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In Vitro Microfluidic Disease Model to Study Whole Blood-Endothelial Interactions and Blood Clot Dynamics in Real-Time

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Cover letter

Dear *JoVE* editorial team,

With pleasure, we would like to submit a protocol entitled: 'An *in vitro* microfluidic disease model to study whole blood-endothelial interactions and blood clot dynamics in real-time.' to be considered for publication in JoVE.

In our protocol, we present an *in vitro* vascular disease model of thrombosis to investigate whole blood interactions with patient-derived endothelium. We emphasize on chronic thromboembolic pulmonary hypertension (CTEPH), a lung disease characterized by extensive thrombus formation in the pulmonary vasculature, wherefore no animal models are available. However, this system is adjustable to the scientific rationale for other disease as it allows the study of endothelial influences on thrombus dynamics. The method is especially suited to evaluate anti-coagulation therapy during the different phases of coagulation.

During hemostasis, endothelial function, blood flow and platelet activation in combined action with the coagulation system determine the formation of a thrombus. However, current studies are often performed on endothelial-platelet or even collagen-platelet interaction, that excludes the secondary hemostasis where activation of the coagulation cascade results in a thrombus. Furthermore, commonly used endothelial cells in such types of assays are commercially purchased and not suitable for disease modeling or patient specific assays. Our protocol will close the translational gap between bench-and-bedside by presenting a method that includes patient specific cells. Our system makes it possible to study thrombus formation on endothelial cells in interaction with whole blood, including single components as platelets, erythrocytes and leukocytes. Besides, we will integrate the use of micro engineered perfusion chambers. The novelty of this is that compared to the most commonly used flow chambers, it is possible to tune specific vascular sizes and geometries that are in the vascular tree, and how this will affect thrombus formation in healthy or diseased state. This modular platform integrates different levels of expertise from engineering, biology, biochemistry and medicine and can be applied to numerous types of studies from drug testing to vascular biology.

This can be used to study thrombus formation, and for the assessment of inflammatory responses in disease modeling. We can study live platelet adhesion and clot formation on endothelial cells and clot resolution over time, but also characterize cell behavior after clot formation via post immunofluorescent analysis for adhesion molecules and integrins. Besides, the patient-specific endothelial cells enable us to use it as a platform to test personal anti-platelet or anti-coagulation therapy for personalized medication.

We would like to thank you for and hope that you share our enthusiasm considering our protocol for publication.

With kind regards and on behalf of the corresponding authors,

Xue Manz

TITLE:

In Vitro Microfluidic Disease Model to Study Whole Blood-Endothelial Interactions and Blood Clot Dynamics in Real-Time

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KEYWORDS:

thrombosis, chronic thromboembolic pulmonary hypertension, endothelial cells, platelets, fibrin, coagulation, blood

SUMMARY:

We present an in vitro vascular disease model to investigate whole blood interactions with patient-derived endothelium. This system allows the study of thrombogenic properties of primary endothelial cells under various circumstances. The method is especially suited to evaluate in situ thrombogenicity and anticoagulation therapy during different phases of coagulation.

ABSTRACT:

The formation of blood clots involves complex interactions between endothelial cells, the underlying matrix, various blood cells, and proteins. The endothelium is the primary source of many of the major hemostatic molecules that control platelet aggregation, coagulation, and fibrinolysis. Although the mechanism of thrombosis has been investigated for decades, in vitro studies mainly focus on situations of vascular damage where the subendothelial matrix gets exposed, or on interactions between cells with single blood components. Our method allows

studying interactions between whole blood and vascular cells.

By utilizing primary human endothelial cells, this protocol provides the unique opportunity to study the influence of endothelial cells on thrombus dynamics and provides valuable insights into the pathophysiology of thrombotic disease when using patient-derived cells. The use of custom-made microfluidic flow channels allows application of disease-specific vascular geometries and model specific morphological vascular changes. The development of a thrombus is recorded in real-time and quantitatively characterized by platelet adhesion and fibrin deposition. The effect of endothelial function in altered thrombus dynamics is determined by postanalysis through immunofluorescence staining of specific molecules.

The representative results describe the experimental setup, data collection, and data analysis. Depending on the research question, parameters for every section can be adjusted including endothelial cell type, shear rates, channel geometry, drug therapy, and postanalysis procedures. The protocol is validated by quantifying thrombus formation on the pulmonary artery endothelium of patients with chronic thromboembolic disease.

INTRODUCTION:

The endothelium forms the inner cellular layer of blood vessels and separates blood from the surrounding tissue. It has been described as a dynamic organ that actively regulates its environment and responds to external stimuli[1]. Because of its direct contact with flowing blood, the endothelium is pivotal in the control of hemostasis and thrombosis and is the primary source of many of the major regulatory molecules that control platelet aggregation, coagulation, and fibrinolysis[2]. Healthy, nonactivated endothelial cells (EC) produce several molecules that counteract platelet activation and prevent coagulation and thrombus formation to maintain blood flow under unstimulated conditions, such as prostacyclin, thrombomodulin, or tissue factor pathway inhibitor (TFPI)[2,3]. This prevents the adhesion of platelets, platelet aggregation, and thrombus formation. Injury or activation of the vessel wall results in a procoagulant endothelial phenotype that initiates localized platelet adhesion and clot formation[2,4]. Upon endothelial damage, activated platelets can adhere to von Willebrand Factor (VWF), a multimeric protein released from ECs, or to exposed binding sites of the underlying subendothelial matrix. Subsequently, molecular changes in platelets and the exposure to tissue factor (TF) initiate the activation of the coagulation system, which induces thrombus formation by fibrin polymerization[5,6]. Together, the resulting clot provides the basis for wound closure by re-endothelialization[7]. Perturbations of the coagulation system may result in bleeding disorders, such as von Willebrand disease, hemophilia, or thrombosis, often consequences of a dysregulated pro- and antithrombotic balance of the endothelial hemostatic pathway[2,3].

The process of hemostasis occurs in both arterial and venous circulation. However, the mechanisms underlying arterial and venous thrombosis are fundamentally different. While arterial thrombosis, as seen in ischemic heart disease, is mostly driven by the rupture of an atherosclerotic plaque under conditions of high shear stress, venous thrombosis mostly develops in the absence of endothelial injury in a condition of stasis[8-10]. A deep vein thrombus may embolize and travel towards the pulmonary arteries, where it causes a pulmonary embolism. This

can result in chronic vascular obstructions leading to significant impaired functional capacities, including the development of chronic thromboembolic pulmonary hypertension (CTEPH)[11-14]. CTEPH is characterized by elevated pulmonary pressure due to obstructions of the pulmonary arteries by thromboembolic material following at least 3 months of anticoagulation therapy[15]. It is postulated that the pulmonary endothelium provides a prothrombotic environment that fosters in situ thrombosis and chronic obstructions of the pulmonary arteries, causing the increase in blood pressure that ultimately can result in heart failure, if untreated[16,17].

Over the past years, various studies have led to the development of assays to examine thrombus formation by measuring platelet function and coagulation[18]. However, most of them either study the interaction of whole blood with single extracellular matrix components like collagens or fibrins, or endothelial function in interaction with single blood components, such as endothelial-platelet or endothelial-leukocyte interaction[19-22]. These assays are most commonly performed with human umbilical cord endothelial cells (HUVEC), as these cells are easily obtained. However, hemostatic genes are differentially expressed across the vascular tree, vessel types, and organ systems[23,24], which makes the use of HUVECs to represent endothelial cells involved in arterial thrombosis or pulmonary embolisms problematic[23].

In addition to EC plasticity, disease-specific hemodynamic alterations and changes in vascular morphology can promote thrombus formation, even when the endothelium is undamaged[25]. Higher shear rates, due to local vasoconstriction or changes in vessel geometry, for example, may result in acute thrombus formation, causing a stenosis that accelerates the cessation of blood flow[26]. The use of custom-made microengineered flow channels allows to specifically design vascular geometries that are representative of the pathology. In this way, it is possible to study the effect of local biomechanical forces on healthy or diseased EC[27].

There are several anticoagulation therapies available for targeting different phases and molecules in the coagulation cascade, which all comprise particular risks and benefits that can be specific to certain disorders. The approach of disease modeling described in this paper is especially suited to test the effects of various anticoagulation and antiplatelet therapies on thrombus dynamics.

The aim is to present a model of thrombosis that includes primary ECs, yielding a versatile model suitable for the analysis of various forms of thrombosis depending on the type of primary ECs that are used. As an illustration, we used pulmonary artery endothelial cells from CTEPH patients in interaction with whole human blood containing all components involved in thrombus formation (platelets, leukocytes, erythrocytes, clotting proteins, and cofactors). This approach can be applied in commercial parallel flow channels or in custom-made microfluidic flow channels with a specific vascular design. As such, the model can eventually be used in the study of thrombus formation and resolution, for the assessment of inflammatory responses in disease modeling, for antiplatelet or anticoagulation therapy, and ultimately for personalized medicine.

This study describes the isolation of primary human pulmonary artery endothelial cells. For the isolation of other primary human endothelial cell types, we refer to previously published

methods, including pulmonary microvascular endothelial cells[25], human umbilical vein endothelial cells[28], and blood circulating endothelial colony forming cells[29].

PROTOCOL:

This study was approved by the institutional Medical Ethical Review Board of the VU Medical Center Amsterdam, The Netherlands (METC VUmc, NL69167.029.19). Primary cell isolation and blood collection of human subjects was performed after informed consent was obtained in accordance with the Declaration of Helsinki.

1. Isolation and culture of primary human pulmonary arterial endothelial cells (PAEC)

1.1. Warm complete endothelial cell medium (cECM) supplemented with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), 1% endothelial cell growth supplements (ECGS), and 1% nonessential amino acids (NEAA), in a water bath at 37 ˚C. Sterilize surgical scissors, forceps, and a scalpel with either a 120 ˚C heat sterilizer or 70% ethanol. Perform the isolation of PAEC in a laminar flow cabinet under sterile conditions.

1.2. Coat a high affinity cell binding 60 mm cell culture dish (see **Table of Materials**) with 2 mL of 5 µg/mL fibronectin and incubate at 37 ˚C for at least 15 min.

1.3. Obtain pulmonary artery (PA) tissue from a surgery (e.g., lobectomy or pulmonary endarterectomy), store it in 4 ˚C cold cord buffer (4 mM potassium chloride, 140 mM sodium chloride, 10 mM HEPES, 11 mM D-glucose, and 1% P/S, pH = 7.3) and keep it on ice until isolation.

1.4. Isolate endothelial cells from PA within 2 h after tissue removal. Take the PA with forceps and put it in a 10 cm Petri dish to wash with PBS. If the PA is still a ring, cut the pulmonary artery open with scissors. Be very careful to not touch the innermost layer of the vessel with the tools, as the endothelium is easily damaged and/or removed.

1.5. Remove the fibronectin from the cell culture dish and add 4 mL of cECM medium.

1.6. Take the PA tissue with forceps and place it in the medium. Meanwhile, carefully scrape the inner layer of the vessel into the medium with a scalpel. Often, lipid accumulations can be seen in the vessel wall. Try to omit these, as they will impact cell outgrowth.

1.7. Keep the cells in culture until small colonies of endothelial cells start to appear.

1.8. Replace the medium every other day. If fibroblasts contaminate the culture, purify it with magnetic affinity cell separation for CD144 with a kit, in accordance to the manufacturer's instructions (see **Table of Materials**). Use the two-column method for the initial purification and the one-column method for every consecutive purification. Generally, a culture is defined pure when flow cytometry detects ≤10% contaminating cells.

1.9. Split cells in ratios of 1:3 to 1:4 until sufficient PAECs are grown for experimental use. If PAECs are ready to use, continue with the next step.

1.10. After purification, primary endothelial cells need to be characterized for the presence of EC specific markers (e.g., VE-cadherin, CD31, Tie1) and for the absence of smooth muscle cells (α-SMA), fibroblast (vimentin), and epithelial (pancytokeratin) markers.

2. Preparation of flow chambers and PAEC monolayers

NOTE: Depending on the hypothesis, use either the commercially available flow chambers (see **Table of Materials** and **Figure 1C Option A**, step 2.1 of the protocol) or a custom-made microfluidic flow chamber (**Figure 1C Option B,** step 2.2 of the protocol).

2.1. Cell seeding of endothelial monolayers in commercially available microslides

NOTE: Commercially available flow chambers are easy to use and provide a laminar flow pattern through the channel that can be used at high shear rates. These microslides are designed to allow multichannel parallel runs and provide an accurate and reproducible flow profile. Because they are optimized for inverted microscopy, it is possible to capture high quality fluorescent images. Furthermore, various dimensions of flow chambers are available in different channel sizes or geometries. The 6 well flow channels are preferred for this assay because these microslides have small dimensions and allow multiparallel runs.

2.1.1. To coat one channel of a 6 well flow slide (3.5 mm width x 0.4 mm height x 17 mm length) use 30 µL of 0.1% gelatin per channel and incubate at 37 ˚C for at least 15 min.

2.1.2. To seed one channel, trypsinize 10 cm^2 of confluent PAECs and spin down at 300 x *g* for 7 min at room temperature (RT).

2.1.3. Suspend PAECs in 600 µL of cECM and pipette 100 µL (1.5 cm^2) of cell suspension in one channel. This covers the microslide through capillary action. If it is going too slow, air bubbles will form. Avoid the formation of air bubbles by using a little bit more force when pipetting.

NOTE: Cell density influences endothelial phenotype. A total of 1.5 cm^2 of confluent cells per channel is overconfluent, because the channel has a surface area of 0.6 cm^2. Before starting the experiment, culture these cells for another 6 days within the channels, when they reach maximum confluency.

2.1.4. Add an additional 50 µL of cECM to the channel to provide sufficient medium for overnight incubation at 37 ˚C, 5% CO_2. Change the medium the next day to wash away unbound cells.

2.1.5. Keep the cells in culture for 6 days at 37 ˚C, 5% CO_2 to allow the PAEC to form a firm, confluent monolayer. Change the medium every other day with 150 µL of cECM.

2.1.6. At day 7, treat the PAEC with 100 µL of 1 µM histamine in ECM and 1% FBS without any other additives for 30 min prior to the assay. The stimulus can be chosen ad libitum. For example, TNF-α has been commonly used as an inflammatory activator.

2.2. Preparation of custom-made microfluidic flow chambers

NOTE: Custom-made flow chambers are adapted to the user's needs because the dimensions and geometries can be easily changed to preference. For example, to mimic a more physiologically relevant vessel, a stenosis can be introduced. This approach allows the use of very small blood volumes as seen in microvascular disease.

2.2.1. Soft lithography of polydimethylsiloxane (PDMS) using patterned wafers

2.2.1.1. Combine PDMS curing agent and prepolymer on a microbalance at a 1:10 ratio and thoroughly mix with a general purpose lab mixer.

2.2.1.2. To remove the resulting air bubbles, degas the PDMS in a desiccator for approximately 1 h.

2.2.1.3. Line a glass Petri dish with aluminum foil and add a few droplets of water before placing the wafer in the lined Petri dish. The wafer serves as the mold for the custom-made channel.

NOTE: The wafers or molds are provided by the clean room facilities. Negative photoresist is patterned onto wafers to create protruding channel-like features with the following typical dimensions: 300 µm width x 270 µm height x 14 mm length.

2.2.1.4. Pour the degassed PDMS onto the wafer placed in a glass Petri dish.

2.2.1.5. Degas the PDMS poured onto the wafer in a desiccator for an additional 15 min to remove any bubbles that might have formed during casting.

2.2.1.6. Remove the Petri dish containing the mold and PDMS from the desiccator and place it in a 60 ˚C oven for a minimum of 4 h to cross-link the PDMS.

2.2.2. Preparation of cross-linked PDMS for bonding to glass slides

2.2.2.1. Remove mold and cross-linked PDMS from the oven and move to a cross-flow hood for dust-free handling of the PDMS.

2.2.2.2. Remove any PDMS from the bottom of the mold using a scalpel and remove the top slab of the cross-linked PDMS from the mold.

2.2.2.3. Line the exposed patterned PDMS slab with adhesive tape to prevent any dust particles to come into contact with the PDMS channels.

2.2.2.4. Cut the PDMS chips to size and punch 1 mm diameter inlets and outlets using a biopsy punch.

2.2.3. Sterilization and bonding of PDMS microfluidic channels and glass slides

2.2.3.1. Clean glass microscopy slides by thoroughly rinsing with ethanol followed by an isopropyl alcohol rinse. After each rinse, thoroughly dry the slides with a nitrogen gun.

NOTE: Alternatively, cover slips can be used if the analysis is conducted using a confocal microscope.

2.2.3.2. Open the plasma chamber and load the cleaned microscopy slides and microfluidic chips without the protective tape.

2.2.3.3. Close the chamber, purge with nitrogen for 1 min, and fill the chamber with filtered air until a pressure of 500 mTorr is reached.

2.2.3.4. Expose the glass slides and PDMS chips to the plasma for 40 s using a power of 50 W and a frequency of 5 kHz.

2.2.3.5. After exposure to the plasma, immediately bond the glass slides and microfluidic chips. Store the devices in sterile Petri dishes.

2.3. Cell seeding of endothelial monolayers in microfluidic channels

2.3.1. Coat the custom-made microchannel by pipetting 10 µL of 0.1 mg/mL collagen Type I or 0.1% gelatin per channel and incubate at 37 ˚C for 30 min.

2.3.2. To seed four microchannels, trypsinize 25 cm^2 of confluent PAECs and spin down at 300 x *g* for 7 min at RT.

NOTE: Only a small percentage of cells will adhere to the surface. Therefore, it is necessary to use an excess of cells to achieve a confluent monolayer.

2.3.3. Suspend PAECs in 20 µL of cECM and use 5 µL of cell suspension for each channel. Because of the small channel dimensions, the capillary forces to fill the microslide are strong enough to prevent air bubble formation.

2.3.4. Change medium after 3–4 h to wash unbound cells.

2.3.5. Keep the cells in culture for 6 days to allow the PAEC to form a firm, confluent monolayer. Because of the small volume, change the medium every day with 150 µL of cECM. Leave the pipette tip filled with medium in the inlet and put an empty tip in the outlet. This will serve as a

reservoir providing sufficient nutrients and growth factors to the cells and prevent the slide from drying out.

2.3.6. On day 7, stain the nuclei in the live cells with Hoechst (1:5,000 in cECM) and incubate for a maximum of 10 min at 37 ˚C and 5% CO_2.

2.3.7. Carefully wash 3x with cECM and treat with 1 µM histamine in ECM + 1% FBS for 30 min prior to the assay. The stimulus can be chosen ad libitum. For example, TNF-α has been commonly used as an inflammatory activator.

2.3.8. Use 1.27 mm diameter 90˚ angled stainless steel connectors to connect the outlet of the PDMS chips to tubing.

3. Preparation of washed human whole blood

3.1. Draw venous blood from subjects in 0.109 M sodium citrate anticoagulant. This can be from healthy or diseased subjects that do not receive anticoagulation treatment. Gently invert the tubes to mix.

3.2. Transfer the blood to a 50 mL tube and add Calcein AM (1:10,000) to fluorescently label blood cells and Alexa488-fibrinogen (15 µg/mL) to conjugate autologous fibrinogen. Let the blood incubate at 37 ˚C for 15 min to allow complete absorption.

3.3. Dilute the blood 1:1 with recalcification buffer (154 mM sodium chloride, 10.8 mM trisodium citrate, 2.5 mM calcium chloride, and 2 mM magnesium chloride) immediately before the start of experiment.

NOTE: Hemodilution with a maximum of 50% did not influence coagulation reaction time[30]. Furthermore, human blood can contain viruses and other agents. Working with blood samples, therefore, carries a risk of infection. It is highly recommended to use appropriate safety measures and to handle the material with care.

4. Assembling the flow system

4.1. Before connecting the tubing, rinse the flow tubes with a 20 mL syringe filled with wash buffer (36 mM citric acid, 103 mM sodium chloride, 5 mM potassium chloride, 5 mM EDTA, and 0.35% wt/vol bovine serum albumin [BSA], pH = 6.5) to prevent clotting of the blood in the tubes.

4.2. Use a new syringe to fill the flow tubes with HEPES buffer (132 mM sodium chloride, 20 mM HEPES, 6 mM potassium chloride, 1 mM magnesium chloride, 1% BSA, and 5.5 mM D-glucose, pH = 7.4) and carefully connect it with an elbow-shaped Luer connector to the microslide with EC in medium. While attaching the connectors to the microchannels, try to prevent the formation of any air bubbles in the slide. Bubbles will damage the endothelium and influence the results of your experiment.

4.3. Set up the flow system as shown in **Figure 1E**. Take up 2 mL of wash buffer in a 20 mL syringe to prevent clotting when the blood enters the syringe. Insert the syringe in the syringe pump and connect the outlet tube with a female Luer connector to this syringe. Put the inlet tube in a blood container.

4.4. Switch on the syringe pump and calculate the flow rate. Flow profiles follow a parabolic pattern in height. Assuming that the blood acts as a Newtonian fluid, use the following formula to calculate flow rate.

$$\tau = \eta \frac{6Q}{h^2 w} \qquad \text{(Equation 1)}$$

where

τ = shear stress $\left[\frac{dyn}{cm^2}\right]$

η = dynamical viscosity $\left[\frac{dyn \times s}{cm^2}\right]$

Q = volumetric flow rate $\left[\frac{mm^3}{s}\right]$

h = channel height $[mm]$

w = channel width$[mm]$

NOTE: For pulmonary arterial flow with blood in the commercial 6 channel microslides with rectangular dimensions with 0.4 mm height and 3.5 mm width, a volumetric flow rate of 25 mL/h was used for 5 min. Due to the differences in the dimensions of the custom-made flow channels, these dynamics will also change. Adjust the variables to make sure the shear stress is equal.

4.5 Define the diameter of the 20 mL syringe to 19.05 mm and set the program to **Withdraw**.

5. Setting up the microscope for image acquisition

5.1. Use a 20x objective and place the microslide onto the stage of the inverted phase contrast fluorescent microscope. Start the microscope software to move the stage in the Z-direction to focus on the cell monolayer.

5.2. Select a region of interest (ROI) in the beginning, middle, and end of every flow channel. The beginning and end should be at least 3 mm away from the inlet and outlet of the channel. Set the blue, green, and red fluorescent filters with a laser power and light intensity that shows minimal background.

6. Perfusion of washed whole blood over PAECs

6.1. If everything is connected as in **Figure 1E** and the microscope is ready for recording, push **Start** to record a video. Push **Start** on the syringe pump to perfuse the blood over the

endothelium.

6.2. As soon as the blood starts flowing over the endothelium, acquire images with the preselected active channels and ROI positions every 15 s for 5 min.

6.3. After 5 min of perfusion, finish recording and stop the syringe pump. Disassemble the flow chamber and very carefully remove the tubing. Often, an air bubble will form if this is done with too much force. Try to prevent this, because this will flush away the endothelium.

7. Fixation and post-analysis of the endothelial cells

NOTE: To exclude false positive platelet adhesion by endothelial damage due to the perfusion experiment, it is necessary to characterize the endothelial cells for their gap formation and monolayer. This can be done by regular immunofluorescence staining for VE-cadherin.

7.1. After perfusion and disassembling of the tubing, wash the microfluidic channel with HEPES. Pipette HEPES into the channel so that it will push the blood to the outlet. Remove the excess with another pipette.

7.1.1. Optionally, wash the channel with PBS^{++} (with 0.5 mM magnesium chloride and 0.9 mM calcium chloride) to prevent precipitation of protein supernatant. This reduces background. However, an extra washing step can change cellular behavior and morphology that could influence post-analysis imaging.

7.2. Remove the HEPES and fix the endothelium with adhered platelets and deposited fibrin by pipetting 37 ˚C warmed 4% paraformaldehyde (PFA) into the channel and incubate for 15 min at RT.

NOTE: Be extra careful when fixing the custom-made microfluidics as these channels are even smaller, which causes higher shear forces in the channel during every handling step.

7.3. Remove the PFA from the channel and wash 3x with PBS. The microslides are now ready for a standard staining protocol to characterize endothelial cell markers such as VE-cadherin, CD31, P-selectin or integrins, Cd42b for adherent platelets, or Cd45 for leukocytes.

7.4. After staining, take at least five images with a regular confocal microscope to characterize colocalization.

8. Image analysis of platelet adhesion or fibrin deposition over time

8.1. Open a flow assay image containing either fluorescently labeled platelets or fluorescent fibrin in ImageJ. Drag image of choice to ImageJ.

8.2. The image is taken in RGB color. For analysis, an 8-bit image is preferred, so transform the

image into 8-bit: **Image** | **Type** | **8-bit**.

8.3. To minimize the background, subtract with a sliding paraboloid, where a rolling ball locally calculates and subtracts background pixels from the original image: **Process** | **Subtract Background** | **Rolling Ball Radius** = **50 pixels** | **Sliding Paraboloid**.

NOTE: The image size is defined in pixels. However, to measure area, it is necessary to set the scale. When the images are taken with a 20x objective with a numerical aperture of 0.45, the microscope has a scale of 2 pixels per µm. Most of the time the necessary information to set the scale is stored in the metadata of the image and can automatically be used by ImageJ: **Analyze** | **Set Scale**.

8.4. Set a threshold to define adhered platelets or deposited fibrin. Use the Triangle method and the threshold will adjust automatically: **Image** | **Adjust** | **Threshold**.

8.5. Analyze the area covered by the platelets or fibrin with the **Analyze Particles** command. Set size at 2-Infinity, as the minimal size of a platelet is 2 µm: **Analyze** | **Analyze Particles**.

8.6. The results will provide the total area in μm^2, with average size of the aggregates in μm^2 and the percentage of covered area.

REPRESENTATIVE RESULTS:

The representative results can be divided into three parts, each representing the respective steps of experimental setup, data collection, and data analysis. Depending on the research question, parameters for each step can be changed. The presented data are applied to study the influence of the pulmonary artery endothelium on thrombus formation.

Experimental Setup

It is well-established that endothelial cells are highly heterogeneous in structure and function, depending on location and time, during health and disease[23]. Various sources of endothelial cells can be used to study endothelial-blood interaction, which in this case were commercially available HUVECs and patient-derived ECs (**Figure 1A**). HUVECs have been the most commonly used in laboratory models, while PAECs are patient-derived isolated cells from the pulmonary circulation. Furthermore, there are also well-established protocols available to isolate microvascular endothelial cells (MVEC) from the pulmonary circulation or blood circulating endothelial colony forming cells (ECFC) that can be used as a source of endothelial cells (**Figure 1A**)[25,28,29]. This protocol introduced a new technique for the isolation of PAECs, therefore representative results were illustrated with PAECs.

After isolation, endothelial cells were characterized by VE-cadherin, CD31, and Tie2 staining to confirm an endothelial phenotype. Characterization for the presence of αSMA and pancytokeratin indicated the absence of a fibroblast or epithelial-like phenotype (**Figure 1B**). After obtaining a highly pure population of ECs, passage 3–5 cells were used to seed either a commercial microslide or custom-made microfluidic flow channels (**Figure 1C**). While the

commercially available microslides are primarily parallel flow chambers, or Y-shaped channels with specifically defined parameters in height or bifurcation angle, the use of microfluidic slides makes it possible to adapt the experimental parameters closer to in vivo vessel geometry and blood flow dynamics[31]. However, custom-made microfluidic sizes are smaller, and small areas tend to suppress cell spread and induce more cell death[32,33]. Using a surplus of cells compensates for the fact that only a small percentage of cells will adhere. This effect was observed when a stenosis was introduced, where endothelial cells showed a more elongated phenotype compared to a parallel microfluidic channel (**Figure D**). Commercial flow chambers have a higher surface area that cells can bind on. This requires fewer cell numbers, which allowed the cells to form a stable monolayer over a week without changing cell phenotype (**Figure 1A**).

To study endothelial cell-blood interaction, whole blood was perfused over an endothelial monolayer. Citrated blood was collected on the day of the experiment and immediately before perfusion recalcified. Cells were stimulated with histamine to induce VWF release and platelet adhesion 30 min prior to perfusion (**Figure 1E**)[34,35]. Because of the small dimensions, the custom-made microfluidic channels allowed use of smaller blood volumes to reach similar blood shear rates over endothelial cells, compared to commercial flow chambers.

Data collection

To investigate thrombus formation on pulmonary artery endothelial cells, Calcein AM-Red fluorescently labeled blood cells and Alexa488-conjugated fibrin were perfused for 5 min at 2.5 dyne/cm^2 (**Figure 2A**–**C**). Adherent blood cells and deposited fibrin emitted a higher fluorescent signal than flowing blood, which was quantified. Images were acquired every 30 s and quantified with ImageJ. It was important to subtract the background to eliminate the autofluorescence of nonadhered platelets. The triangle algorithm for thresholding has been used to define minimal background. It allowed for measurement of small platelet aggregates (**Figure 2D**).

Under nonstimulated conditions, there was no binding of platelets and fibrin to the endothelium. To promote VWF release and platelet binding, PAECs were stimulated with histamine, which resulted in an immediate increase of platelet adhesion reaching a plateau after 2.5 min. At this time, platelets started to secrete autocrine factors that induced platelet aggregation and fibrinogen cleavage into fibrin. Fibrin was deposited after 3 min and formed a stable aggregate with platelets after 4 min (**Figure 2E**).

To investigate whether this effect could be inhibited by a direct oral anticoagulant (DOAC), blood was treated with 10 nM dabigatran. Dabigatran was added to the blood dilution, where it inhibited Factor IIa in the coagulation pathway, and prevented the cleavage of fibrinogen to form fibrin fibers. When dabigatran-treated blood was perfused overstimulated PAECs, clot formation could be directly inhibited mainly by delaying fibrin deposition (**Figure 2F**). Furthermore, platelet adhesion was also decreased compared to perfusion of untreated blood (**Figure 2E**–**F**).

Data analysis

To study the influence of various endothelial sources on thrombus formation, the cellular changes upon 5 min of blood perfusion were analyzed. The endothelium was fixed and adherent

platelets were labeled with Cd42b before imaging under a confocal microscope. This provided a more detailed analysis for colocalization of platelets and fibrin that could indicate dysfunctional coagulation factors in the blood. The influence of endothelial cells in clot formation was determined by standard immunofluorescent staining. Endothelial cell-cell contacts were maintained, as confirmed by VE-cadherin staining, indicating that the blood clots formed on an endothelial monolayer rather than on the underlying matrix between endothelial gaps (**Figure 3**). Furthermore, the use of different cell sources resulted in different patterns of thrombus formation on the endothelium. HUVECs are venous endothelial cells and showed less platelet adhesion and fibrin deposition, while diseased primary PAEC from CTEPH patients showed increased platelet adhesion and more fibrin deposition compared to healthy PAEC. This suggests that the endothelium of CTEPH patients provides a prothrombotic environment that might result in increased thrombus formation (**Figure 3**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic overview of the protocol. (**A**) Various sources and different types of endothelial cells were isolated and cultured for usage in a microfluidic flow channel for perfusion experiments. Representative brightfield images of different types of endothelial cells. Scale bar = 50 µm. (**B**) Isolated cells were characterized by immunofluorescent staining to confirm an endothelial phenotype. Scale bar = 50 µm. (**C**) Cells can be seeded in either commercial flow slides or custom-made microfluidic channel. (**D**) Representative brightfield images of HUVEC grown in different channel geometries. Scale bar = 50 µm. (**E**) Experimental setup of blood perfusion experiments. Citrated blood was collected, diluted with saline buffer, and perfused over endothelial cells with a syringe pump. The lung and umbilical vein in this figure were modified from Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic Licence. http://smart.servier.com/

Figure 2: Image acquisition and quantification of thrombus formation. (**A**) Representative time-lapse imaging of adhered Calcein AM-Red labeled platelets and deposited Alexa488-conjugated fibrin at 1, 3, and 5 min after whole blood perfusion over unstimulated PAECs (**B**) and over histamine stimulated PAECs. (**C**) Representative time-lapse images of platelet adhesion and fibrin deposition at 1, 3, and 5 min of perfusion with whole blood incubated with dabigatran perfused over histamine-stimulated PAECs. Scale bar = 50 µm. (**D**) Schematic overview of image quantification in ImageJ. (**E**) Quantification of platelet adhesion and fibrin deposition for every 30 s on an unstimulated and histamine stimulated endothelium. (**F**) Quantification of the effect of dabigatran on thrombus formation quantified by platelet adhesion and fibrin deposition. Data are represented as mean ± SD, n = 3.

Figure 3: Representative confocal images of flow experiments to characterize thrombus formation in platelet adhesion and fibrin deposition on endothelial cells. Endothelial cell-cell contacts were characterized by VE-cadherin and measured in control PAECs, patient-derived CTEPH PAECs, and HUVEC. Scale bar = 50 µm.

DISCUSSION:

Coagulation is a result of the interplay between the endothelium and blood components. This in

vitro assay presents a method to investigate thrombogenic properties of endothelial cells under flow. Various types of primary human endothelial cells can be used, facilitating in situ thrombosis in an organ and patient-derived manner. In this study we illustrated the use of this protocol comparing thrombogenic properties of pulmonary artery ECs isolated from healthy donors versus CTEPH patients. Live thrombus formation was studied by perfusion of whole blood over an activated endothelium, while the effect of a DOAC was tested as an antithrombotic agent.

Besides the use of commercially available microchannels, as shown in the representative results, the introduction of the custom-made microfluidic channels enables the study of the influence of vascular geometry changes on thrombus formation. For example, flow decreases at a branch point or stenosis results in an increase in shear stress and more platelet activation[36]. However, a significant limitation of these custom-made microfluidic channels is the requirement of high cell numbers to form a stable monolayer of endothelial cells as described in step 2.3.2. This may form a limiting factor when patient-derived cells are scarce. The strengths of the commercially available microslides are that surface areas are modified for cell culture and growth areas are bigger, thereby allowing endothelial cells to form a confluent and stable monolayer. On the other hand, a bigger surface area will result in a bigger lumen. According to **Equation 1**, this requires higher blood volumes to reach similar flow rates as in the custom-made microfluidics, which are typically smaller.

An improvement of this protocol could be to investigate live endothelial cell loss. Endothelial cell damage in this protocol is only measured at the end of the experiment. For live tracking, ECs can be tagged with mCherry VE-cadherin, for example[37]. However, as this would need a highly optimized protocol with efficient virus transfection, Electric Cell-substrate Impedance Sensing (ECIS) could be used as an alternative to study endothelial integrity and barrier function[38]. Perfusion over special ECIS flow channels allows longitudinal monitoring of endothelial barrier integrity under flow. These specific ECIS features allow for parallel measurements of endothelial barrier properties and thrombus formation. Alternative ways for parallel EC barrier measurements, especially in the custom-made arrays include the use of fluorescent dextrans in the perfusate, which diffuse out of the lumen, depending on the EC barrier properties.

A limitation of the described protocol is that endothelial cells are removed from the body and cultured on tissue culture plastic, which is a stiff substrate. Cells adapt to their biophysical environment. This could possibly affect endothelial response to platelet activation, as there is an association between platelet activation and wall stiffness[39]. Despite these adaptations to culture plastics, cells may keep disease-specific characteristics that can be identified in direct comparison with ECs derived from healthy donors as shown in **Figure 3**. Even though under culture, control, CTEPH-PAEC, and HUVEC show a different pattern of platelet adhesion after 5 min of blood perfusion.

In contrast to other protocols, this system uses whole blood while other described protocols study endothelial interaction with a single blood component such as platelets and leukocytes[19-21]. Besides, there have been more advanced microfluidic models developed that allow the study of endothelial function in a vascular model with a round vessel and soft extracellular matrix.

However, these are optimized with HUVECs[40-42]. The novelty of the described protocol is the use of primary endothelial cells combined with whole blood, bringing the modelling of in situ thrombosis one step closer to in vivo conditions. Having optimized the protocol for the use of patient-derived endothelial cells and patient-derived blood further optimizes disease modelling in vitro, allowing the assessment of personalized thrombus formation and drug treatment.

The described protocol can be applied to study the effect of anticoagulation therapies on patient-derived cells. While we used dabigatran to evaluate thrombus formation, it is also possible to use other direct oral anticoagulants, such as rivaroxaban, which directly inhibits factor Xa in the coagulation cascade. Direct-platelet inhibitors like clopidogrel or aspirin can be studied as well, as these act on the primary phase of hemostasis where platelets bind to endothelial cells. Ultimately, the thrombogenic capacities of patient-specific endothelial cells in interaction with the patient's own blood can be used to predict the personal effect of anticoagulation therapy on the patient. Furthermore, a knockdown of specific proteins can provide information about endothelial protein function during coagulation.

There are some steps in the described protocol that are critical for a successful perfusion experiment. First, during the isolation of primary cells, it is necessary to obtain a highly pure endothelial cell phenotype. If there is still contamination with fibroblasts, higher rates of endothelial cell death occur upon shear, which increases thrombus formation. Second, it is important that the endothelial cells form a stable confluent monolayer. If this is not the case, a slight change in shear stress can cause endothelial damage and activation of the coagulation cascade, or platelets can start binding to the basement membrane, which will provide false positive thrombus formation. Third, it is essential to prevent air bubbles, as those can damage the endothelium and influence the results as well.

Moreover, after recalcification of the citrated blood with calcium chloride and magnesium chloride, it is important to immediately start the perfusion experiment. Recalcification induces a rapid response in platelet activation and thrombus formation, resulting in fast clotting in the sample.

In conclusion, we describe a highly versatile protocol to study whole blood-endothelial cell interactions during thrombosis. Describing the use of patient-derived endothelial cells and whole blood, the protocol facilitates the study of in situ thrombosis in various disease conditions. It is a tool for understanding the role of endothelial-blood interaction in this context.

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DISCLOSURES:

The authors declare no conflict of interest.

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Figure 1 in AI

Figure 1

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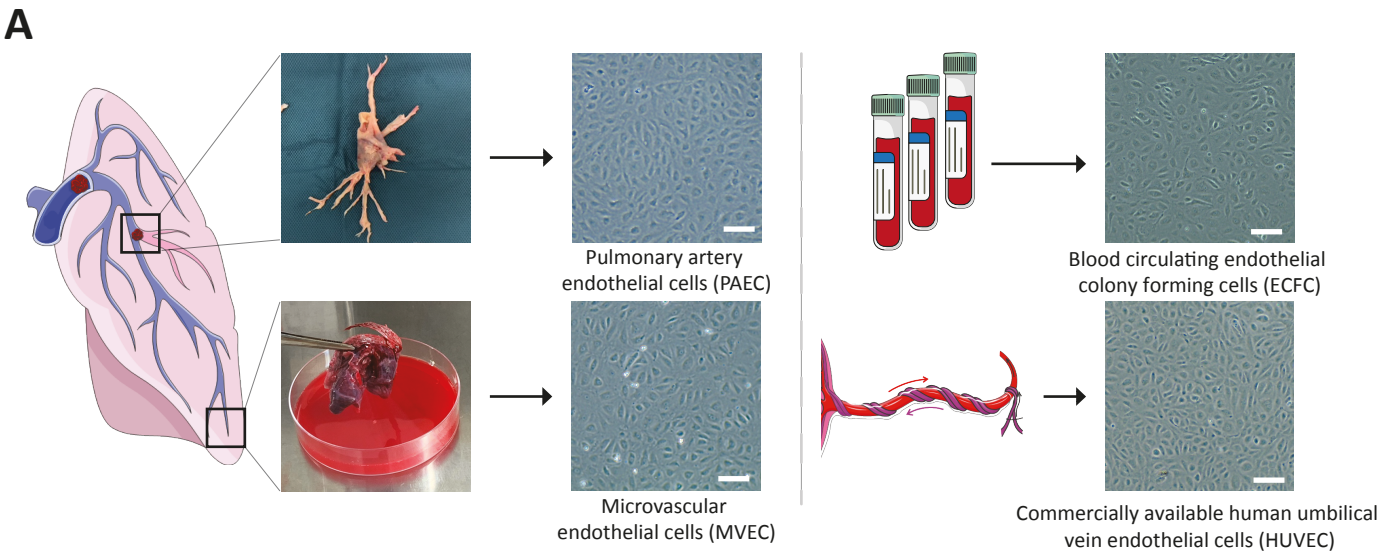


Figure 1

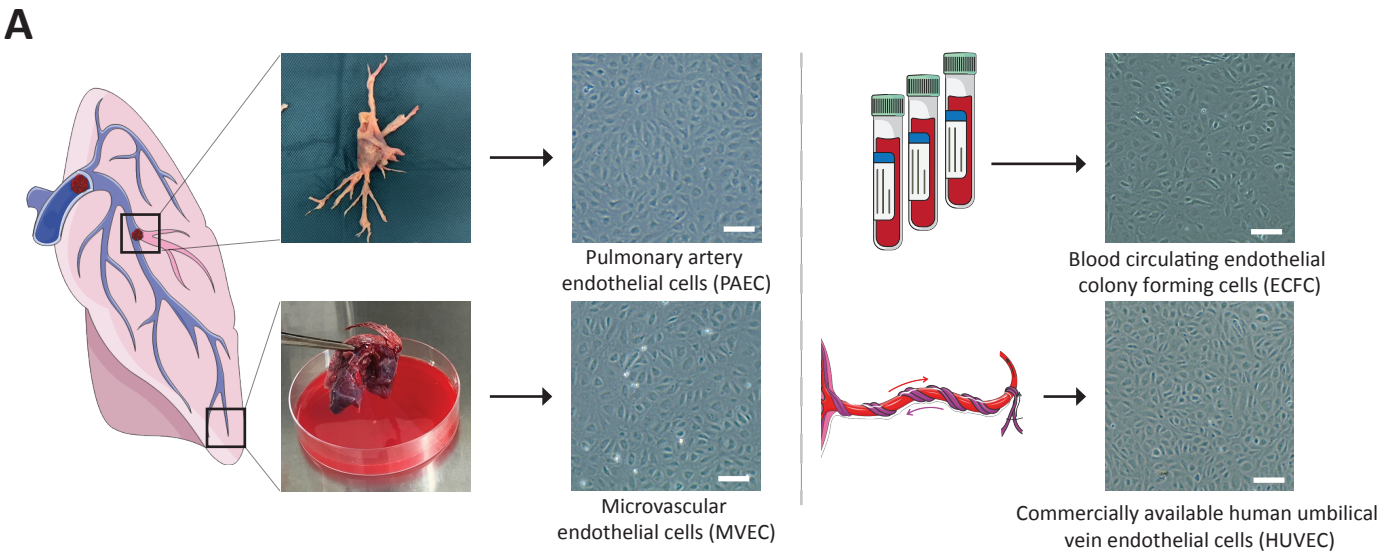
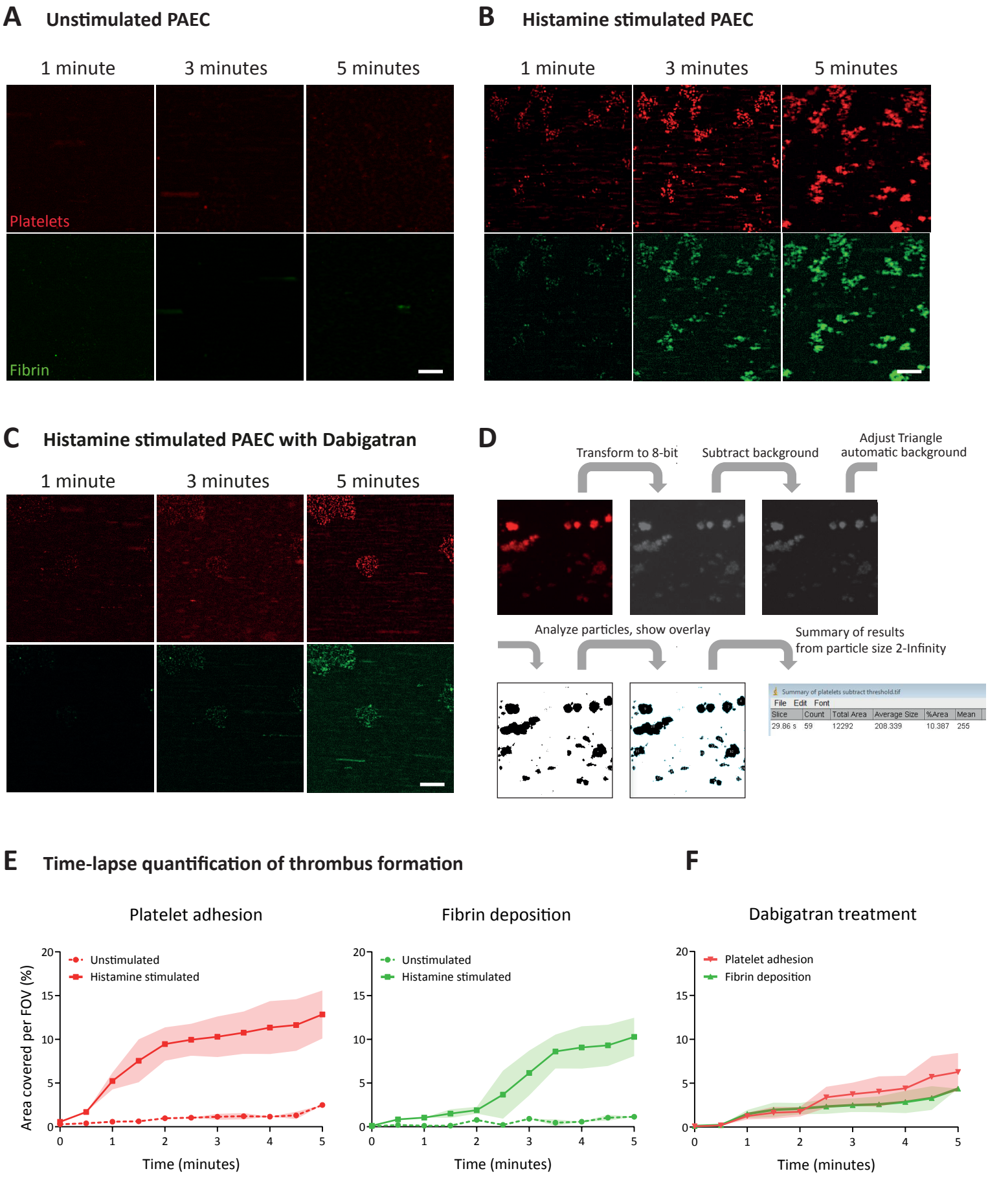


Figure 2



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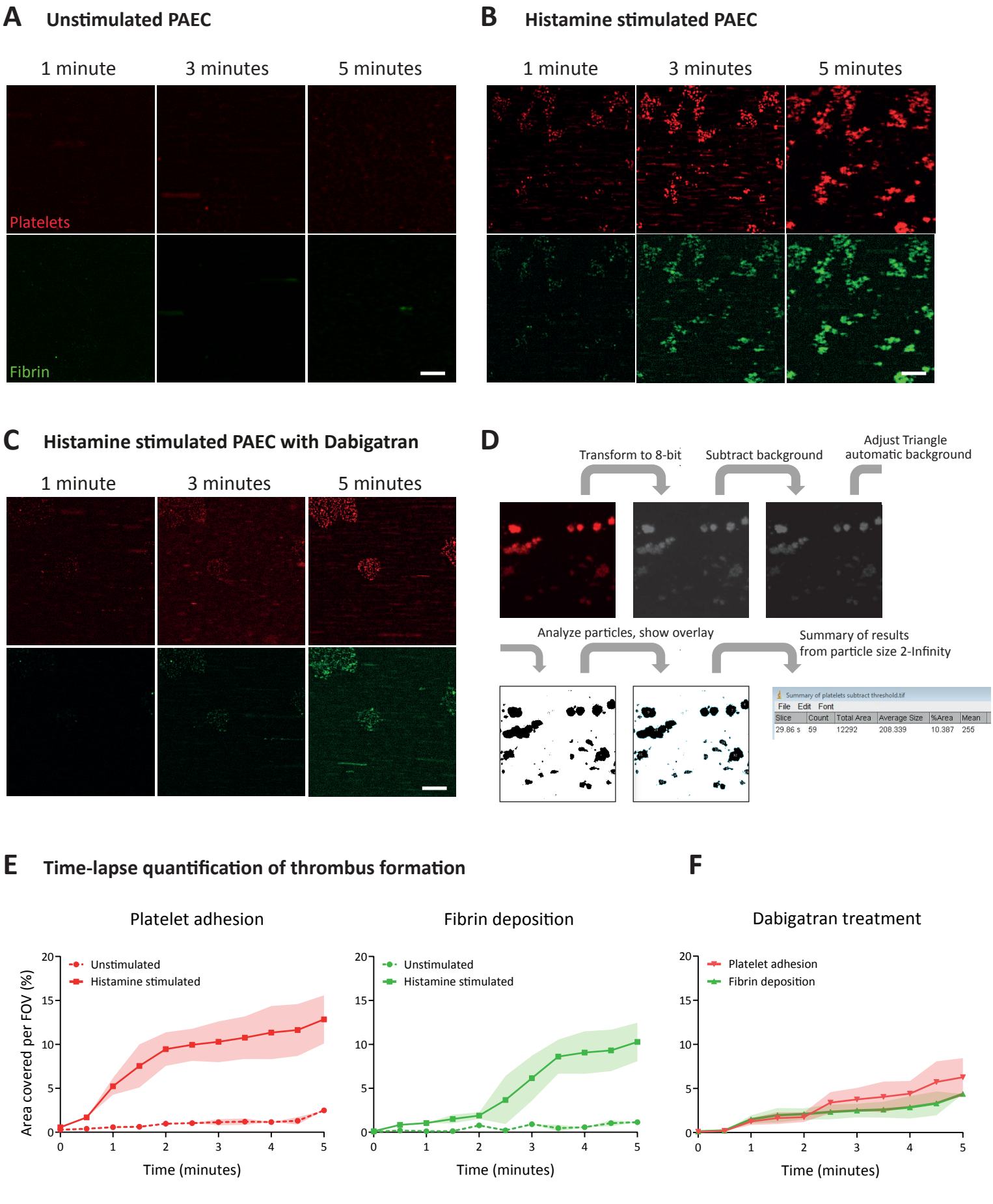

Figure 3

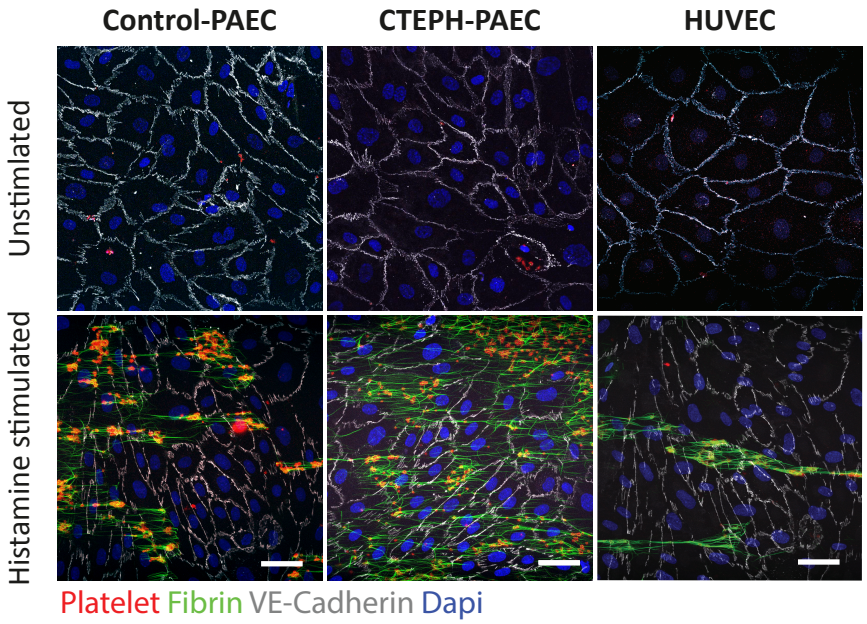

Figure 3

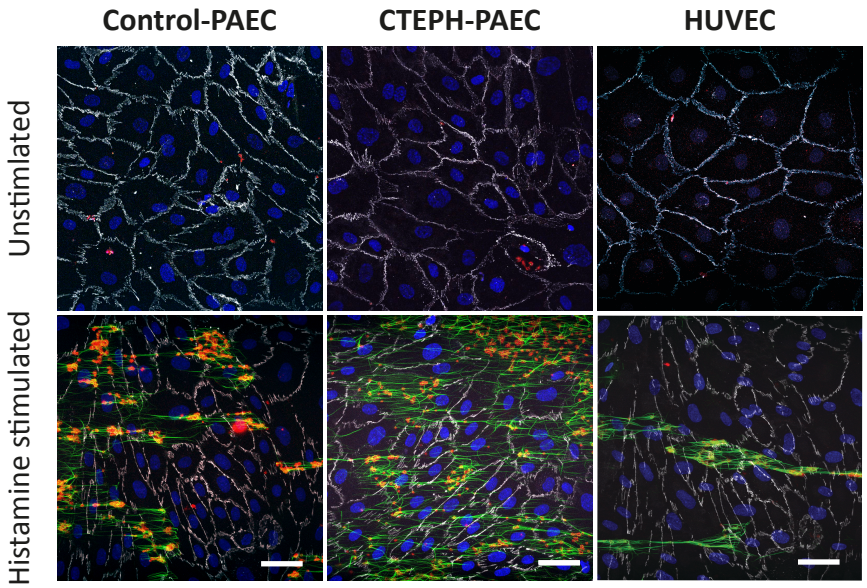

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials_revisie_v1.xlsx

Name of Material/Equipment	Company	Catalog Number
20 mL syringe	BD Plastipak	300613
20X objective	Olympus	
2-Propanol (IPA)	Boom	76051455 . 5000
Aladdin Syringe Pump	Word Precision Instruments	AL-4000
Alexa488-Fibrinogen	Invitrogen	F13191
Alexa647 Goat anti Rabbit	Invitrogen	A21245
Biopsy punch 1 mm diameter, Integra Miltex	Ted Pella	15110-10
Bovine Serum Albumin (BSA)	Sigma Aldrich	A9647
CaCl2	Sigma Aldrich	21115
Calcein AM-Red	Invitrogen	C3099
CD42b-APC	Miltenyi Biotec	130-100-208
Citric Acid	Merck	244
Collagen Typ I	Corning	354249
Corning CellBIND Surface 60 mm Culture Dish	Corning	3295
Cross-flow hood	Basan	
Desiccator	Duran	24 782 69
D-Glucose	Merck	14431-43-7
EDTA	Invitrogen	15575-038
Endothelial Cell Medium (ECM)	ScienCell	1001
Fibronectin	Sigma Aldrich	F0895
Flow tubings	ibidi	10831, 10841
Gelatin	Merck	104070
HEPES	Sigma Aldrich	H4034
Hoechst 33342	Invitrogen	H1399
ibidi µ-Slide VI 0.4 flow chambers	ibidi	80606
ImageJ	ImageJ	v1.49
KCl	Merck	7447-40-7
Lab oven	Quincy	10GC
LS720 Fluorescent microscope	etaluma	LS720
Luer connector	ibidi	10802, 10825
MACS magnetic beads (anti-CD144)	Miltenyi Biotec	130-097-857
MgCl2	Sigma Aldrich	M1028

Microscopy slides, 76 x 26 mm	Thermo Scientific	AAAA000001##12E
Negative photoresist, SU-8	Microchem	
Non-Essential Amino Acids (NEAA)	Lonza	13-114E
Paraformaldehyde (PFA)	Merck	818715
Penicillin/streptomycin (P/S)	Gibco	15140-122
Phosphate Buffered Saline (PBS)	Gibco	14190-094
Plasma chamber, CUTE	Femto Science	
Polydimethylsiloxane (PDMS), Sylgard 184	Dow	101697
sodium citrate blood collection tubes	BD Vacutainer	363048
trisodium citrate	Merck	6448
Trypsin-EDTA (0.05%), phenol red	Gibco	25300-045
VE-Cadherin (D87F2)-XP	Cell Signaling	2500

Comments/Description

15 ug/mL

0.180555556

1:1000
1:100

0,1 mg/mL

0.1%

1:1000

Male elbow connectors, and Female tube connectors
1:5

4% PFA in PBS
1%

1:300

Thank you for revising our manuscript. The review process substantially improved readability and clarity of the manuscript. Please find below the line-by-line response to each of the editorial and reviewer comments.

Editorial Comments:

Q1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

A1: Thank you for this comment. We proofread the manuscript to correct spelling and grammatical errors.

Q2: **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. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:
1) 1.3: How long after surgery? How was the artery handled immediately post surgery? What is the cord buffer temperature?
2) 4.3: Describe all steps in text within the protocol.

A2: We have edited the manuscript and provided more details when needed. Please refer to the marked-up version of the manuscript.

Q3: **Protocol Numbering:** Please add a one-line space after each protocol step.

A3: A one-line space has been added after each protocol step.

Q4: **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. 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 highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

A4: We highlighted the following section:
1. isolation of primary pulmonary artery endothelial cells
2.1. seeding of PAECs in commercially available microslides
3. preparation of whole blood sample
4. perfusion of whole blood over primary PAEC

Q5: **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.

A5: We completely rewrote the discussion according to the proposed format.

Q6: **References:** Please spell out journal names.

A6: Journal names were spelled out.

Q7: **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 CellBIND®, ibidi, ibidi µ-slide V, MESA+ institute, falcon tube,
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

A7: We have deleted commercial language.

Q8: If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

A8: Our figures are original and not copied or published previously. The lung and umbilical vein images in Figure 1A are modified from Servier Medical Art. The images are licensed under a Creative Common Attribution 3.0 Generic Licence. http://smart.servier.com/. We referenced to it in the legend of Figure 1 in the manuscript.

Comments from Peer-Reviewers:

The line numbers from the revised manuscript are provided for the line-by-line discussion.

Reviewer #1:

Manuscript Summary:
This manuscript by Manz and colleagues describes a method for culturing endothelial cells inside commercial and custom microfluidic channels and perfusing diluted whole blood over these cells to monitor thrombus formation. The major strength of the manuscript is the description of the isolation and culture of primary pulmonary artery endothelial cells from patients. There are some technical details that could use further clarification and non-standard nomenclature that should be addressed prior to publication as described below.

Major Concerns:

1. To call this a model of thrombosis is misleading. The assays are not performed until occlusion of the channel, the hallmark of thrombosis. It would be more accurate to describe as a model to measure the thrombogenicity of a given cell line / set of primary cells.

We have changed the name to a model to study thrombogenic properties of primary endothelial cells and adjusted the manuscript in accordance. It is worth mentioning that the system can be used to study thrombus formation, e.g. in the stenotic custom-made slides, by adjusting duration of blood perfusion.

2. Lines 110-113. Changes in hemodynamics and shear stress almost always lead to changes in endothelial cell phenotype, so the state the 'endothelium is unaffected' is misleading.

Line 113 – we changed ‘unaffected’ to ‘undamaged’. The endothelium is activated, but still forms a monolayer as confirmed by VE-cadherin staining.

3. Line 158. Please provide more information on how to detect the level of purity of isolated cells and when you would need to use a MACS type assay.

Line 173 – section 1.8. We added to the manuscript how we detect he level of purity. Generally, a culture is defined pure when flow cytometry detects ≤10% contaminating cells. Examples of a pure culture are given in Figure 1A.

4. Line 273. The rationale for diluting the whole blood 1:1 with buffer needs to be stated explicitly. Platelet adhesion is a function of hematocrit. By diluting the blood to low hematocrits characteristic of anemia the dynamics of thrombus formation will be significantly different than with undiluted whole blood at physiologic hematocrits.

Line 336 – section 3.3. Hemodilution with a maximum of 50% does not influence coagulation reaction, and in that case we need less blood from the patient.

Tobias, M. D., Wambold, D., Pilla, M. A. & Greer, F. Differential effects of serial hemodilution with hydroxyethyl starch, albumin, and 0.9% saline on whole blood coagulation. Journal of Clinical Anesthesia. 10 (5), 366-371, (1998)

5. Line 294. This equation is incorrect. The units are do not work out (the left hand side is not equal to the right hand side). The shear rate is equal to the viscosity multiplied by the shear rate. The relationship between the shear rate and flow rate depends on the geometry of the devices, but in general involves dividing the flow rate by the product of geometric parameters of the cross-sectional that result in units of volume (e.g. width x height^2/6 for channels with high aspect ratios). If the authors used this equation, then they have miscalculated their shear stresses.

Line 358 – section 4.4. Initially we used the equation provided by the manufacturer of the slides we used. I agree that the units left and right do not fit, where factor A was undefined. After some discussion we decided to use the standard equation for a rectangular shaped channel to calculate shear stress and defined them as follows:

$$\tau = \frac{\eta \times 6Q}{h^2 w}$$

τ = shear stress $\left[\frac{dyn}{cm^2}\right]$
η = dynamical viscosity $\left[\frac{dyn \times s}{cm^2}\right]$
Q = volumetric flow rate $\left[\frac{mm^3}{s}\right]$
h = channel height $[mm]$
w = channel width$[mm]$

By using this formula, the calculations are correct.

6. There are too many grammatical and spelling errors to enumerate. Please have this manuscript copy edited.

Thank you for this remark. We thoroughly proofread the manuscript to ensure that there are no spelling and grammatical errors.

7. Line 350. The micrometer to pixel conversion depends on the resolution, which is a function of not only on the objective magnification, but its numerical aperture (NA). Please list the NA of the objective used.

Line 444 – we added the numerical aperture of 0.45 to the manuscript.

8. Line 389. What are the characteristics of 'stressed phenotype' that the reader should see in Figure 1?

Line 486 – Stressed has been changed to elongated. Especially in the stenotic region, cells show a more stretched and elongated phenotype in Figure 1D.

9. Line 403. The statement that endothelial cell type is more important than the microfluidic

channel geometries is perhaps an overstatement. Owing to the Fahreus and Fahreus-Lindquist effects, the channel size, especially at this length scale, can strongly influence hematocrit and viscosity, which in turn affects endothelial cell-platelet interactions.

We agree with you and removed our statement.

10. What concentration of dabigatran used and why it chosen?

Line 515 – We used a concentration of 10 nM after we did a titration where it turned out that a dosage of 10 nM was minimal toxic and showed maximal effect in reduction of fibrin aggregates.

11. It would be useful if the authors could compare and contrast their protocol with similar ones in the literature for thrombus formation or platelet adhesion on cultured endothelial cells: Michels et al., J. Vis. Exp. (126), e55917, 2017; Barendrecht et al. J. Vis. Exp. (125), e55658, 2017; Vejen et al. J. Vis. Exp. (134), 2018; Myers et al, J. Vis. Exp. (64), e3958, 2012.

To our opinion, this is discussed in the introduction (line 102 – 110) and discussion (line 607 – 615)

Minor Concerns:
1. There are instances where the authors confuse coagulation and platelet function: Line 396, VWF may weakly promote activation of the contact pathway, but I think the authors intend to mean that VWF promotes platelet adhesion. Line 510, Platelet inhibitors act on platelets (not coagulation), maybe 'primary phase of hemostasis'.

Line 493, we changed the sentence to that histamine induces VWF release and consequently platelet adhesion.
Line 621, we changed that platelet inhibitors act in the primary phase of hemostasis.

2. Line 316. What are the locations of the 3 ROI's relative to the channel inlet?

Line 385 - ROI positions are chosen in the beginning, middle and end of every channel. The beginning and end should be at least 3 mm away from the in- and outlet of the channel.

3. Line 476. No data was presented that shows thrombus formation in 'vascular geometries' (e.g. stenosis), so perhaps modify this statement.

Line 567 - We modified our statement that we presented an in vitro assay as a method to investigate thrombogenic properties of endothelial cells under flow.

Reviewer #2:

Manuscript Summary:
The manuscript describes a method to assess thrombosis on microfluidic straight channels lined with primary endothelial cells derived from patients

Major Concerns:
Since the major novelty and emphasis is on the patient-specific aspect of the endothelial cells, this protocol should include 1 or 2 methods that assess the "patient-specificity" of the endothelial cells. For example, barrier function, gap measurement, and adhesion molecules on the endothelium

Thank you for your comment. We agree with your comment and removed patient-specificity from the manuscript as the emphasis of our protocol is on characterizing thrombogenic potential of endothelial cells rather than barrier function and gap formation. These are certainly important cell properties that influence thrombus formation, and we would refer you to other protocols that measures for example barrier function (reference 38). To define patient-specificity is outside the scope of the presented protocol. We did experiments to assess barrier function and measured adhesion molecules to be different from healthy control endothelium, but this is in a manuscript in preparation.

Minor Concerns:
Please provide protocol on measurements made to characterize the quality of the endothelial lumen formed in this device.

The channels we have used in our protocol do not form a lumen. Endothelial cells form a monolayer on the bottom of the flow slide.
We have shown in Figure 3 how we assess the quality of the EC monolayer by immunofluorescence staining for VE-cadherin.

Reviewer #3:

Manuscript Summary:

In this manuscript, the authors describe their protocol that leverages microfluidics (commercial and custom made via microfabrication/photolithography) to study the influence of endothelial cells on thrombus dynamics in a pathobiologically meaningful context. They demonstrate the usage of these systems by describing experiments involving he development of a thrombus that is recorded in real-time and quantitatively characterized by platelet adhesion and fibrin deposition.

The effect of endothelial function in altered thrombus dynamics is determined by via post hoc analysis through immunofluorescence staining of specific molecules. The authors have constructed a well written protocol that can be useful for the fields of bioengineering and hematology and they should be commended for their efforts. However, several significant issues need to be addressed to enable this article to be accessible to the target audiences

Major Concerns:

First, the authors should better place their system, protocol, and experiments in better context of the field. For example, papers by Zheng et al PNAS, Tsai et al JCI 2012, and numerous other groups who study thrombosis and vascularized fluidic systems should be cited and discussed.

We have referenced similar papers in the introduction (Line 102 – 110) and discussion (Line 607 – 615) and cited the recommended papers in our manuscript.

Importantly, while use of patient endothelial cells is extremely important, attaining patient pulmonary artery tissue is not trivial and few investigators have this capability at their institutions. As such, the authors should include other protocols and ways to obtain patient endothelial cells - from other patient types, other anatomic regions, etc. The goal is to provide several or a generalized protocol that most researchers will be able to adapt and apply at their own institution.

That is an important suggestion and we have adapted the manuscript and included references that refer to protocols to isolate HUVEC, MVEC and ECFCs (Figure 1A)(Line 141, 470). Since we introduced the isolation of PAECs as main technique, we focused the protocol on PAEC isolation and performed experiments with these cells but agree that our protocol is meant to be modular and adjustable to the needs of the scientist.

Moreover, as culture of primary patient cells is non-trivial and more detailed protocol regarding this process is needed including what quality metrics the authors use to ensure that the cultured cells are in fact functional endothelial cells and have not altered phenotype (e.g. become fibroblast-like), ceased exhibiting endothelial attributes, or become pro-inflammatory due to culture conditions

Reviewer 1, Q3 also asked for a quality control of purity of cell population. In accordance with this, we have added an immunofluorescence staining in Figure 1B. ECs were positive for

VE-cadherin, CD31 and Tie2, which are endothelial specific proteins. Furthermore, these cells are negative for αSMA, which is a smooth muscle cell marker and pancytokeratin for epithelial cells (Line 475).

Some discussion of the attributes of the different commercially available microfluidic systems should be compared, contrasted, and discussed to help the readers decide which might be most suitable for their specific research.

We provided information in Line 196 why we decided to use a 6-well flow channel. We were not allowed to use commercial language, and therefore decided not to further explain and compare the available microslides.

In depth discussion of how altering the geometry of the microfludics affects the shear is needed and ideally this section should offer the readers some guidance on how to design their channels and what the consequences regarding the flow dynamics are.

We have added more detailed discussion about how the geometry of microfluidics changes the shear (line 577). For example, flow decreases at branch points or increases at sites of stenosis or vasoconstriction consequently alter platelet activation. Please refer to the updated equation 1 for more details on how the channel design changes shear stress. Parameters to consider are viscosity of the flow through, geometry and diameter of the channel, and flow rate.

Minor Concerns:

1) endothelial cell concentration at "seeding" of the microfluidics is likely important and should be included

We seed 1.5 cm^2 of confluent cells per channel, this is overconfluent as the channel has a surface area of 0.6 cm^2. We further culture these cells for 6 days within the channels, where they reach maximum confluency before starting the experiment.

2) the blood samples seem to be citrated and the recalcified. is there any other anticoagulation used? if so, this should be discussed. if not, the sample will only be usable for a short time period before clotting in the sample tube begins, rendering the experiment to be unreliable and this should be discussed.

No there are no other anticoagulants used in the blood samples (line 326, section 3.1). However, the tubes are pre-treated with wash buffer that contains 5 mM EDTA to prevent clotting in the flow tubes (line 342, section 4.1). However, endothelial cells are not washed with this buffer, and therefore can interact with the blood to form a clot. We further elaborate on the use of citrated blood in the discussion as a critical step for the protocol (Line 636)