal results when the antibodies were diluted in the above-mentioned alkaline carbonate buffer. Based on previous results, the recommended blocking solutions and the amount of antibodies are suitable for many applications. However, different experiments might require individual optimization of the antibody combination, antibody concentration, incubation time, and constitution of the blocking buffer. As an alternative, a phosphate-buffered system with a neutral pH range might be suitable or even preferred as an incubation buffer. In case of weak fluorescence signals, the concentration of secondary antibody should be increased. If too much unspecific fluorescence background noise is detected, the amount of blocking substances should be increased.

6.2.4 For VWF-immunofluorescence staining, wash the channel slide using a 1 mL Luer syringe by injecting 200 µL of the washing solution 3x and incubate the slide with the 1:50 diluted VWF-specific antibody for 30 min at RT. Afterwards, wash the channel slide again 3x with 200 µL of the washing solution and incubate the slide with the 1:100 diluted AlexaFluor488-conjugated mouse-specific antibody for 30 min at RT. Finally, wash the channel slide again 3x with 200 µL of the washing solution.

NOTE: The AlexaFluor-fluorophores are sensitive to bleaching. Therefore, the slide should be protected by keeping it in a dark chamber during the incubation steps with the fluorophore-conjugated antibodies.

6.2.5. For immunodetection of the pneumococci, incubate the slide with a 1:100 diluted pneumococcus-specific rabbit antibody for 30 min at RT. Afterwards, wash the channel slide 3x with 200 µL of the washing solution and incubate the slide with 1:100 diluted AlexaFluor568-conjugated rabbit-specific antibody for 30 min at RT. Wash the channel slide again 3x with 200 µL of the washing solution.

6.2.6. To stain the cellular actin cytoskeleton with fluorescent phalloidin, permeabilize the HUVEC by incubation with 120 µL of 0.1% Triton X-100 for 5 min at RT. Wash the channel slide 3x with 200 µL of the washing solution and incubate the slide with 120 µL of 1:1,000 diluted AlexaFluor350-conjugated phalloidin. This incubation step will visualize the polymerized actin cytoskeleton and allow monitoring of the cell shape and possible stress-induced morphological changes.

6.2.7. Wash the channel slide 3x with 200 µL of the washing solution. Finally, wash the slide 4x with 200 µL ddH_2O and visualize the green fluorescent VWF strings, the red fluorescent bacteria, and the blue fluorescent actin cytoskeleton using the appropriate filter settings on the fluorescence microscope.

REPRESENTATIVE RESULTS:

Culturing primary HUVEC in a constant unidirectional flow results in the formation of a confluent and tightly packed cell layer that promotes the generation of cellular WPBs filled with the mechanosensitive VWF[13,14]. This protocol describes the use of an air pressure pump-based, pulseless recirculation system for infection analysis that requires mimicking the shear stress situation in the human blood flow.

This system enables a defined, software-controlled setting of flow conditions. The flow scheme in **Figure 1** illustrates the principal workflow, starting with the precultivation of primary endothelial cells (**Figure 1**, **Inset 1**) and the precultivation of pneumococci (**Figure 1**, **Inset 2**). The applied microfluidic system (**Figure 1**, **Inset 3**) is composed of a special channel slide that is connected via a Luer adapter to perfusion tubing with two medium reservoirs. The perfusion tubing set is placed on a fluidic unit that serves as a stand and employs the perfusion tubes as a valve system. For flow cultivation, the fluidic unit with the medium-filled perfusion set and the connected channel slide are placed in a CO_2 incubator (**Figure 1**, **Inset 3**). The fluidic unit is connected to an air pressure pump via air tubing. The air in the tubing must pass through a drying bottle containing silica beads for the removal of moisture from the perfusion reservoirs before the air is repumped into the pump system (**Figure 1**, **Inset 3**). The air pressure pump is controlled by computer software (PumpControl v1.5.0) that enables the setting of a continuous, defined flow rate depending on the diameter of the channel slide, the length and diameter of the perfusion tubing set, and the viscosity of the medium used (**Figure 1**, **Inset 3**). The secretion of VWF by WPB exocytosis of confluently grown HUVEC is induced by histamine-stimulation[8] applied into the medium circulation in the perfusion tubing using an injection port (**Figure 1**, **Inset 4**). At a minimum shear stress of 10 dyn/cm^2, the released VWF proteins multimerize and form long protein strings reaching lengths of more than 100 µm (**Figure 1**, **Inset 4**,**5**,**6** and **Figure 2A**). These protein strings are microscopically detected and visualized after the injection of fluorescein isothiocyanate (FITC)-conjugated VWF-specific antibodies into the medium. The circulating antibodies enable the immunofluorescence detection of the cell surface-bound VWF strings in real time (**Figure 1**, **Inset 5**)[8].

The established microfluidic-based cell culture infection approach using primary endothelial cells mimics the situation of a locally inflamed vasculature upon infiltration of the blood circulation by bacterial pathobionts such as *Streptococcus pneumoniae*. Examples of the visualization and quantitative analysis of bacterial interaction with differentiated vascular cells under shear stress using microfluidic endothelial cell cultivation are shown (**Figure 1**, **Insets 5**,**6**). RFP-expressing pneumococci were injected into the flow and after 30 min of circulation, the first signs of bacterial attachment to VWF strings of histamine-stimulated HUVEC were microscopically detected (**Figure 1**, **Insets 5**,**6** and **Figure 2A**,**B** white arrows)[8]. Thus, the use of RFP-expressing pneumococci enabled the quantification of bacterial attachment to the VWF strings on the endothelial cells without the requirement of bacterial antibody detection.

Histogram overlay plots of the fluorescence intensities were generated using the evaluation software provided by Leica (i.e., LasX) to visualize the colocalization of RFP-expressing pneumococci with the VWF strings detected with green fluorescent antibodies. This enabled a quantitative analysis of the colocalization probability at specific regions of interest (ROI) within the fluorescence image. Using histogram overlays of bacterial signals in combination with the fluorescence signals of the VWF, overlapping fluorescence peaks for both could be

visualized and thereby confirmed pneumococcus attachment to the VWF strings. The bacterial attachment resists the continuously applied shear stress for a minimum of 25 min (**Figure 2B**)[8].

In sum, the continuous flow cultivation of primary HUVEC enabled VWF secretion and the generation of long VWF protein strings that serve as adhesion sites for circulating bacteria[8].

FIGURE LEGENDS:

Figure 1. Workflow for analyses of bacterial attachment to VWF strings using microfluidic endothelial cell cultivation. Workflow of the principal experimental steps beginning with the precultivation of primary endothelial cells to subconfluency in cell culture flasks. Prior to cell cultivation in the flow, cells are seeded into a gelatin-coated channel slide (**Inset 1**). Bacteria are grown on agar plates followed by cultivation in a complex liquid medium to mid-log phase (**Inset 2**). For microfluidic cell culture, a channel slide with endothelial cells is connected to the perfusion tubes of a fluidic unit of the pump system and subjected to constant flow for cell differentiation (**Inset 3**). The generation of the VWF-strings (green arrow) was induced by histamine injection at a shear stress of 10 dyn/cm^2 and microscopically monitored by immunofluorescence detection using FITC-labelled VWF-specific antibodies (**Inset 4**). After injection of RFP-expressing bacteria to the circulating medium, pneumococcus attachment (red arrow) to the VWF-strings was microscopically visualized in real time by fluorescence emission at 450 nm (**Inset 5**). After fixation of the cells using PFA, differential immunofluorescence staining provides the visualization of bacterial attachment at specific infection time points (**Inset 6**). The images of the pump system and the HUVEC cell layer described in steps 1, 3, and the image of the injection port (**Inset 4**) are included. The immunofluorescence images shown in **Insets 4** and **5** have been modified and used with permission from Jagau et al.[8]. The lengths of the scale bars are indicated at the lower right.

Figure 2. Representative immunofluorescence images of pneumococcus binding to the VWF strings generated in continuous flow on the surface of histamine-stimulated HUVEC. (**A**) The generation of the VWF strings was microscopically quantified after exposing confluently grown HUVEC to shear stress using a microfluidic pump system at 10 dyn/cm^2. FITC-conjugated VWF-specific antibodies detected the VWF strings. White arrows point to RFP-expressing pneumococci with red fluorescence attached to long VWF strings. (**B**) Bacterial attachment to the green fluorescent VWF strings was microscopically observed for up to 2 h in constant flow (white arrows) and was confirmed by software-based evaluation of fluorescence intensities of a defined ROI. Real time images were taken with the fluorescence equipment of a confocal laser scanning microscope (SP8, Leica). Scale bars = 10 µm. This figure has been modified and used with permission from Jagau et al.[8].

DISCUSSION:

The simulation of bacterial interaction with mechanosensitive host proteins such as VWF requires a perfusable cell culture system that enables the generation of a defined, unidirectional, and continuous flow of liquids, thereby generating reliable shear stress. Several microfluidic pump systems have already been described. A comprehensive review from Bergmann et al. summarizes the key aspects of different two- and three-dimensional cell culture models[17].

The microfluidic technology is a very young technique started in the early 1990s with the development of controllable, reproducible, and perfusable microenvironments in a micrometer and even in a nanometer scale[18]. The presented microfluidic approach can also be applied to study the broad range of bacterial adhesion mechanisms and involved proteins. It is best suitable for any interaction that involves mechanoresponsive adhesion components such as VWF, which in general are not approachable using standard cell culture techniques. For example, it is known that the conformation of several extracellular matrix proteins differs depending on their location. Within the blood system, glycoproteins like fibronectin circulate in a globular conformation, whereas in the extracellular matrix the proteins appear as multiconnected di- and multimerized scaffold[19,20]. Moreover, several distributors of microfluidic equipment offer standardized channel slides precoated with different types of collagen (e.g., subendothelial collagens or other plasma-derived proteins). These channel slides are suitable for visualization and quantitative analyses of bacterial adhesion to specific extracellular matrix proteins in different flow situations.

Different microfluidic systems can be categorized based on the different materials used for microdevice production. In this regard, the glass/silicone-based platforms differ from the polymer-based and the paper-based platforms[21]. Polymer-based channel slides are produced with an elastically supported surface (ESS), generally require a surface coating for cell attachment during flow experiments. As an alternative to a coating with adhesion-supporting components such as collagen, the surface of some commercially available channel slides is physically modified, which creates a hydrophilic and adhesive surface suitable for most cell types. Moreover, some channel slides are generated using substrates like polydimethylsiloxane (PDMS), which is oxygen-permeable and also enables the culture of blood vessel cells on the inner surface of microchannels in fluidic pump systems[22,23].

The microfluidic system that was used in these studies served as an efficient and reliable system that was applied for the analysis the interaction of *Staphylococcus aureus* with multimerized VWF fibers after culture of human endothelial cells in flow[24,25]. This microfluidic system was a closed circular perfusion system that enabled the analysis of infection by pathogenic bacteria under biosafety 2 conditions. Moreover, the channel slides were suitable for microscopic monitoring during flow and are available with different precoatings (e.g., gelatin, poly-L-lysine, or collagen-IV) that ensure cell adhesion and a high degree of experimental reproducibility. This protocol uses a microfluidic system (see **Table of Materials**) for the establishment of a perfusable infection model for *S. pneumoniae,* thereby mimicking the flow situation within the human vascular system[8].

The described general procedure of bacterial cell attachment in this microfluidic system can also be conducted with other types of pump systems generating a defined and continuous flow in a sterile environment. For microfluidic purposes, mainly four types of flow control systems are used: i) peristaltic pumps and recirculation pumps, which are used in this protocol, ii) syringe pumps, iii) pressure controllers, and iv) pressure controllers with flow switch matrices. Each kind of flow control system has advantages and drawbacks depending on the specific microfluidic application and the ability to carry out microscopic visualization in real time. In most applications requiring a continuous circulation of the samples, recirculation pumps are combined with software-based pressure controllers to ensure a defined flow situation. This is also optimized in the pump system demonstrated here. The syringe-based pump systems can be subdivided into “classic” syringe pumps, which generate flow oscillation,

and “pulseless” microfluidic syringe pumps. These syringe pump-based systems are generally easy to use, but flow control in dead-end channels is challenging using syringe pumps. Moreover, flow changes inside the chip might take some time, and a flow meter is required to determine the flow rate. Even pulseless syringe pumps might generate periodic pulsation on the flow rate due to the step-by-step motor of the syringe pump. Another two syringe pump device is able to generate an “infuse and withdraw”-based fluidic movement that applies defined shear forces on cell surfaces and is suitable for microdialysis applications even in a PC-independent manner. This system must be combined with microtubing for connection of chambers or channel slides for cell cultivation followed by microscopic analyses.

The abovementioned pressure controllers are flow control systems that pressurize the tank containing the sample, which is smoothly injected in a microfluidic chamber or on a chip. The pressure controllers can establish a pulseless flow and also provide flow rates in combination with flow meters. A combination of pressure controllers with flow switch matrices enable a fast flow switch with no back flows. The recirculating system presented here enabled the generation of shear stress values typically reported for the human vascular system[26]. In vivo vascular wall shear stress has been estimated from wall shear rates as derived from non-invasively recorded velocity profiles, and whole blood viscosity in large arteries and plasma viscosity in arterioles[26]. Reneman and Hoeks recorded velocity profiles in large arteries by using a specially designed ultrasound system and in arterioles via optical techniques using fluorescent flow velocity tracers[26]. An average shear stress of 11-13 dyn/cm^2 is reached in the carotid artery, as opposed to only 4-5 dyn/cm^2 in the brachial artery. Peak values of up to 25-70 dyn/cm^2 have been monitored for the carotid artery. The shear stress values of small and middle veins ranges between 0.1 and 0.5 dyn/cm^2. In the microfluidic system described here, the applicable shear stress value depends on the selected perfusion tubing diameter, the height of the channel slide, and the viscosity of the fluidic medium used. The selected fluidic setting was composed of a slide with 0.4 cm in height (volume of 100 µl) in combination with a perfusion set of 50 cm in length and 1.6 mm in diameter and the medium viscosity was 0.0072 [dyn·s]/cm^2. This setting is appropriate for a shear stress range between 3.5 and 31.2 dyn/cm^2 at flow rates of 3.8 mL/min−33.9 mL/min. In addition, the pressure pump software control can apply a pulsed medium flow that could mimic a pulsed arterial blood flow.

The successful, reliable, and reproducible use of this combined microfluidic infection method requires some precautions that must be kept in mind. During the infection process under microfluidic conditions, the cell layer might be exposed to cytotoxic or cytolytic bacterial compounds such as pneumolysin, which affect the eukaryotic cell viability and weaken the cell adherence to the slide surface. Therefore, keeping a constant flow is required throughout the whole course of the experiment and the integrity of the cell morphology must be frequently monitored. Moreover, the flow culture medium should provide all essential nutrients to the endothelial cells to ensure a tight surface attachment of the cells throughout the infection experiment. Nevertheless, it must be noted that medium supplements contain substances that might interfere or inhibit the interaction between the bacteria and specific host proteins. In studies of pneumococcus-VWF interaction for example, heparin must be depleted from the cell culture medium, because it inhibits the binding of pneumococci to VWF[8].

Another critical step in flow culture of endothelial cells is the maintenance of tight cell adhesion, which depends on the overall cell vitality and on the level of differentiation. Shear flow-resistant cell adhesion of primary endothelial cells was only achieved when cells were

strictly kept in subconfluency before exposure to shear stress. On the other hand, the production of WPB directly depends on the confluency of the endothelial cell layer promoting tight cell-cell-contacts[13]. The benefit of the microfluidic cell culture of primary endothelial cells covers the strongly induced cell proliferation that quickly leads to the formation of a confluent cell layer under flow conditions. The cells are tightly attached to each other, are collectively oriented in the direction of flow, and represent a highly differentiated phenotype that is required to produce secretory WPB. These WPB serve as storage vesicles for VWF, vasodilation activators, and cytokines that are exocytosed as protein bursts upon histamine stimulation or pneumolysin activity[27,13]. Therefore, the generation of a highly adhesive, confluent cell layer of differentiated primary endothelial cells in low proliferation passages is an indispensable prerequisite for efficient VWF release and the formation of VWF strings on the cell surface and the analyses of bacteria-VWF-interaction in flow conditions. Thus, it has to be noted that the differentiation of the endothelial cells took 48 h of flow cultivation at a minimum and required a constant shear flow without any variations in the pump pressure. Any variations might lead to a sudden medium blast, which would flush the cells out of the slide. Moreover, during microscopic visualization the microfluidic slide and the medium reservoirs of the pump system needed to be kept at a temperature of 37 °C as this represents the temperature optimum of the human cells.

Immortalized human cell lines are suitable for many scientific experiments and are often employed in cell culture infection studies. These cell lines share some general technical advantages, such as low or moderate culture requirements and unlimited proliferation, which enables passaging several hundred times without significant changes in morphology or receptor profiles. However, these cell lines represent a solitary monoculture and are subjected to artificial two-dimensional growth conditions.[28] The missing physiological source tissue environment results in substantial changes of functional and morphological cell phenotype with every passage of the culture[29]. Prior to other bacterial adhesion experiments, the profile of surface-exposed cell-type specific marker proteins and receptors of human primary lung endothelial cells at different cell culture passages was determined. The flow cytometry analysis revealed a strongly reduced expression of specific surface proteins such as the platelet endothelial cell adhesion molecule 1 (PECAM1) within eight rounds of cell passaging. Moreover, the expressed integrin receptor profile was significantly changed in higher cell passages in favor of a cell type-unspecific integrin receptor pattern and a reduced cell type-specific integrin receptor pattern (Bergmann et al., unpublished data). These results are particularly relevant for analyses of pathogen-host interactions in infection biology studies. With the aim of sustaining the phenotypic cell characteristics and a high level of functional differentiation during microfluidic cell culture at the time of bacterial infection, primary endothelial cells from multiple donors were chosen in this case and used only up to five passages at maximum in order to keep the phenotypic characteristics as specific as possible[8].

The combined visualization of bacteria and specific cell surface structures during flow requires an optimized fluorescence staining protocol. Using different combinations of directly labelled proteins, fluorescence protein-expressing bacteria, and fluorescence-conjugated antibodies, immunofluorescence staining procedures can be specifically adapted to the visualization targets. These procedures enable a defined and clear microscopic visualization and also a differentiation and quantification of bacterial adherence and internalization[3,13,30,31]. The immunofluorescence-based detection of internalized bacteria requires a cell permeabilization step such as a short Triton X-100 incubation, which might lead to cell detachment. Therefore,

the detection of bacterial internalization processes occurring in a flow culture should be visualized after flow incubation using PFA-crosslinked samples. For real time visualization of bacteria in flow, the use of genetically modified bacteria expressing fluorescence proteins enables a quick and targeted microscopic detection. RFP-expressing pneumococcus strains that were generated using an efficient genetic construct designed by Kjos and Veening[16,8] were used in this experimental study. For detection of VWF strings in flow, different VWF-specific fluorescein-labelled antibodies were tested and could be used. To obtain an optimal signal response at a minimized unspecific background, the adequate antibody concentration was consistently titrated for each applied antibody.

For microscopic live cell imaging, the fluidic unit and the channel slide are removed from the CO_2 incubator and placed into a chamber prewarmed to 37 °C at the microscope. This chamber could not be adjusted to 5% CO_2. Without a carbonate-buffered atmosphere, HUVEC morphology and bacterial fitness remained intact for up to 180 min, which is enough for analysis of VWF-mediated bacterial adherence. If longer infection times and microscopic monitoring outside of a CO_2 incubator are required, a buffered cell culture medium should be used in order to compensate for pH shifts due to inadequate CO_2 concentration. Alternatively, the whole system could be placed back into the CO_2 incubator in between the steps of a time series of microscopic visualization.

In addition to the fluorescence detection in real time, the bacterial infection of the endothelium within the channel slide can be stopped and preserved by medium exchange with paraformaldehyde (PFA) as a fixation substance. After fixation in the flow, the optimized and stepwise immunofluorescence staining of the cell surface within the channel slide enables the generation of valuable microscopic snapshot visualizations and provides a versatile combined structure detection of specific cellular compounds involved in the interaction between bacteria and host cells. The scientific benefit of this combined method is that the PFA-treated channel slide can be stored and used for a post-flow immunofluorescence staining of the structures of interest. The PFA treatment inactivates the bacteria and thus arrests the expression of RFP-protein. Therefore, a pneumococcus-specific antiserum was generated in rabbit for bacterial immune detection and visualization was performed using a rabbit-specific AlexaFluor568-conjugated secondary antibody[8]. As already mentioned, the use of different antibody combinations requires an exact optimization of antibody amounts and the blocking buffer composition. Otherwise, unspecific background signals and cross-detection effects might lead to artificial staining results. An optimized immunofluorescence staining procedure can easily be used for the detection of many different cellular targets such as the actin cytoskeleton or endosomal markers[13,31].

This procedure can be adapted to create a more complex tissue environment that mimics physiology from a histologic, physiological, and functional point of view. The presented infection analyses can be efficiently used to answer several scientific questions at the same time by using an in line-connection of several channel slides to one perfusion set. This extended set up would facilitate the analyses of different cell types, cell confluences, and different slide-coatings within the same flow setting in parallel and allow a direct comparison of bacterial infections of different cell types. Moreover, the serial in line connection and combined analyses of several channel slides also provides the possibility of time series experiments, which can be applied for gene expression profiling (e.g., the analysis of infection time-dependent virulence factor gene expression).

The infection analyses can also be extended to a constant laminar flow condition lasting several days or even weeks in order to analyze cellular response in conditions mimicking a long-term chronic infection phase. In addition to the presented single-cell type culture, some examples of heterotypic cell culture in microfluidic cell culture devices have already been reported[32,33]. This allows for high throughput pharmacological studies and might ultimately result in using microfluidic cell culture systems for regenerative purposes as well[34].

In summary, the microfluidic system in combination with immune staining procedures served as a valuable model for the analysis of pathomechanisms between bacteria and host cells in an environment that simulates the conditions within the vascular system.

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DISCLOSURES:
The authors have nothing to disclose.

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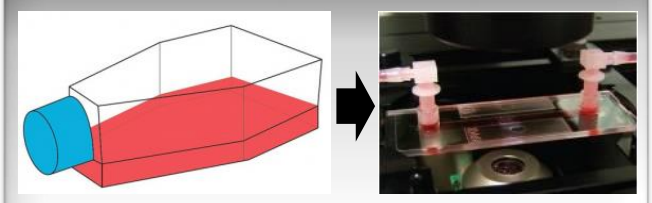
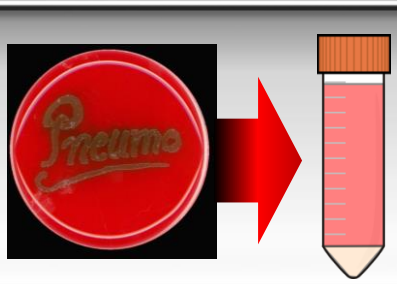
Figure 1

Click here to access/download;Figure;Figure1FlowSchemeRev.pdf

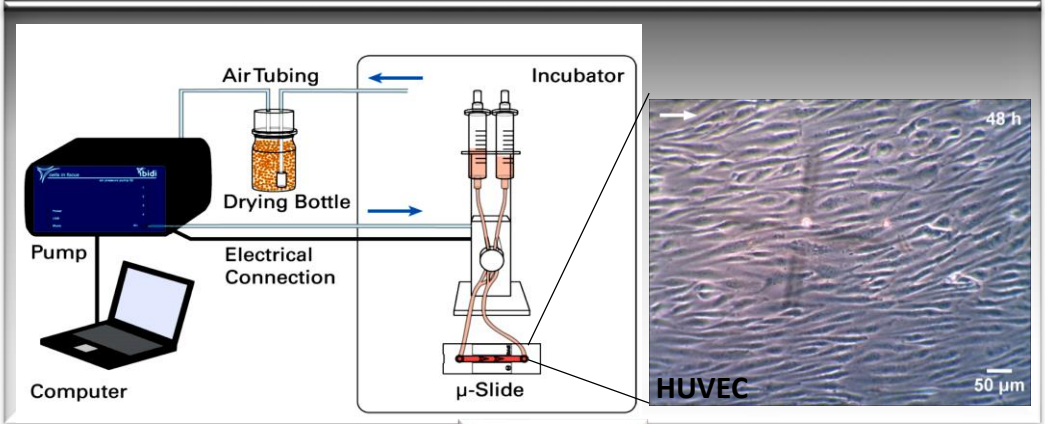
1. Precultivation of primary endothelial cells



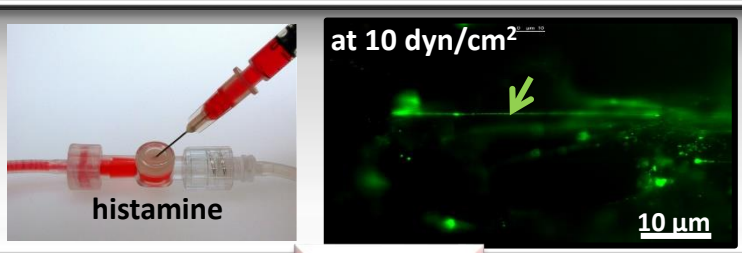
2. Precultivation of *Streptococcus pneumoniae*



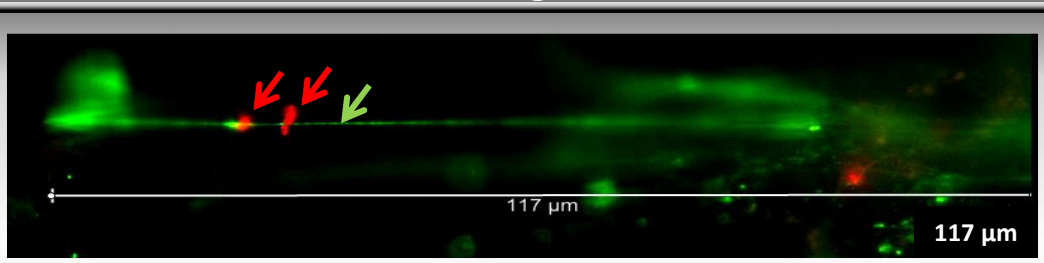
3. Endothelial cell cultivation of HUVEC under microfluidic conditions



4. Induction of VWF-release & visualization of multimerized VWF strings



5. Microscopic evaluation of bacterial attachment to VWF strings in real time



6. Microscopic evaluation of bacterial attachment to VWF-strings after sample fixation

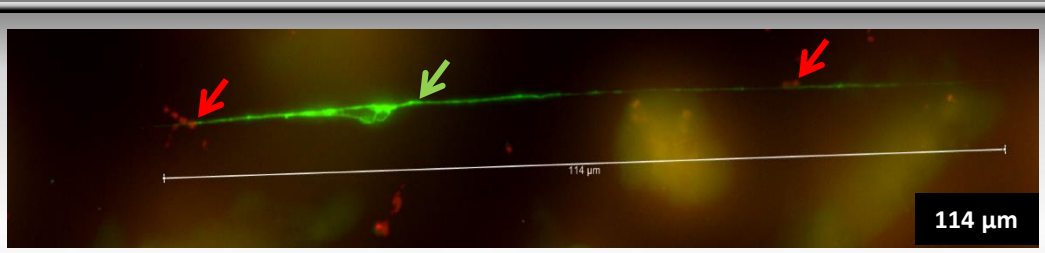
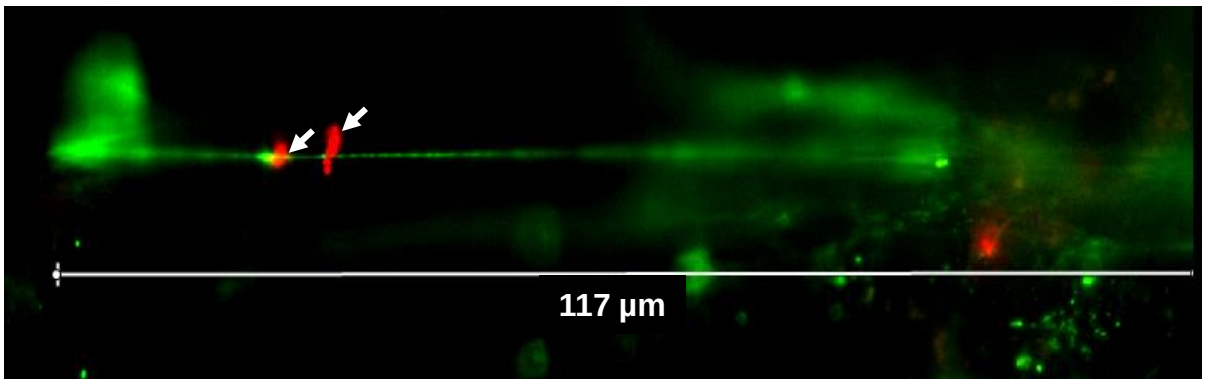
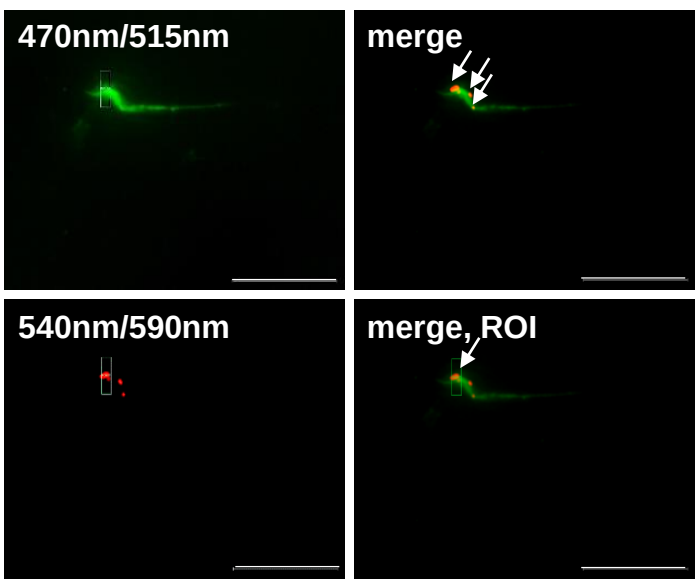


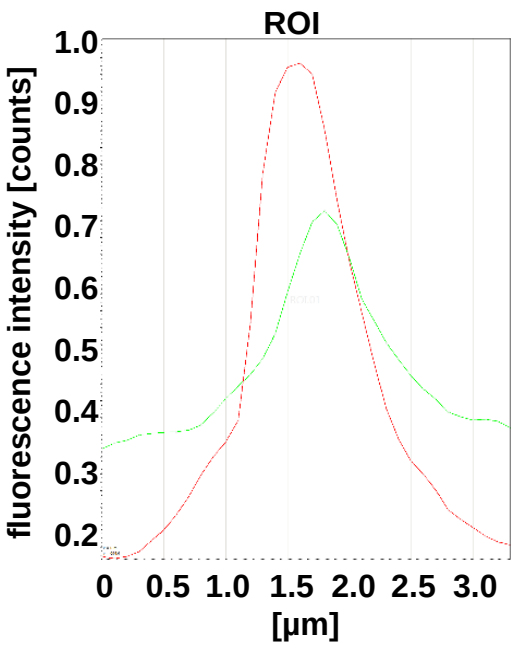
Figure 2 Click here to access/download;Figure;Figure2Rev.pdf

A



B





Name of Material/Equipment	Company	Catalog Number
1 mL Luer-syringe	Fisher Scientific	10303002
2 mL Luer-syringe	Sarstedt	9077136
Accutase	eBioscience now thermo fisher	00-4555-56
AlexaFluor350-conjugated Phalloidin	Abcam	ab176751
AlexaFluor488-conjugated goat-derived anti-mouse antibody	Thermo Fisher Sientific	A11001
AlexaFluor568-conjugated goat-derived anti-rabbit-antibody	Thermo Fisher Scientific	A-11011
Bacto Todd-Hewitt-Broth	Becton Dickinson GmbH	BD 249210
Bovine Serum Albumin (BSA)	Sigma Aldrich	A2153-25G
Cell culture flasks with filter	TPP	90026
Centrifuge Allegra X-12R	Beckman Coulter Life Sciences	392304
Centrifuge Allegra X-30	Beckman Coulter Life Sciences	B06314
Centrifuge Z 216 MK	Hermle	305.00 V05 - Z 216 M
Chloramphenicol	Carl Roth GmbH + Co. KG, Karlsruhe	3886.2
Clamp for perfusion tubing	ibidi	10821
CO_2-Incubator	Fisher Scientific	MIDI 40
CO_2-Incubator	Sanyo	MCO-18 AIC
Colombia blood agar plates	Becton Dickinson GmbH	PA-254005.06
Computer	Dell	Latitude 3440
Confocal Laser Scanning Microscope (CLSM)	Leica	DMi8
Di Potassium hydrogen phosphate (KH_2PO_4)	Carl Roth GmbH + Co. KG, Karlsruhe	3904.1
Drying material	Merck	101969
ECGM supplement Mix	Promocell	C-39215
ECGMS	Promocell	C-22010
Endothelial Cell growth medium (ECGM, ready to use)	Promocell	C-22010
Fetal Calf Serum (FCS)	biochrome now Merck	S 0415
FITC-conjugated goat anti-human VWF antibody	Abcam	ab8822
Fluidic Unit	ibidi	10903
Gelatin (porcine)	Sigma Aldrich	G-1890-100g
histamine dihydrochloride	Sigma Aldrich	H-7250-10MG
Human Umbilical Vein Endothelial Cell (HUVEC)	Promocell	C-12203 Lot-Nr. 396Z042

Human VWF-specific antibody derived from mouse (monoclonal)	Santa Cruz	sc73268
Injection Port	ibidi	10820
Light microscope	Zeiss	Axiovert 35M
Luer-slides $I^{0.4}$ (ibiTreat472microslides)	ibidi	80176
Magnesium sulfate ($MgSO_4$, unhydrated)	Sigma Aldrich	M7506-500G
Microfluidic Pump	ibidi	10905
Neubauer cell counting chamber	Karl Hecht GmbH&Co KG	40442002
Paraformaldehyde 16% (PFA)	Electron Microscopy Sciences	15710-S
Perfusion Set	ibidi	10964
Phosphate-buffered saline (PBS)		
Plastic cuvettes	Sarstedt	67,741
Pneumococcus-specific antiserum	Pineda	
Polystyrene or Styrofoam plate		
Potassium chloride (KCl)	Carl Roth GmbH + Co. KG, Karlsruhe	6781.1
Pump Control Software (PumpControl v1.5.4)	ibidi	v1.5.4
reaction tubes 1.5/ 2.0mL	Sarstedt	72.706/ 72.695.500
reaction tubes with 50 mL volume	Sarstedt	62,548,004
RFP-expressing pneumococci	National Collection of Type Cultures, Pul	10,319
serological pipets 5, 10 mL	Sarstedt	86.1253.025/ 86.1254.025
Sodium Carbonate (Na_2CO_3, water free)	Sigma Aldrich	451614-25G
Sodium dihydrogen phosphate (NaH_2PO_4)	Carl Roth GmbH + Co. KG, Karlsruhe	P030.2
Spectral Photometer Libra S22	Biochrom	80-2115-20
Sucrose	Sigma Aldrich	S0389-500G
Triton X-100	Sigma Aldrich	T9284-500ML
Yeast extract	oxoid	LP0021

Comments/Description

with 1 mL volume for gelatin injection using the luer-connection of the slides
For piepting/injecting fluids into the luer connections of the channel chamber slides
protease mix used for gentle detachment of endothelial cells
no concentration available from the manufacturer, stock solution is sufficient for 300 tets, company recommends to use 100 µl of a 1:100
stock concentration: 2 mg/mL for immunostaining of human VWF in microfluidic slide after PFA fixation, green fluorescence

stock concentration: 2 mg/mL for immunostaining of pneumococci in microfluidic slide after pFA fixation, red fluorescence
complex bacterial culture medium
solubilized, for preparation of blocking buffer
subcultivation of HUVEC in non-coated cell culture flasks of 25cm^2 surface
spinning down of bacteria (volumes of > 2mL)
spinning down of eukaryotic cells
spinning down of bacteria (volumes of less than 2 mL)
used in a concentration of 0.2 /mL for cultivation of pneumococci transformed with genetic construct carrying red fluorescent protein and
for holding the liquid in the tube bevor connecting the slide to the pump system
incubator size is perfectly adapted to teh size of the fluidic unit with connected channel slide and was used for flow cultivation at 37°C and
for incubation of bacteria and cells in a defined atmosphere at 5% CO_2 and 37°C
agar-based complex culture medium for *S. pneumoniae* supplemented with 7% sheep blood
Comuter with pressure pump software
An inverse microscope with a stage covered by a heatable chamber and with a fluorescence unit equipped with fluorescence filter, Xenon-
used for PBS buffer
orange silica beads for drying used in a glass bottle with a tubing adaptor
supplement mix for ECGM -medium, required for precultivation of endothelial cells: 0.02 mL/mL Fetal calf serum, 0.004 mL/ mL endothelia
ECGM supplemented with 5 % [w/w] FCS and 1 mM $MgSO_4$ to increase cell adhesion
culture medium of HUVECs, already supplemented with all components of the supplement mix
supplement for cell culture, used for infection analyses
stock concentration: 10 mg/mL, for immunodetection of globular and multimerized VWF in flow
fluidic unit for flow cultivation
for precoating of microslide channel surface
for induction of VWF secretion from endothelial Weibel Palade Bodies
primary endothelial cells from pooled donor, stored crypcoserved in liquid nitrogen

stock concentration: 200 µg/mL for immunostaining of VWF in microfluidic slide after PFA fixation
for injection of histamin or bacteria into the reservoir tubing during the flow circulation
inverse light microscope for control of eukaryotic cell detachement and cell counting using a 40 x water objective allowing 400 x magnificat
physically modified slides for fludic cultivation (µ–Slide $I^{0.4}$Luer with a channel hight of 0.4 mm, a channel volume of 100 µl, a growth area c
For preparation of ECGMS medium
air pressure pump
microscopic counting chamber for HUVECs
for cross linking of samples
Perfusion Set Yellow/Green has a tubing diameter of 1.6 mm, a tube length of 50 cm, a total working volume of 13.6 mL, a dead tube volur
the solution was prepared using the following chemicals: 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4 , pH 7.4
(2 x optic) for OD measurement at 600 nm
raised in rabbit using heat-inactivated *Streptococcus pneumoniae* NCTC10319 and D39, IgG-purified using proteinA-sepharose column.
this is a precaution step to avoid cold stress on the cells seeded in the channel slides. Any type of styrofoam such as packaging box-materia
used for PBS buffer
Computer software for controlling the pressure pump, setting the flow conditions and start/end the flow
required for antibody dilutions

Streptococcus pneumoniae serotype 47 expressing RFP fused to ahistone-like protein integrated into the genome
for pipeting larger volumes
for preparation of 100 mM Sodium Carbonate buffer
used for PBS buffer
measurement of optical density (OD) of bacterial suspension at 600 nm
for preparation of blocking buffer
Used in 0.1% end concentration diluted in dH_20 for eukaryotic cell permeabilization after PFA fixation
bacteria are cultivated in THB supplement with 1% [w/w] yeast extract = complete bacterial cultivation medium THY

) dilution, blue fluorescence (DAPI-filter settings)

chloramphenicol resistance cassette

5% CO_2

light source (SP8, DMi8) and DFC 365 FX Kamera (1392 x 1040, 1.4 Megapixel)

ıl cell growth supplement, 0.1 ng / mL epidermal growth factor, 1 ng / mL basic fibroblast growth factor, 90 µg / mL heparin, 1 µg / mL Hy

tion

of 2.5 cm and a coating area of 25.4 cm^2) suitable for all kinds of flow assay, the physical treatment generates a hydrophilic and adhesive s

ne of 2.8 mL and a reservoir size of 10 mL. combined with the µ-slide L0.4Luer, at 37°C and a viscosity of 0.0072 dyn x s/cm^2 a flow rate ra

al can be used. The plate might by 0.5 cm thick and should have a size of 20 $cm^{2.}$

drocortisone

surface.

ange of 3.8mL/min up to 33.9 mL/min and shear stress between 3.5 dyn/cm^2 and 31.2 dyn/cm^2 can be reached. with 50 cm lenght for mic

:rofluidic

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Author(s): Hilge Jagger, Ina-Kristin Behrens, Michael Steinert, and Simone Bergmann

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Revision to Manuskript **JoVE60323**

Entitled: "Pneumococcus infection of primary human endothelial cells in constant flow"

Authors: Hilger Jagau, Ina-Kristin Behrens, Michael Steinert, and Simone Bergmann

Dear Dr. DSouza,

I thank you very much for sending the comments and the instructions for the revision of our manuscript. The editorial and the reviewer comments helped a lot to improve the quality of the manuscript. Please find below my line-by-line responses to the comments of the reviewers. The corrected text phrases were marked in green and the protocol steps selected for the video are marked in yellow. In addition to the revised manuscript I also attached the filled table of materials and Figure 1 and 2 in a better quality. Moreover, we have generated screen shots of important software settings and added the screen shot images as supplementary figures 1-4. I hope that all comments were clearly addressed.

Kind regards,

Simone Bergmann

Response to Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Response: Grammar and spelling of the whole manuscript was approved.

• **Textual Overlap:** Significant portions show significant overlap with previously published work. Please re-write the text on lines 101-116, 433-438, 442-446, 465-467, 492-507, 535-538 to avoid this overlap.

Response: Each of the above mentioned text parts were rewritten, clarified and extended (please refer to green text marks in the manuscript).

• As HUVECs are used, please include an ethics statement before your numbered protocol steps indicating that the protocol follows the guidelines of your institutions human research ethics committee.

Response: The ethics statement was included before the numbered protocol steps with the following sentence: "**Ethic statement:** The microfluidic cell cultivation was performed with primary human umbilical vein endothelial cells (HUVEC), which were purchased from the Company Promocell. The company isolated the cells with informed consent of the donor. This was approved by the Ethics Committee of Doctors Chamber of the Federal State Baden-Wuerttemberg with the reference number: 219-04."

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. 1) Mention microscope magnification in 5.1.

Response: For microscopic visualization, a HC PL APO CS2 63 x/1.40 oil objective was used, which enables a 630-fold magnification of the sample. We have included this information in the protocol step 5.1.3 and provided the details in table of materials.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

Response: The protocol steps for the video are highlighted in yellow. They will present the most important steps in a cohesive narrative and they follow a logical flow.

- **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

  Response: The discussion was critically approved and the paragraphs were subdivided into the above mentioned headings in a slightly changed order. Moreover, some technical aspects were added and discussed to more detail (please refer to the additional text passages marked in green).

- **References:** Please spell out journal names.

  Response: all journal names were spelled out.

- **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are ibidi®, (µ-slides; ibidi®, falcon, Luer0.4 265 µ-slide, ibiTreat 472 ®microslides, etc

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  Response: all commercial sounding terms were deleted or exchanged with generic terms, such as "channel slide" instead of µ-slide and "tube" instead of "falcon".

- **Table of Materials:** Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as

reagents, cell lines, culture media, microscope, slides, µ slides, culture plates, bacterial strains, agar, FCS, ECGM, microfluidic pump, spectrometer, cell counter, antibodies along with their concentrations and RRIDs, etc.

Response: I apologize for not sending the filled table- this was my mistake. Please find attached the Excel-file with the filled table of materials.

• Please define all abbreviations at first use.

Response: all abbreviations were defined at first use and abbreviations were also clarified to detail in table of materials.

• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.

Response: standard abbreviations were used only.

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Response: for the images shown equipment in Figure 1, we submitted a permission for image publication from the company as PDF File. In Figure 1 and 2 we have reused images from our publication in Frontiers in Microbiology. This Journal has published the permission to reuse result figures according to their editorial policies. Please refer to https://www.frontiersin.org/legal/terms-and-conditions.

<u>Responses to Comments from Peer-Reviewers:</u>

Reviewer #1:

Manuscript Summary: The manuscript form Jagau et al. describes the application of a unidirectional pump flow system to investigate the adhesion of pneumococci to endothelial

cells. The manuscript is clearly written and provides helpful information in order to perform comparable experiments. However several experimental steps required better explanation. The focus of the paper is the investigation of VWF.

- Would it be also possible to investigate other adhesion mechanisms/molecules?

  Response: The presented microfluidic approach can also be applied to study other adhesion mechanisms and proteins. In particular, it is best suitable for any interaction which involves mechanoresponsive adhesive components, which are not approachable using standard cell culture techniques. For example, it is known that the conformation of several extracellular matrix proteins differs depending on the specific location. Within the blood system, some glycoproteins such as fibronectin circulate in a more globular conformation, whereas the proteins appear as multi-connected di- and multimerized scaffold in the extracellular matrix. This option has been included in the discussion part into the chapter headed with the title "Significance with respect to existing methods".

- **Major Concerns:**

  The presented concepts of VWF exposure which are explained in the introduction are not clear. The authors should distinguish between the VWF/collagen matrix exposed after detachment of the endothelial cells (vascular injury) and the active release of VWF from the endothelium after stimulation promoting the formation of VWF strings on the endothelial cell surface.

  Response: The introduction has been focused to the details, which are relevant for the described new approach and clarified.

- Is degas the correct term? Degasing of fluids is frequently performed to remove any gas from solutions. In the present paper, cell culture medium was equilibrated at 5% CO2 probably to adjust the pH of the CO2 sensitive culture medium? Moreover I did not understand why the authors equilibrate the slides prior to the coating with non-equilibrated gelatine solution. Wouldn't it make more sense to perform the coating prior to the CO2 equilibration?

  Response: The term "to degas" derives from the application manual from the pump system company. In order to clarify this term and procedure, we have described the degassing process more to detail in 3.2.1. and explained the reason for it using the following sentences: "Equilibrate a channel slide, a perfusion set of 1.6 mm in diameter and 50 cm in length (see table of materials) and an aliquot of ECGM-medium supplemented with 5% FCS and 1 mM $MgSO_4$ (ECGMS) for 24 h in an incubator with 5% CO_2- atmosphere to reduce the amount

of air bubbles. If materials or liquids have been stored at RT or in the refrigerator, gases dissolved in the plastic and liquids will be released when heated up in the incubator during the experiment. Gas bubbles will then appear. Degassing all plastic components before the experiment will eliminate this effect. The gelatin solution is only applied in a very low volume which does not requires a degassing.

Note: Each time you take the system out of the incubator, the process of gas absorption begins again. Therefore work quickly at RT and never leave the fluidic unit outside the incubator for longer time periods."

- The authors tried to smoothly adapt the cell to the flow conditions by starting the under-flow-culture at 5 dyn/cm² for 30 minutes. Do the authors have any evidence that this is true? It is difficult to believe that 30 minutes are enough to allow an adaptation.

  Response: We started the flow culture at a shear stress of exactly 5 dyn/cm^2 because the software-based pump pressure control enables the exact setting of shear stress depending on the used perfusion set, channel chamber height and medium viscosity. In our previous analyses we experienced that HUVEC cells tend to detach if we directly start with 10 dyn/cm^2. Instead, the cells remain attach to the chamber surface if this stepwise protocol beginning with 5 dyn/cm^2 is applied. After 30 min we increased the shear stress to desired 10 dyn/cm^2, which is the required shear stress value for VWF string formation. I added this information in a note to protocol step 3.2.4.

- Did the authors cultivate the cells under flow also in the presence of 5% CO2 or only at 37°C. Currently, the reader would get the impression that those experiments (under flow cultivation and microscopy) were performed at 37°C only which would mean that the pH of the culture medium would increase to an alkaline pH rapidly. If so, experiments should be performed in a CO2 independent buffer system as most physiological processes are strongly pH dependent. Shift from an almost neutral physiological pH (~7.4) to an alkaline environment may affect the biology of bacterial adhesion and many other things

  Response: We thank the referee for this helpful advice. Indeed, we cultivated the cells under flow using a CO_2-incubator at 37°C and 5% CO_2 in order to avoid changes of the buffer pH. We have used a small CO_2-incubator from the company Fisher Scientific for this incubation step, because the size of this incubator is perfectly adapted to the size of the fluidic unit with a connected channel slide. But any other type of CO_2-incubator can be used for this step. I added this important information to protocol step 3.3.7. For the time of infection analyses

and microscopic visualization, we had to remove the fluidic unit from the incubator due to the limited length of the perfusion tubing. The fluidic unit was placed in a small heating chamber prewarmed to 37°C and the channel slide was fixed with clamps on the microscope stage, which is totally covered by a temperature incubation chamber (3.3.6). We prewarmed the chamber for 24 h to 37°C. For this step, the use of a CO_2-independent buffer system would allow longer infection times than 180 min. This step is also critically discussed in the discussion part in section "Limitations of the technique".

- The authors postulate that their experimental setup allows the visualisation of bacterial internalisation, which would be of high interest. However, the provided data showed bacterial VWF-binding only.

  Response: According to our studies, the VWF-mediated attachment of pneumococci does not induce bacterial uptake into HUVEC, therefore we cannot provide images with internalized bacteria. But in general, the described differential immunostaining procedure is well applicable for a differential detection of bacterial attachment in combination with bacterial internalization after PFA-fixation of the infected cells cultured together with bacteria under flow. This differential immunostaining just requires an additional incubation step with Triton X-100 to permeabilize the eukaryotic cells followed by incubation with primary bacteria-specific antibodies and a fluorescence-conjugated secondary antibody. Since this statement is not clear, I have rewritten this part in the discussion in section "Limitations of the technique" and explained it more to detail. I have removed the term "internalization" from the heading of the figure legend to figure 1 and I have also rewritten the last sentence of the summary.

- The authors prewarm the stage of a confocal laser scanning microscope, finally they used epifluorescence microscopy for their experiments?

  Response: The used confocal laser scanning microscope is additionally equipped with fluorescence filter and a Xenon light source. The optic and the software are shared by the two systems. The fluorescence settings are used for the flow visualization because the adjustment to image changes is much faster than the settings of the laser optic. This might be a bit irritating to the readership, therefore we have clarified it in protocol step 4.2.1.

- How did the authors visualise the HUVEC? They stated that they "create snap shots of at least 50 representative field views, each containing approximately 10 morphologically intact

HUVEC". According to the reported microscope settings it is not clear how that was possible.

Response: This is also a very important control, which I immediately clarified in the respective protocol step. In addition to the fluorescence monitoring, we always monitor the HUVEC cell layer using the bright field modus of the microscope in order to control cell morphology and layer integrity. In this regard, the epifluorescence camera of the microscope was used for optical monitoring of several fields of view in short time. I included this important information in the protocol to step 3.3.8.

- Immunofluorescence staining was performed in a 100 mM Na_2CO_3 buffer containing 4% sucrose. Please provide the pH value of the buffer. As most researches would prepare such staining with PBS it would be helpful to explain this unusual buffer choice.

  Response: The pH of the buffer was set to 9.2. I have explained and clarified this choice in a note written to protocol step 6.2.3.: "In the described immune fluorescence setting, the antibody detection delivered optimal results if the antibodies were diluted in the above mentioned alkaline carbonate buffer. According to our experience, the used blocking substances and the recommended amount of antibodies are suitable for many applications. But each experimental set up might require individual optimization of antibody combination, antibody concentration, incubation time, and constitution of the blocking buffer. As alternative, a phosphate buffered system with neutral pH range might be as suitable as incubation buffer or even better. In case of weak fluorescence signals the concentration of secondary antibody should be increased. If too much unspecific fluorescence background noise is detected the amount of blocking substances should be increased."

- **Minor Concerns:**

  I encourage the authors to add detailed information of the used materials and reagents. Provided information such as in line 266, "…(green/yellow) …" are difficult to understand for researches that are not familiar with the ibidi pump system.

  Response: I am thankful for this comment. The perfusion tubing sets from this system differ in length and diameter and are differentially colored. This term was deleted and clarified to detail in the material list.

- The authors wrote several times "access oxygen". Is that correct?

Response: this term is irritating and was deleted.

- The authors should exchange dyne/cm² to dyn/cm²
  Response: the term has been exchanged.

- Line 329, resolution of the camera?
  Response: The images were taken at 1392 x 1040 resolution at 1.4 Megapixel using a DFC 365FX Camera at the microscope. This information was included in list of materials in information to the CLSM.

- Please replace the word "probe" by "sample" (section 6)
  Response: The word has been exchanged.

- Please correct line 394.
  Response: the line was corrected.

- The quality of the provided images should be improved. Bacteria are partially difficult to discriminate from the background and figure labels are sometimes too small and thus difficult to read (e.g. figure 2b).
  Response: Images and labels of Figure 2 were enlarged and improved.

Reviewer #2:

Manuscript Summary: The experiments is a very nice visualization of a pathologic interaction between bacteria and the surface of endothelial cells, mediated in blood flow via mechanosensitive proteins.

The use of a commercial system made the experimental conditions very reproducible.
I am not an expert in biology, so I cannot give any opinion about works/do not works from the biological and molecular point of view.

I have some experience in the setting up of microfluidic experiments for cell adhesion assays or shear stress solicitations of cells in microfluidic conditions.

The experiments is very interesting and the visualization of the phenomena seems to be clear and evident.

The protocol is well explained, logical steps are well defined, cause- effect relation of each step is well defined and described.

Major Concerns:

The description of the set up is, on the other hand, very polarized on the use of the ibidi system so quantities, time intervals, flow rates, size of the chambers/ channel / slides are not very well defined because related to the ibidi systems features.
As I underlined in the following comments, I suggest completing the description of the system at the aim to let the reader be able to reproduce the experiment in a customized set up, if he/she may desire.

The ibidi system gives solid and reproducible experimental conditions but, from the microfluidic point of view, set up and conditions are quite easy to be reproduced in a normal customized, self-made setup, as long as the sizes, volumes, coatings, actions and 'numbers' are explicit.

- 265 - 3.2.1 what kind of ibidi microslide? There are different products on the website.
  Response: We have clarified the material description to detail including the special order number in the material list. We have used a "Luer$^{0.4}$ ibi treat I µ-slide" for the presented approach.

- 273 - explicit the degassing procedure (it is described on the ibidi datasheet but it takes time so better if you put it explicitly in the protocol)
  Response: We have described the degassing procedure more to detail in a note to protocol step 3.2.1 and explained the reason for it using the following sentences:
  "Note: This procedure is recommended by the manufacturer to degas the plastic equipment and to prewarm the medium, the perfusion tubes and the reservoirs. If materials or liquids have been stored at RT or in the refrigerator, gases dissolved in the plastic and liquids will be released when heated up in the incubator during the experiment. Gas bubbles will then appear. Degassing all plastic components before the experiment will eliminate this effect. Each time you take the system out of the incubator, the process of gas absorption begins again. Therefore, work quickly at RT and never leave the fluidic unit outside the incubator for longer time periods."

- 277 - it is not clear to me what is a styropor plate

Response: We have clarified this step by adding a note and changing the sentence in chapter 3.2.3. “Place the gelatin-coated channel slide on a thin polystyrene or styrofoam plate to prevent decrease of the slide temperature….”

“Note: Placing the channel slide on the cold metal surface of the clean bench could decrease the temperature of the slide bottom thereby generating cold stress to the endothelial cells….”

- 280 - better description of ' angular '

  Response: We have exchanged the term by explaining more to detail.

  The new sentence of the note to chapter 3.2.3 is:

  “Note: During cell pipetting hold the slide a bit upwards to let air bubbles ascent and be removed from inside the slide.”

- 289 - better to put a description of this set otherwise the experiment would not be reproducible without the ibidi experimental set. ('Connect the degassed green/yellow'?)

  Response: We have clarified the material descriptions of each component of the pump system to detail and also included the description and the special order number in the material list.

- 291 - it is not clear to me what is the meaning of 'to get rid of access oxygen'

  Response: this term is irritating and was deleted and the required process was clarified.

- 299- 'start the flow at 5 dyne/cm2 ' is not the correct description of a flow. Please put the description of the flow rate in microL/min or ml /min, flow that generates the shear stress

  Response: We have included the equations for calculation of shear stress and the correlation between flow rate and shear stress (according to Cornish 1928) more to detail in the introduction part. In our setting, the flow rate at 10 dyn/cm^2 corresponds to 10.86 mL/min. I have added this information in 3.3.2 and 3.3.4. In addition, we have included screen shots of each critical step of setting the pressure pump control software as supplemental figures 1-4.

- 304 - the flow speed - the velocity of the flow - again it is better to explicit the flow rate in in microL/min or ml /min

  Response: this system is using shear rate values, but using the equations described in introduction, the flow rates can be calculated and adapted to other pump systems. We have included this information to protocol steps 3.3.2 and 3.3.4 as mentioned above.

- 373 - 387-394 : when you say ' rinse' do you mean by means of a pipette?
  Response: the term “rinse” was deleted and the process was clarified in chapter 3.3.1 by adapting the sentence:
  “…flush the whole system with the liquid medium by starting the continuous medium flow with the pressure pump using 40 mbar pressure.”
  In chapters 6.2.2 - 6.2.6 the term “rinse” was exchanged with: “Wash the PFA-incubated channel slide three times using a 1 mL Luer syringe for injecting 200 µL 100 mM Na_2CO_3…”

- 436 - it is not clear to how long the flow in the microslide should stay on. How long is the experiment, how much buffer and solutions should be prepared to set up the experiment and observe the bacteria doing what you described.
  Response: Of course, this information is really necessary. We have added the information about time of flow cultivation (48 h), time of infection experiment and microscopic monitoring (2 h) and the required amount of buffers and solutions to the respective text passages of the protocol steps (2.2, 3.1, 4.1, 5.12, 6.2 and 6.2.1). The added information is marked in green. We also have included all required components in the list of materials.

- 472 - it is clear what the system does but not how the system is composed, dimensions, parts, functions.
  Response: In order to clarify the composition and purpose of this pump system, I have included a paragraph in the result section in which I describe to detail the single components of the system and the function of each of the components according to the work flow depicted in Figure 1.

- 478 – here, I may explicit what condition you mean (range of velocitiy, range of shear stress…)
  Response: In 2008, Reneman and Hoeks published *in vivo* measurements of vascular wall shear stress. I have included this values in the discussion and have set these values in correlation to the selected fluidic setting. (Please refer to the text in the discussion section marked in green beginning in line 700).

- 559 - amount of antibody ? it is mentioned the importance of the use of the right quantity to avoid visualization problems but I cannot find the guidelines to determine how to determine this quantity.

  Response: The use of an optimized antibody staining is indeed a critical step for the quality of immunofluorescence staining in general. In most cases, the manufacturer recommend a working concentration and a suitable blocking substance, which works fine for many applications. But sometimes, pretesting might be necessary using different amount of antibody in combination with different blocking substances, and incubated for different time periods to find the optimal staining procedure. Sometimes, antibodies from different sources or clone derivatives had to be compared before the optimal one is found, which is specific and sensitive enough. I have added some advice to the use of antibodies and constitution of blocking buffer in the protocol step 6.2.3 and clarified this topic in the troubleshooting section of the discussion. But this issue is not within the focus of this microfluidic-based infection protocol and a detailed description of this process would require a whole new protocol outline and would exceed the limitations of this manuscript.

- Fig 1

  Insert n°3 - it is not clear the composition of the system, the use of the tube connected to the drying bottle.

  Insert n°4 it is not clear how the insertion of bacterial solution or histamine solution can be perturb the SS in the device (or maybe not but it is not clear).

  Response: Thank you for this important comment. I have clarified the composition of the system including all insets of Figure 1 and the work flow in general in the results part. I also described the purpose and mode of connection of a glass bottle filled with silica particles for drying the air of the pressure pump in chapter 3.3.1.

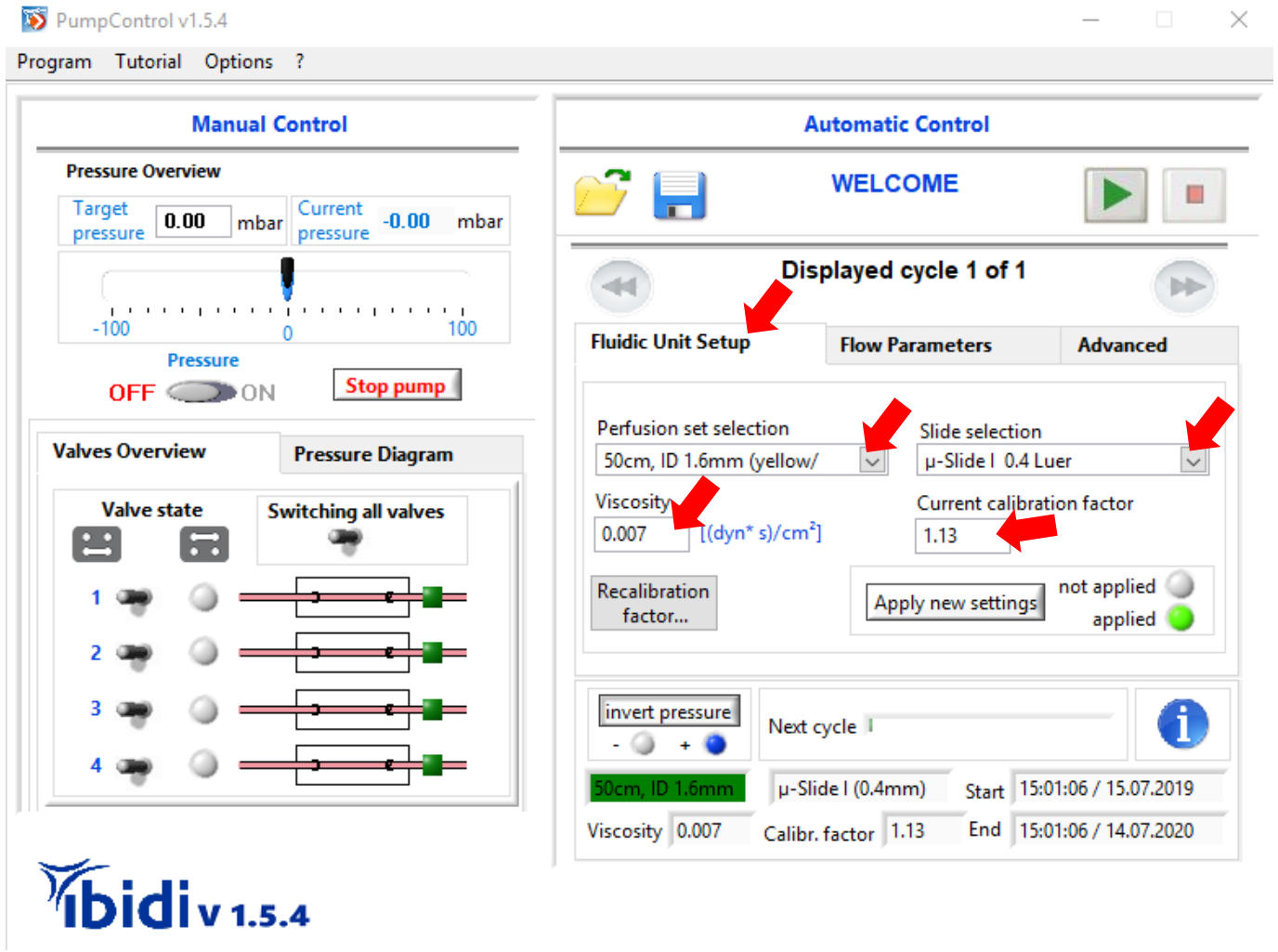
- Table of materials is empty, should definitely not to be so.

  Response: I am very sorry, this was my mistake. I have added an Excel file with the filled table of materials to the resubmission.

Supplemental Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);SupplFig1.pdf

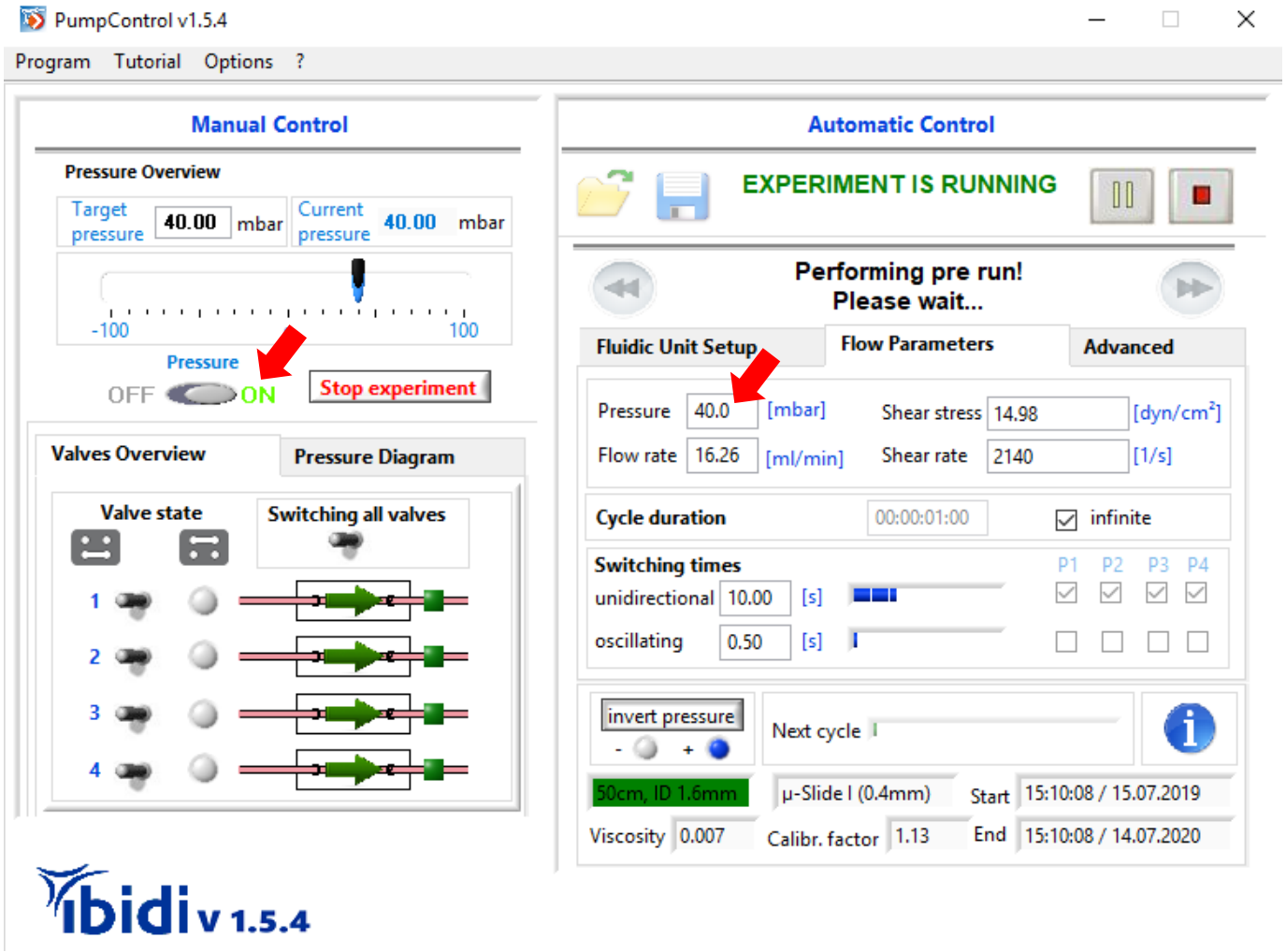
Suppl. Fig. 1:
Screenshot to 3.2.1 „Fluidic Unit Setup“



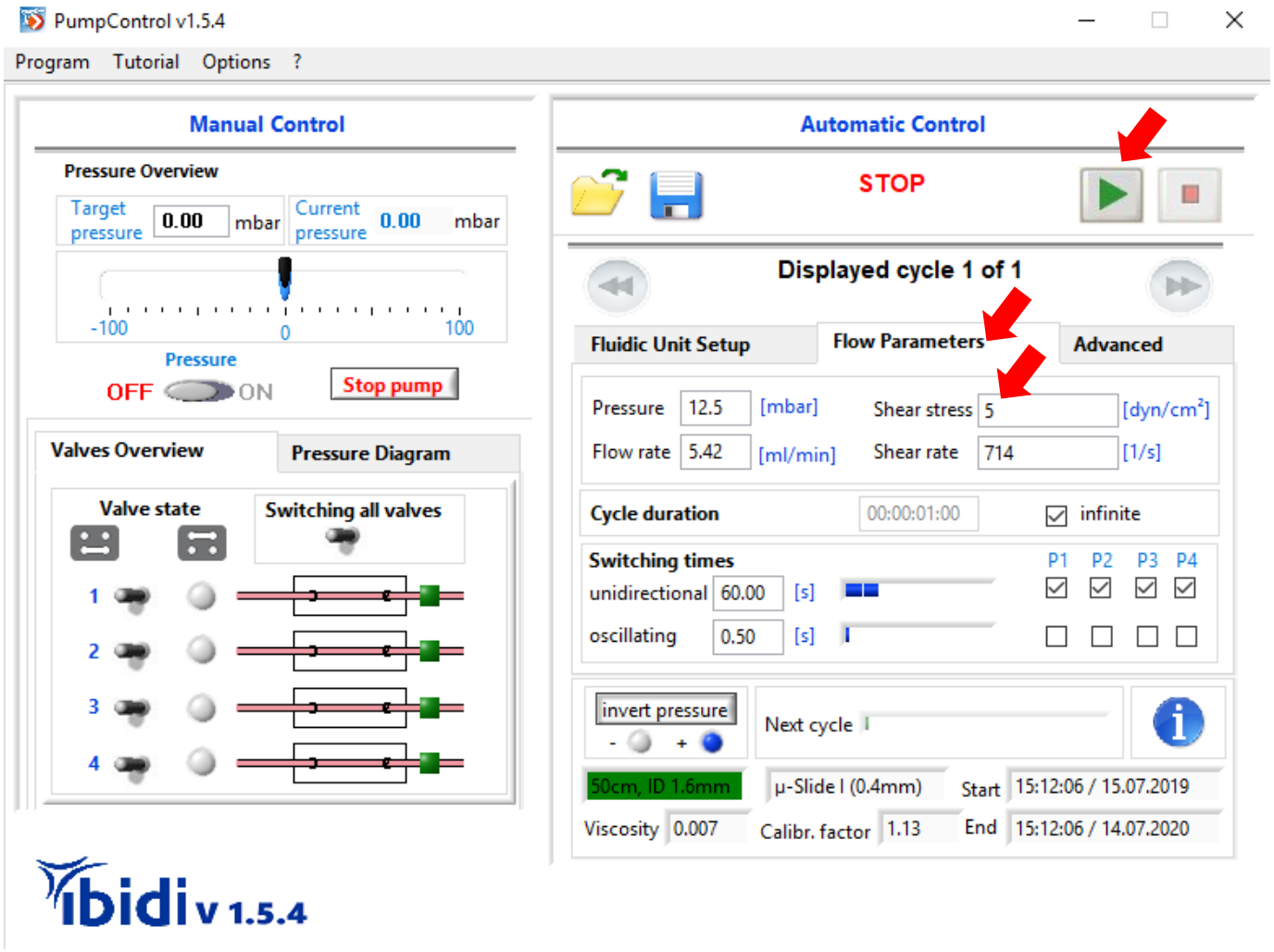
Supplemental Figure 2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);SupplFig2.pdf

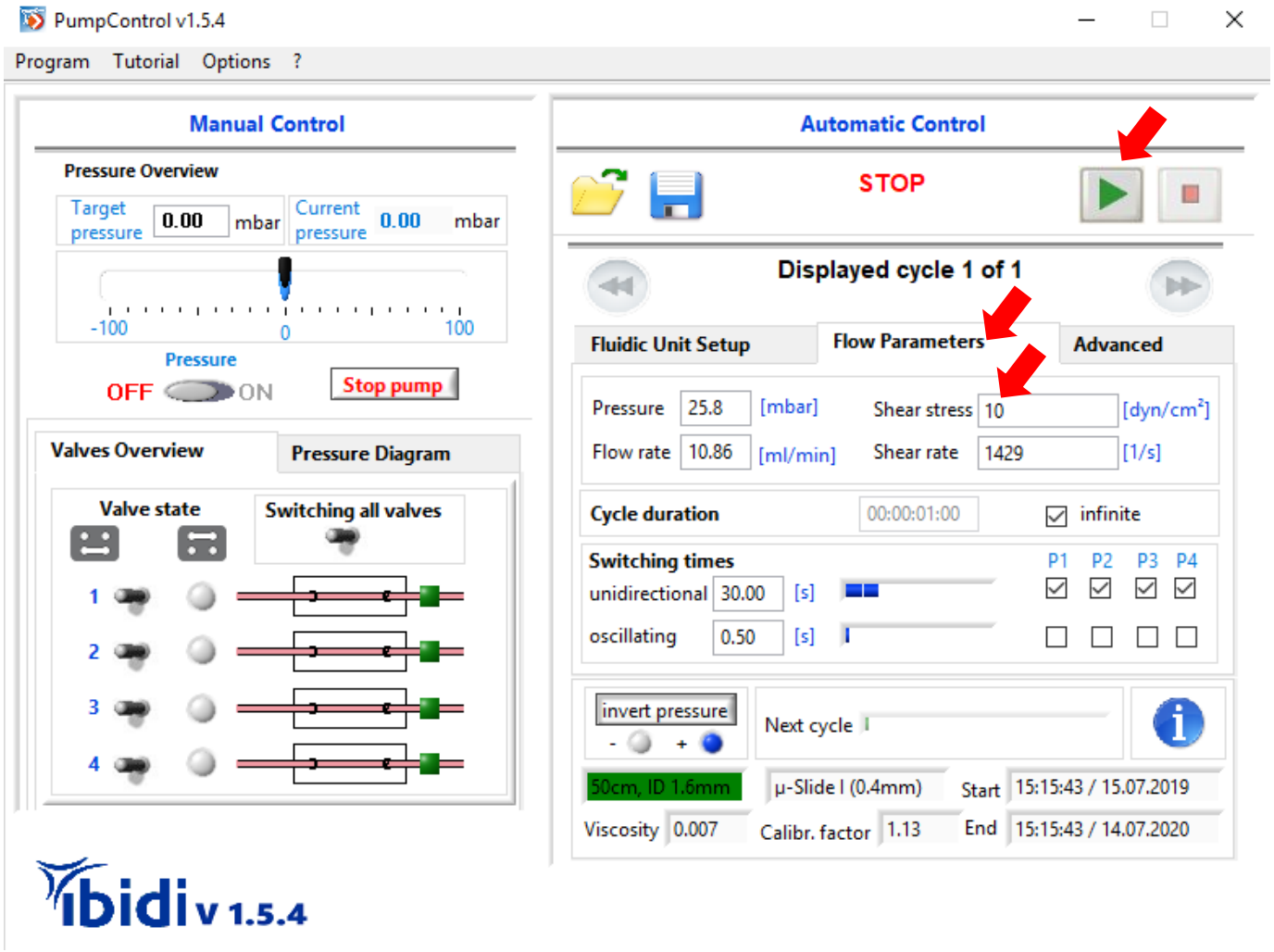
Suppl. Fig. 2:
Screenshot to 3.2.1 „Flow Parameters“



Suppl. Fig. 3:
Screenshot to 3.2.2 „Flow Parameters“



Suppl. Fig. 4:
Screenshot to 3.2.4 „Flow parameters“



Dear Fr. Bergmann,

In your JoVE publication „Figure1FlowScheme“ (see attached) you use three images of ibidi:

- µ-Slide I Luer on a microscope
- System overview of the ibidi Pump System
- Use of Inline Luer Injection Port

With this letter, we officially permit the publication of these ibidi images in JoVE.

If you have further questions, please do not hesitate to come back to us.

Best regards,

Susanne Seifert

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