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TITLE:

Standardized and Scalable Assay to Study Perfused 3D Angiogenic Sprouting of iPSC-Derived Endothelial Cells In Vitro

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3D cell culture, microfluidics, in vitro, human induced pluripotent stem cells, endothelial cells, angiogenesis, gradients, high-throughput screening, assay development

SUMMARY:

This method describes the culture of iPSC-derived endothelial cells as 40 perfused 3D microvessels in a standardized microfluidic platform. This platform enables the study of gradient-driven angiogenic sprouting in 3D, including anastomosis and stabilization of the angiogenic sprouts in a scalable and high-throughput manner.

ABSTRACT:

Pre-clinical drug research of vascular diseases requires in vitro models of vasculature that are amendable to high-throughput screening. However, current in vitro screening models that have sufficient throughput only have limited physiological relevance, which hinders the translation of findings from in vitro to in vivo. On the other hand, microfluidic cell culture platforms have shown

unparalleled physiological relevancy in vitro, but often lack the required throughput, scalability and standardization. We demonstrate a robust platform to study angiogenesis of endothelial cells derived from human induced pluripotent stem cells (iPSC-ECs) in a physiological relevant cellular microenvironment, including perfusion and gradients. The iPSC-ECs are cultured as 40 perfused 3D microvessels against a patterned collagen-1 scaffold. Upon the application of a gradient of angiogenic factors, important hallmarks of angiogenesis can be studied, including the differentiation into tip- and stalk cell and the formation of perfusable lumen. Perfusion with fluorescent tracer dyes enables the study of permeability during and after anastomosis of the angiogenic sprouts. In conclusion, this method shows the feasibility of iPSC-derived ECs in a standardized and scalable 3D angiogenic assay that combines physiological relevant culture conditions in a platform that has the required robustness and scalability to be integrated within the drug screening infrastructure.

INTRODUCTION:

In vitro models play a fundamental role in the discovery and validation of new drug targets of vascular diseases. However, current in vitro screening models that have sufficient throughput only have limited physiological relevance[1], which hinders the translation of findings from in vitro to in vivo. Thus, to advance pre-clinical vascular drug research, improved in vitro models of vasculature are necessary that combine high-throughput screening with a physiologically relevant 3D cellular micro-environment.

Within the last decade, significant progress has been made to increase the physiological relevance of in vitro models of vasculature. Instead of culturing endothelial cells on flat surfaces such as tissue-culture plastics, endothelial cells can be embedded in 3D scaffolds, such as fibrin and collagen gels[2]. Within these matrices, the endothelial cells show a more physiologically relevant phenotype associated with matrix degradation and lumen formation. However, these models only demonstrate a subset of the many processes that occur during angiogenic sprouting as important cues from the cellular microenvironment are still lacking.

Microfluidic cell culture platforms are uniquely suited to further increase the physiological relevance of in vitro models of vasculature. For example, endothelial cells can be exposed to shear stress, which is an important biomechanical stimulus for vasculature. Also, the possibility to spatially control fluids within microfluidics allows the formation of biomolecular gradients[3-6]. Such gradients play an important role in vivo during the formation and patterning during angiogenesis. However, while microfluidic cell culture platforms have shown unparalleled physiological relevancy over traditional 2D and 3D cell culture methods, they often lack the necessary throughput, scalability and standardization that is required for drug screening[7]. Also, many of these platforms are not commercially available and require the end-users to microfabricate their devices prior to use[8]. This not only requires manufacturing apparatus and technical knowledge, but also limits the level of quality control and negatively affects reproducibility[9].

To date, primary human endothelial cells remain the most widely used cell source to model angiogenesis in vitro[10]. However, primary human cells have a number of limitations that hinder

their routine application in screening approaches. First, there is a limited possibility to scale up and expand primary cell-derived cultures. Thus, for large scale experiment, batches from different donors need to be used, which result in genomic differences and batch-to-batch variations. Second, after a few passages, primary endothelial cells generally lose relevant properties when cultured in vitro[11,12].

Endothelial cells derived from human induced pluripotent stem cells (iPSC) are a promising alternative: they resemble primary cells, but with a more stable genotype that is also amenable to precise genome editing. Furthermore, iPSCs are able to self-renew and thus can be expanded in nearly unlimited quantities, which make iPSC-derived cells an attractive alternative to primary cells for usage within in vitro screening models[13].

Here, we describe a method to culture endothelial cells as perfusable 3D microvessels in a standardized, high-throughput microfluidic cell culture platform. Perfusion is applied by placing the device on a rocker platform, which ensures robust operation and increases the scalability of the assay. As the microvessels are continuously perfused and exposed to a gradient of angiogenic factors, angiogenic sprouting is studied in a more physiological relevant cellular microenvironment. While the protocol is compatible with many different sources of (primary) endothelial cells[14,15], we focused on using human iPSC-derived ECs in order to increase the standardization of this assay and to facilitate its integration within vascular drug research.

PROTOCOL:

1. Device preparation

1.1. Transfer the microfluidic 384-well plate to a sterile laminar-flow hood.

1.2. Remove the lid and add 50 µL of water or phosphate-buffered saline (PBS) to each of the 40 observation wells (**Figure 1b**, well B2) using a multichannel or repeating pipette.

NOTE: The protocol can be paused here. Leave the plate with the lid on in the sterile culture cabinet at room temperature (RT).

2. Prepare gel and coating

2.1. Prepare 2.5 mL of 10 µg/mL fibronectin (FN) coating solution. Dilute 25 µL of 1 mg/mL fibronectin stock solution in 2.5 mL of Dulbecco's PBS (dPBS, calcium and magnesium free). Place the solution in the water bath at 37 °C till use.

2.2. Prepare 100 µL of collagen-1 solution. Add 10 µL of HEPES (1 M) to 10 µL of $NaHCO_3$ (37 g/L) and mix by pipetting. Place the tube on ice and add 80 µL of collagen-1 (5 mg/mL) to yield a neutralized collagen-1 concentration of 4 mg/mL. Use a pipette to mix carefully and avoid the formation of bubbles.

2.3. Add 1.5 µL of collagen-1 solution (4 mg/mL) to the gel inlet of each microfluidic unit (**Figure 1b**, well B1). Make sure the droplet of gel is placed in the middle of each well in order for the gel to enter the channel (see **Figure 2a**).

NOTE: Phaseguides prevent filling of the adjacent channels and enables gel patterning. Correct gel loading can be confirmed under a brightfield microscope by observing the meniscus formation through the 'observation window' (well B2) or by flipping the plate upside down. If the gel did not completely fill the channel, an additional droplet of 1 µL can be added.

2.4. Place the microfluidic plate in an incubator (37 °C, 5% CO_2) for 10 min to polymerize collagen-1.

NOTE: Timing of the polymerization is crucial; due to the low volumes used in microfluidics, evaporation can already be observed after 15 min of incubation, which results in gel collapse or shrinkage.

2.5. Take the plate out of the incubator and transfer to a sterile laminar-flow hood.

2.6. Add 50 µL of 10 µg/mL FN coating solution to the inlet well of the top perfusion channel of each microfluidic unit (**Figure 1b**, well A1). Press the pipette tip against the side of the well for correct filling of the well without trapping air bubbles (see **Figure 2b**). The channel should fill, and the liquid should pin on the outlet (well C1) without filling the outlet well.

2.7. Place the plate in the incubator (37 °C, 5% CO_2) for at least 2 days.

NOTE: The protocol can be paused here, as the collagen-1 gel together with the coating mixture is stable for at least 5 days in the incubator. If the coating mixture is refreshed, longer periods could be possible, but this has not been tested. Crucial is the level of FN-coating, as this prevents the dehydration of the collagen-1 gel.

3. Cell seeding/microvessel culture

3.1. Add 5 mL of fetal calf serum and 2.5 mL of pen/strep to 500 mL of basal endothelial cell culture medium and filter sterilize using a bottle top filter with 0.22 µm pore size. This medium is now referred to as basal medium.

3.2. Prepare vascular growth medium: Add 3 µL of 50 µg/mL vascular endothelial growth factor (VEGF) and 2 µL of 20 µg/mL basic fibroblast growth factors (bFGF) to 5 mL of basal medium.

3.3. Thaw the frozen iPSC-ECs rapidly (<1 min) in a 37 °C water bath and transfer to a 15 mL tube and dilute in 10 mL of basal medium.

3.4. Count the cells.

NOTE: A single vial contains 1 million cells in 0.5 mL with >90% viability.

3.5. Centrifuge the tube at 100 x *g* for 5 min. Aspirate supernatant without disturbing the cell pellet and resuspend in basal medium to yield a concentration of 2 x 10^7 cells/mL.

3.6. Transfer the microfluidic 384-well plate from the incubator to a sterile laminar-flow hood.

3.7. Aspirate FN-coating solution from the perfusion inlet (well A1). Add 25 µL of basal medium in the inlet wells (well A1).

3.8. Add a 1 µL droplet of cell suspension to each top perfusion outlet well (**Figure 1b**, well C1). The droplet should flatten in a few seconds (see **Figure 3a,b** for an illustration of this 'passive pumping' method).

NOTE: Check under a microscope whether the seeding is homogeneous. If not, add another 1 µL in the outlet and wait till the droplet flattens.

3.9. Incubate the microfluidic well-plate for 1 h at 37 °C, 5% CO_2. After this, the cells should have adhered. If not, wait another 30 min.

3.10. Remove the basal medium from the top perfusion inlet wells (**Figure 1b**, well A1). Add warm vessel culture medium in the top perfusion inlet and outlet (**Figure 1b**, wells A1 and A3). Place the plate on the rocker platform (set on 7° angle, 8 min rocking interval) in the incubator (37 °C, 5% CO_2).

3.11. Image the plate using a brightfield microscope with automated stage at day 1 and 2 post-seeding to confirm cell viability. After 2 days, a confluent monolayer should have formed against the collagen-1 scaffold.

NOTE: If the channels do not appear to be equally confluent, the microvessels can be cultured for an additional 24 h.

4. Study angiogenic sprouting including tip- and stalk cell formation

4.1. Prepare 4.5 mL of angiogenic sprouting medium by supplementing basal medium with 4.5 µL of VEGF (10 µg/mL stock), 4.5 µL of phorbol 12-myristate-13-acetate (PMA) (20 µg/mL stock), and 2.25 µL of sphingosine-1-phosphate (S1P) (1 mM stock).

4.2. Prepare 8.5 mL of vessel growth medium (basal medium supplemented with 30 ng/mL VEGF and 20 ng/mL bFGF).

4.3. Aspirate medium from the wells and add 50 µL of fresh vessel culture medium in the top perfusion inlet and outlet wells and gel inlet and outlet wells (**Figure 1b**, wells A1, A3, B1 and B3).

4.4. Add 50 µL of angiogenic sprout mixture to each of the bottom perfusion channel inlet and outlet wells (**Figure 1b**, wells C1 and C3).

4.5. Place the device back in the incubator (37 °C, 5% CO_2) on the rocker platform in order to form a gradient of angiogenic growth factors.

4.6. Image 1 day and 2 days after addition of the angiogenic growth factors using a brightfield microscope with automated stage.

Note: Continue culturing the microvessels to study anastomosis (go to section 5) or fix and stain the microvessels to quantify sprouting length and morphology at day 2 and day 6 (go to section 6).

5. Study anastomosis and sprout stabilization

5.1. Image using a brightfield microscope with automated stage.

5.2. Add 1 µL of fluorescently labeled albumin (0.5 mg/mL) to the top perfusion inlet (**Figure 1b**, well A1) and mix using a 50 µL pipette.

5.3. Transfer the plate to a fluorescent microscope with automated stage and incubator set at 37 °C. Set microscope at 10x objective and correct exposure settings (e.g., tetramethylrhodamine [TRITC]-channel, 20 ms exposure). Acquire time-lapse images every minute for 10 min.

5.4. Remove the plate from the microscope and transfer the plate to a sterile laminar-flow hood.

5.5. Remove all medium from the wells and replace both the vessel culture medium and angiogenic sprouting medium in the corresponding wells (see steps 4.3 and 4.4).

5.6. Place the device back into the incubator (37 °C, 5% CO_2) to continue angiogenic sprouting.

5.7. Repeat steps 5.1−5.6 to study the permeability at day 6.

6. Fixation, staining and imaging

6.1. Aspirate all culture media from all the wells.

NOTE: Residual medium or liquids in the microfluidic channels does not influence the fixation due to its low volume of 1−2 µL.

6.2. Add 25 µL of 4% paraformaldehyde (PFA) in PBS to all the perfusion inlet (**Figure 1b**, A1 and C1) and outlet wells (**Figure 1b**, A3 and C3). Incubate for 10 min at RT. Place the device under a slight angle (± 5°) to induce flow (e.g., by placing one side of the plate on a lid).

6.3. Aspirate PFA from the wells. Wash all the perfusion inlets and outlets twice with 50 µL of Hank's balanced salt solution (HBSS). Aspirate HBSS from wells.

6.4. Permeabilize at RT for 10 min by adding 50 µL of 0.2% nonionic surfactant to all the perfusion inlets and outlets.

6.5. Aspirate the nonionic surfactant from wells. Wash the perfusion channels twice by adding 50 µL of HBSS to all perfusion inlet and outlet wells. Aspirate HBSS from wells.

6.6. Stain the nuclei using Hoechst (1:2000) and F-actin using phalloidin (1:200) in HBSS. Prepare 2.2 mL for 40 units, and add 25 µL to each perfusion inlet and outlet well. Place the plate under a slight angle and incubate at RT for at least 30 min.

6.7. Wash twice with 50 µL of HBSS in all the perfusion inlets and outlets.

6.8. Directly image using a fluorescent microscope with automated stage or store the plate protected from light at 4 °C for later use.

REPRESENTATIVE RESULTS:

The microfluidic 3D cell culture platform consists of 40 perfused microfluidic units (**Figure 1a,b**), which is used to study angiogenic sprouting of perfused microvessels against a patterned collagen-1 gel (**Figure 1c**). These microvessels are continuously perfused and exposed to a gradient of angiogenic growth factors (**Figure 3a-d**). The angiogenic sprouts can be either studied 2 days after gradient exposure or cultured for more than 5 days after gradient exposure to study anastomosis and sprout stabilization (see timeline, **Figure 1d**).

Seeding the iPSC-ECs using the passive pumping method should result in homogenous seeding densities (**Figure 4a,b**). Culture under continuous perfusion resulted in confluent microvessels in 2 days, with the cells completely lining the circumference of the microfluidic channel and the formation of a confluent monolayer against the patterned collagen-1 gel.

Exposure to a gradient of angiogenic factors resulted in directional angiogenic sprouting of the microvessels within the patterned collagen-1 gel (**Figure 5a-g**). Clear tip cell formation and invasion into the collagen-1 gel was visible 24 h after addition of the angiogenic gradient, while stalk cells including lumen formation were visible after 48 h (**Figure 5a**).

After fixation and staining, the capillary network can be visualized using phalloidin to stain F-actin and using Hoechst 33342 to stain the nucleus (**Figure 5b,c**). These sprouts can be quantified (e.g., shape and length[14]). Without addition of growth factors, no invasion into the collagen-1 gel should be observed (**Figure 5d**). Confocal imaging was used to determine the sprout diameter and to confirm lumen formation (**Figure 5e-g**).

The sprouts continue to grow towards the direction of the gradient and reach the opposite perfusion channel within 3–4 days after addition of angiogenic growth factors. This results in

remodeling of the vascular network, with a clear reduction in the number of angiogenic sprouts (**Figure 6a**). Lumen formation was assessed by perfusion of the vascular network with fluorescently labeled macromolecules (e.g., albumin or dextrans). Perfusing the microvessels with 0.5 mg/mL labeled albumin before and after anastomosis revealed a clear difference in sprout permeability after 10 min (**Figure 6b-e**), which suggests that the capillaries stabilize and mature after anastomosis.

FIGURE AND TABLE LEGENDS:

Figure 1: Microfluidic cell culture protocol for iPSC-derived microvessels. (**a**) The bottom of the microfluidic cell culture device is shown displaying the 40 microfluidic units that are integrated underneath the 384-well plate. Larger view displays one of the 40 microfluidic units. (**b**) Each microfluidic unit is positioned underneath 9 wells with 3 inlet wells and 3 outlet wells. The microfluidic channels are separated by ridges ('phaseguides'), which enable the patterning of hydrogels in the central channel ('gel channel') while there is still contact with the adjacent channels ('perfusion channels'). (**c**) Method to culture a perfused microvessel within the microfluidic device, which is used to study gradient driven angiogenic sprouting through a patterned collagen-1 matrix. (**d**) Timeline for studying angiogenic sprouting and/or anastomosis. This figure has been modified from van Duinen et al.[14].

Figure 2: Loading procedures for gel and medium. (**a**) Examples of correct and incorrect gel deposition. Correct filing results in a patterned collagen-1 gel in the middle channel, which is subsequently polymerized. (**b**) Examples of correct and incorrect filling of the wells. Wells are filled in the order of 1−4 to prevent air-bubble trapping within the microfluidic channels.

Figure 3: Continuous hydrostatic pressure driven flow and gradient stabilization. (**a**) Hydrostatic pressure differences between wells result in passive levelling and flow within the microfluidic channels. (**b**) Placing the device on a rocker platform set at 7° and 8 min cycle time results in continuous, bi-directional perfusion within the microfluidic channels. (**c**) Gradients are formed by introducing two different concentrations within the wells, which are continuously refreshed by passive leveling. (**d**) Gradient visualization using fluorescein isothiocyanate (FITC)-dextran. Bi-directional flow stabilizes the gradient up till 3 days. Scale bar = 200 µm. This figure has been modified from van Duinen et al.[14].

Figure 4: Passive pumping method for cell seeding. (**a**) Passive pumping is driven by pressure differences that are caused by differences in surface tension. This results in a flow from the droplet (high internal pressure) towards the reservoir (low internal pressure). (**b**) Time lapse of a droplet (gray outline) that is placed on top of the inlet (white outline) of the microfluidic channel (blue outline). Right after addition (**Figure 4b, i**), the droplet on top of the inlet shrinks (**Figure 4b, ii**: 1 s after addition; **iv**: 2 s after addition), which results in a flow towards the outlet. This continues until the droplet meniscus is pinned by the inlet (**Figure 4b, iv**). Scale bar = 400 µm.

Figure 5: Robust 3D sprouting of iPSC-EC microvessels. (**a**) Sprouting of iPSC-EC over time. Microvessels were grown for 48 h (right) and then stimulated with an angiogenic cocktail

containing 50 ng/mL VEGF, 500 nM S1P, and 2 ng/mL PMA. The first tip-cells that invade the collagen-1 scaffold (middle) are visible 24 h after exposure. The first lumens are visible (arrows) 48 h after exposure (right) while the tip-cells have migrated further in the direction of the gradient. (**b**) Array of 15 microvessels that were stimulated with VEGF, S1P and PMA for 2 days and stained for F-actin (yellow) and nuclei (blue). Scale bar = 200 µm. (**c**) Stimulated microvessel (positive control). (**d**) Unstimulated microvessel (negative control). (**e**) The maximum projection of a single capillary within the gel. (**f**) Same as (**g**) but focused on the middle. Dotted line indicates the position of the orthogonal view in panel g. Scale bars (a-d: 200 µm; e-g: 20 µm).

Figure 6: Visualization of angiogenic sprout permeability before and after anastomosis. (**a**) Anastomosis with basal channel triggers pruning and maturation of angiogenic sprouts. Closeup of capillary bed at 2, 4, 6 and 7 days after stimulation with angiogenic growth factors. (**b**) Angiogenic sprouts after 2 days after addition of angiogenic growth factors. Angiogenic sprouts are formed within gel, but are not yet connected to the bottom perfusion channel. (**c**) Perfusion of the microvessel with 0.5 mg/mL albumin-Alexa 555 solution. Fluorescent images obtained at 0 and 10 min. (**d,e**) Same as in panels b and c, but after 7 days of stimulation. Sprouts are connected to the other side and formed a confluent microvessel in the basal perfusion channel. Scale bars = 100 µm.

Table 1: Troubleshooting common errors.

DISCUSSION:

This method describes the culture of 40 perfusable endothelial microvessels within a robust and scalable microfluidic cell culture platform. Compared to traditional 2D and 3D cell culture methods, this method shows how a physiological relevant cellular microenvironment that includes gradients and continuous perfusion can be combined with 3D cell culture with adequate throughput for screening purposes.

One of the major advantages over comparable microfluidic assays is that this method does not rely on pumps for perfusion but uses a rocker platform to induce continuous perfusion in all microfluidic units simultaneously. This ensures that the assay is robust and scalable: plates can be stacked on a rocker platform. Importantly, all microfluidic units remain individually addressable, which allows this method to be implemented within drug screening including the generation of a dose-response curve. Furthermore, without a pump, imaging and medium replacement is far simpler with less risk of (cross)-contamination.

Another advantage of this method is usage of a standardized, pre-manufactured platform, while comparable microfluidic cell culture platforms need to be fabricated by the end-users. This availability facilitates the adoption of this assay among other academic and pharmaceutical research groups, leading to standardization. Also, unlike microfluidic prototypes, the 384-well plate interface ensures compatibility with the current lab equipment (e.g., aspirators, plate handlers and multichannel pipettes), facilitating the integration within the current screening infrastructure.

There are several critical steps in performing this assay. The collagen-1 gel should completely fill the gel channel. During gel loading, this filling can be observed by inspecting the microfluidic channels either through the observation window (**Figure 2a**) or by flipping the plate upside down (as shown in **Figure 1a**). While filling, the collagen gel should remain in the center channel, without flowing into adjacent perfusion channels. We noticed that the quality of the collagen-1 gel is crucial for proper assay performance. Collagen-1 batches with too high viscosity will lead to incomplete filling of the gel channel. After 10 min of polymerization at 37 °C, the gel should be homogenous and clear. If collagen-1 is not stored properly (e.g., due to fluctuating temperatures in the fridge), collagen will polymerize within the channels with clearly visible fiber formation. This can result in invasion of the ECs into the gel without addition of angiogenic factors, but without proper lumen development.

When the cells are seeded, the fibronectin coating solution is removed from the wells, leaving only the microfluidic channels filled with coating solution. Aspiration of the coating solution from microfluidic channels could cause gel disruption or gel aspiration. The cell suspension needs to replace/displace this coating solution. This works best when the cell suspension is seeded using the passive pumping method, as directly pipetting the cell suspension into the channels show less reproducible seeding densities.

As the microvessels form a stable monolayer against the gel, these small differences only result in different times needed for reaching confluency. Thus, the assay start point is determined by confluency rather than culture time. If necessary, the culturing time can be extended until a clear monolayer has been formed against the gel.

Within the wells, air bubbles can be trapped by incorrect filling of the wells (see **Figure 2b**). These air bubbles will restrict the flow of medium, even when the device is placed on a rocker platform, and result in collapse of the microvessel and improper gradient formation. Pressing the pipette tip against the side wall of the wells will increase the success of completely filling the well. If an air bubble is trapped within the wells, it can be removed by gently inserting a sterile pipette tip into the glass bottom. Air bubbles can also occur within the microfluidic channels. When medium has been removed from the wells, evaporation of medium is noticeable from the microfluidic channels after 30 min (due to the microliter volumes within the channels). Thus, medium changes are preferably performed as quick as possible. When medium is added in a channel with evaporated medium, air bubbles will be trapped within the microfluidic channels. These air bubbles within the microfluidic channels can be removed manually by placing a P20 pipette directly on either the inlet or outlet and forcing medium through the microfluidics from the opposite well. Successful removal of the air bubbles results in a small but noticeable decrease in volume in the other well. **Table 1** lists common errors and how to troubleshoot them.

The lack of a pump is a limitation when continuous imaging is required, as the rocker platform limits the user to image at sequential time intervals. Furthermore, the perfusion of medium in this platform consists of bi-directional flow with low levels of shear stress, while vasculature in vivo is exposed to unidirectional flow with higher levels of shear stress. While we do not observe negative effects of the bi-directional flow with regards to the angiogenic sprouting, flow is an

important biomechanical stimulus and preferably controlled. However, while there are commercially available pump setups, interfacing with the 384-well plate remains challenging and pump setups severely hamper the scalability of this assay.

The possibility to use iPSC-ECs to study angiogenic sprouting opens up new opportunities in disease modeling and drug research. In contrast to primary ECs, these cells can be generated in nearly limitless quantities with a stable genotype and by using genome editing techniques, cells can be generated that including gene knockouts and knock-ins. However, as the protocols to differentiate ECs from iPSC are relatively new, it is still unclear what leads to iPSC-ECs that best reflect primary ECs and which subtypes of EC are or can be generated. Also, there are still remaining questions regarding their relevancy. For example, do iPSC-ECs still exhibit the plasticity that is typical for endothelial cells? And to what degree do iPSC-derived cells respond to and interact with their cellular microenvironment? The standardized platform presented here could be used to answer some of these questions in order to further validate the usage of iPSC-derived ECs in vitro.

The most straight-forward future direction for this assay will be the integration of other cell types that play an important role during angiogenesis, such as pericytes and macrophages. This will facilitate the ability to study the role of macrophages during anastomosis between sprouts or the adherence of pericytes after capillary formation. Also, it is possible to culture various other cell types within or against an extracellular matrix (e.g., we have shown the culture of neurons and various epithelial structures such as proximal tubules and small intestines), which can be combined with the vascular beds generated using this method. Finally, it will be interesting to study angiogenic sprouting in synthetic hydrogels, as their defined composition further increases the standardization of the assay and allows tuning of stiffness and binding motives that affect cell-matrix interactions.

In conclusion, this method shows the feasibility of iPSC-derived ECs in a standardized and scalable 3D angiogenic assay that combines physiological relevant culture conditions in a platform that has the required robustness and scalability to be integrated within the drug screening infrastructure.

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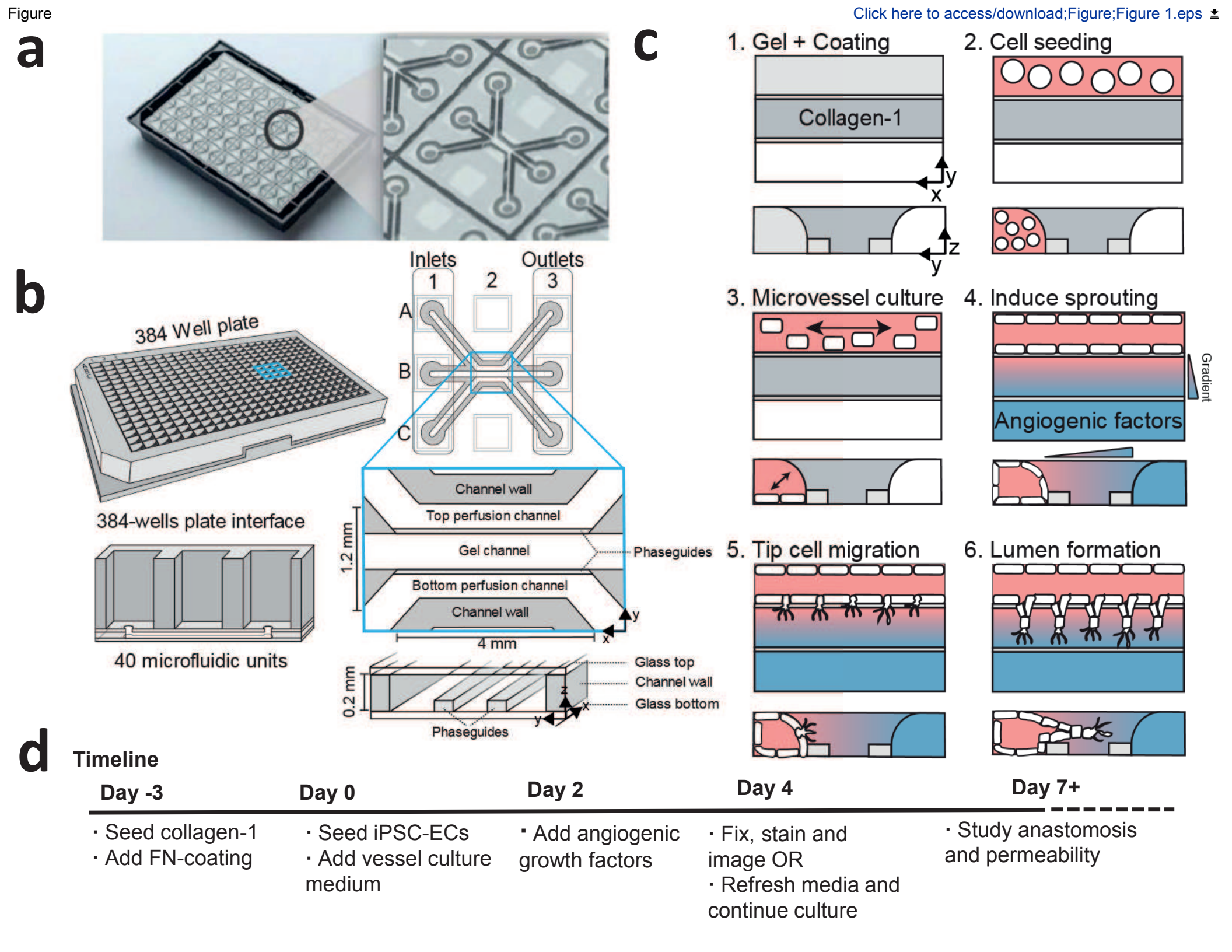
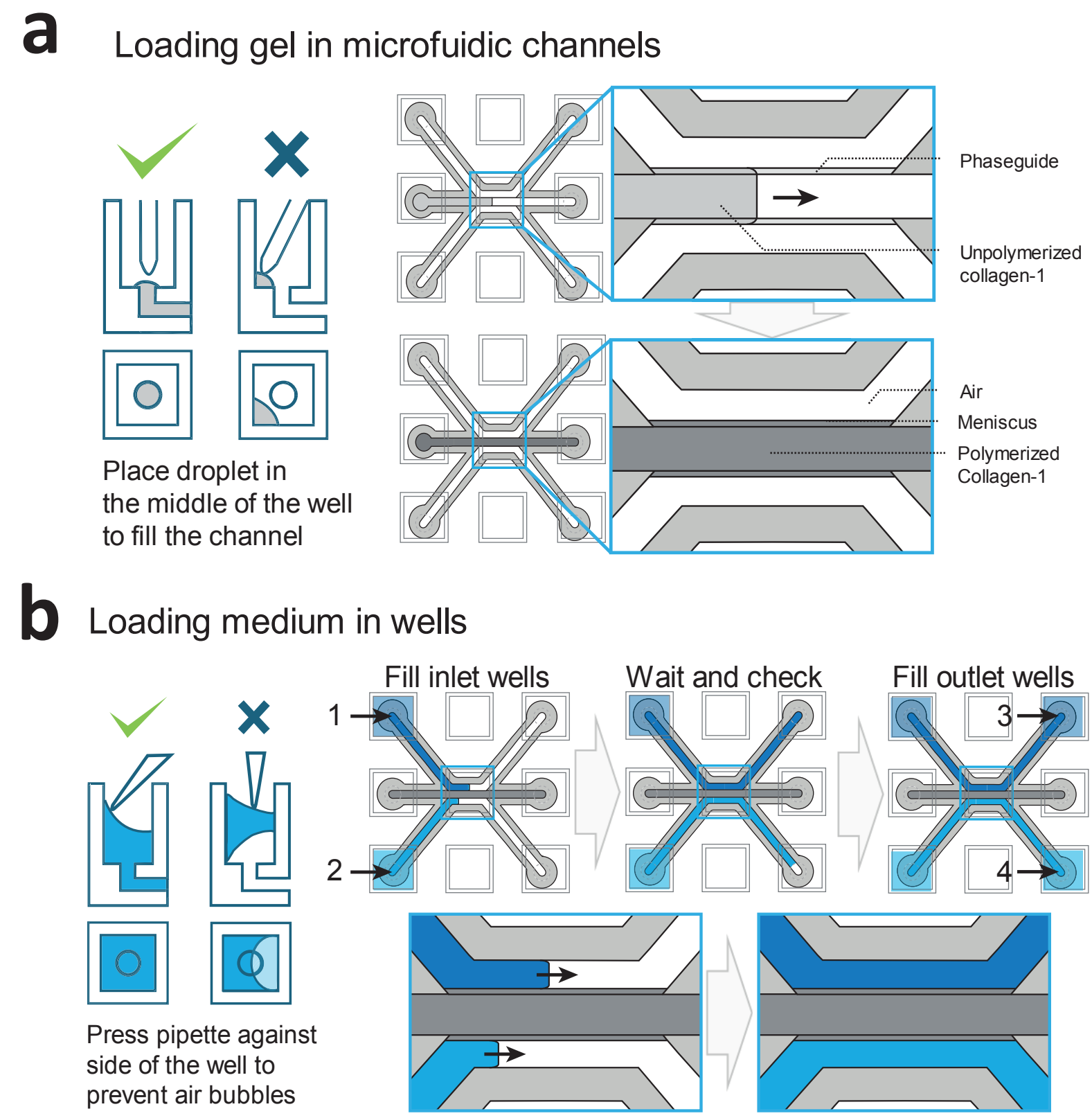
Figure

Click here to access/download;Figure;Figure 1.eps





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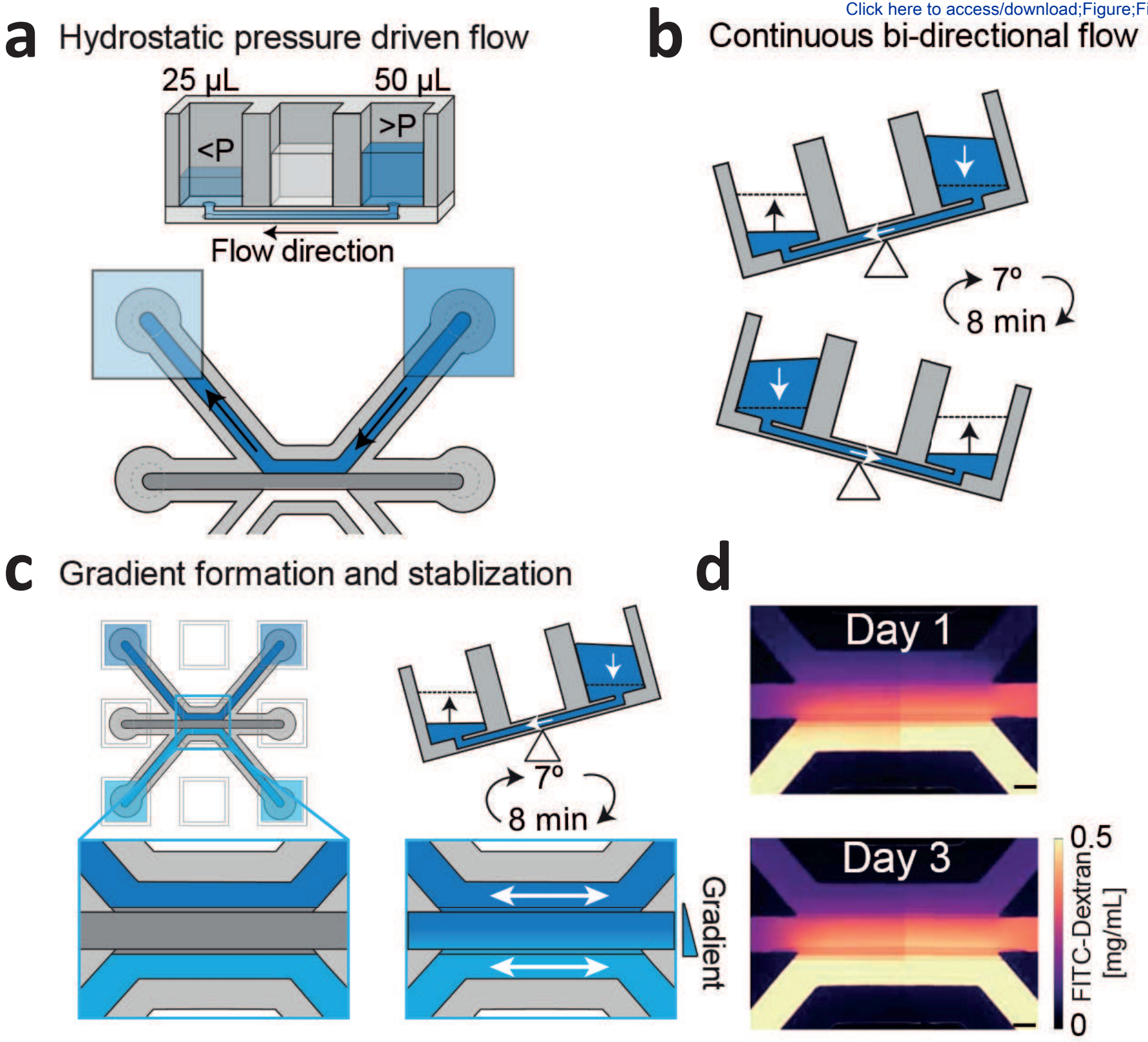

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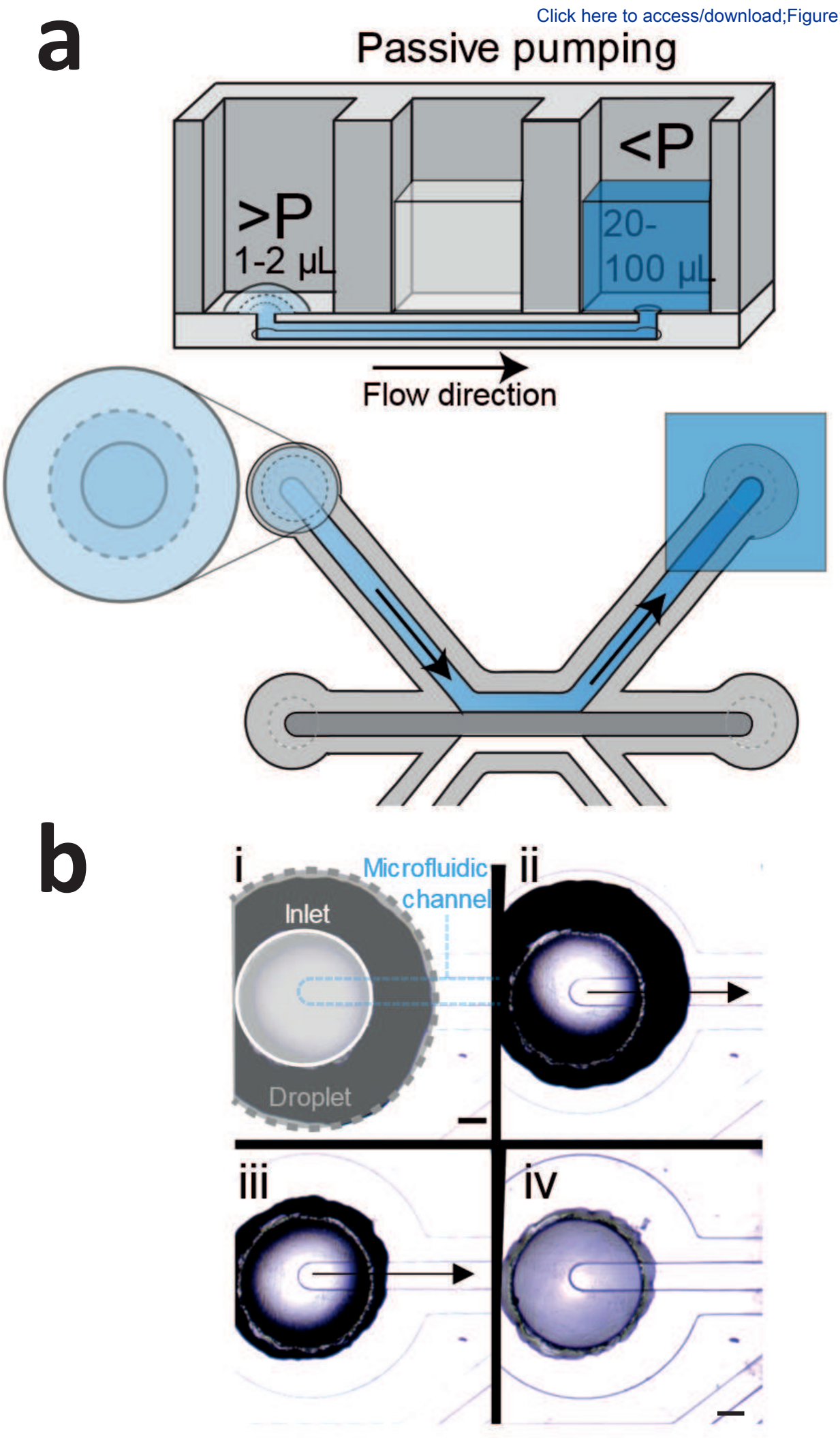

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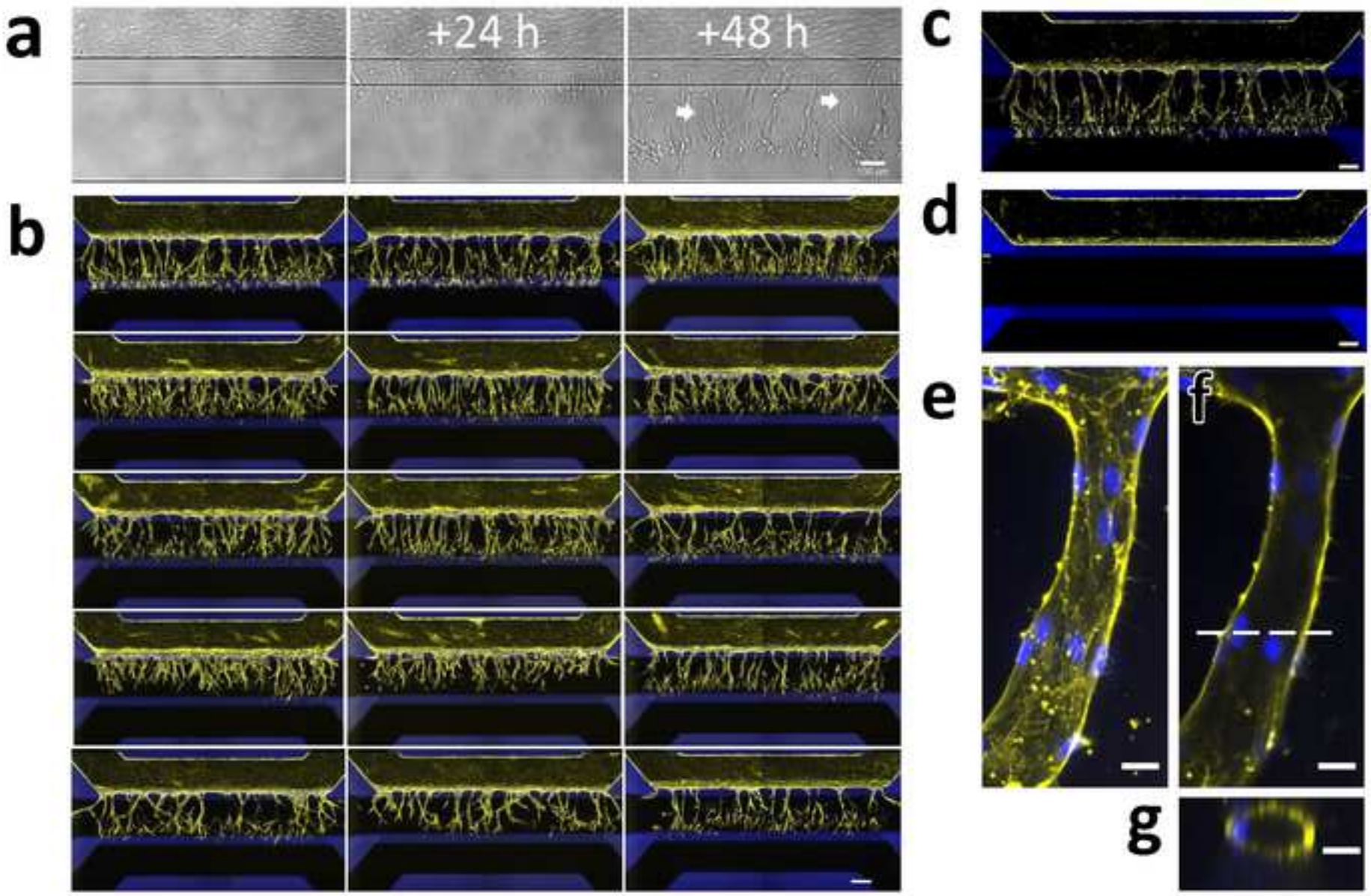

Figure

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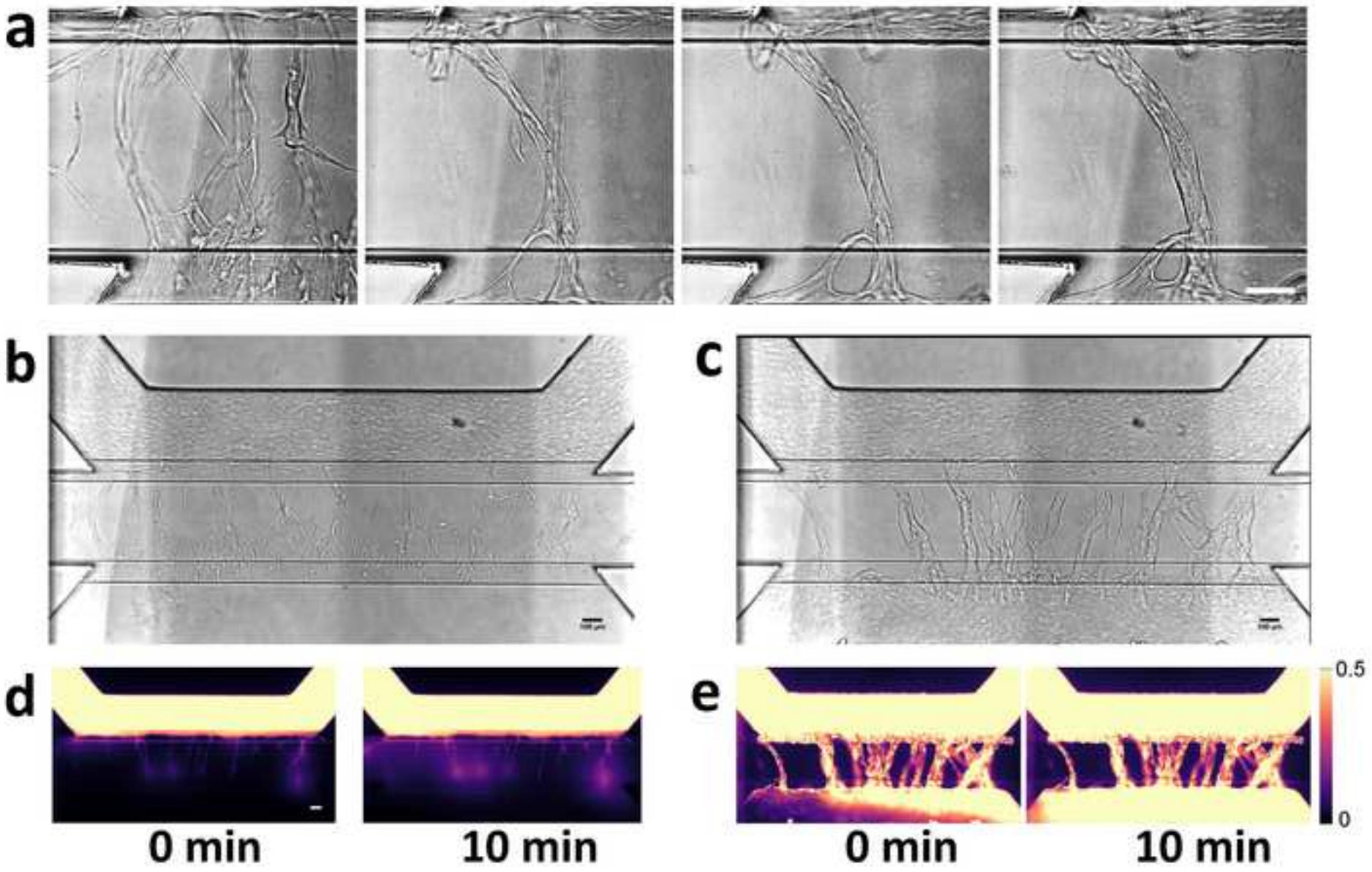

Problem	Cause
Collagen-1 does not enter or fill the channel completely	Collagen-1 droplet is not placed on top of the inlet
	Volume of collagen-1 is too low
	Collagen-1 is too viscous
Collagen-1 flows into perfusion channels	Collagen-1 is pipetted directly into the inlet of gel channel
Collagen-1 is not clear/fiber formation	Collagen-1 is not stored properly
	$NaHCO_3$ and HEPES are not mixed well before adding collagen-1
Droplet does not shrink using passive pumping method	Droplet adheres to side of the well
No sprouting is observed	Growth factors are not added or aliquots are not stored properly
	Air bubble blocks perfusion/ gradient formation
	Volume differences between wells
Cells not viable	Plate not placed on rocker platform/rocker platform turned off
	No perfusion possible due to presence of air bubbles
No lumens are formed, cells migrate as single cells	Angiogenic sprouting mixture was added before a monolayer was formed
Major variation in sprouting density	Differences in cell densities after seeding

Solution
Carefully place the droplet on top of the inlet from the gel channel
Use 1.5 µL of the gel to fill the channel completely
Use another batch of collagen-1
Carefully place the droplet on top of the inlet from the gel channel
Store collagen-1 at 4 °C, do not freeze
Carefully mix the $NaHCO_3$ and HEPES by pipetting before adding the collagen-1
Aspirate droplet and add new droplet on top of the inlet
Make sure the outlet well is filled with at least 20 µL of medium
Prepare fresh angiogenic sprouting medium
Remove air bubbles using a P20 or P200 pipette
Volumes in all wells need to be equal in order to form a linear gradient
Make sure rocker platform is on and has the right cycle time/angle (8 min/7°)
Remove air bubbles using a P20 or P200 pipette
Wait an additional 24 h before adding the angiogenic growth factors
Check if cell density is homogenous and comparable between microfluidic units. Add another droplet of cell suspension if necessary

Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
0.2-10 µL Electronic repeater pipette	Sigma	Z654566-1EA	
1 M HEPES	Gibco	15630-056	
3-lane OrganoPlate	Mimetas	4003-400B	
4% PFA in PBS	Alfa Aesar	J61899	
Basal culture medium	NCardia		
Collagen-1	Cultrex	3447-020-01	
Combitips Advanced Biopur 2.5 mL	VWR	613-2071	
dPBS	Gibco	14190-094	
Eppendorf Repeater M4 pieptte	VWR	613-2890	
Fibronectin	Sigma-Aldrich	F4759-1MG	1 mg/mL in milliQ water
HBSS	Gibco	14025-050	
Hoechst	Molecular Probes	H3569	10 mg/mL solution in water
iPSC-derived endothelial cells	NCardia		
$NaHCO_3$	Sigma	S5761	37 g/L in milliQ water
OrganoPlate Perfusion Rocker Mini	Mimetas		Rocker platform used to provide flow
Pen/Strep	Sigma	P4333	
Phalloidin	Sigma	P1951	1 mg/mL in DMSO
PMA	Focus-Biomolecules	10-2165	2 µg/mL in PBS containing 0.1% DMSO
S1P	Ecehelon Biosciences	S-2000	1 mM in methanol containing 1% acetic a
TRITC-albumin	Invitrogen	A34786	
VEGF	Peprotech	450-32-10	50 µg/mL in water containing 0.1% BSA

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Title of Article:	Standardized method to study 3D angiogenic sprouting of perfused hiPSC-derived endothelial microvessels in vitro
Author(s):	V. van Duinen, W. Stam, V. Borgdorff, A. Reijerkerk, V. Orlova, P. Vulto, T. Hankemeier and A.J. van Zonneveld

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Dear Editor,

We thank you and the reviewers for the time and effort that they have spent on our manuscript. We found the comments and suggestions very helpful and have revised the manuscript accordingly. Please, find our responses to each comment below.

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3. Figure 2: Please change the time unit “hr” to “h”.

Done

4. Title: Please avoid the use of abbreviations.

We changed the title from

“Standardized method to study 3D angiogenic sprouting of perfused hiPSC-derived endothelial microvessels in vitro”

to

“Standardized and scalable assay to study perfused 3D angiogenic sprouting of iPSC-derived endothelial cells in vitro”,

as the abbreviation iPSC-derived endothelial cells is, in our opinion, unambiguous in the field of biomedical research.

5. Affiliations: Please provide an email address for each author.

See email addressed below. How will this be published in the article as there is usually only a single corresponding author?

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6. Summary: Please shorten it to no more than 50 words.

Done

7. Please define all abbreviations before use.

Done

8. Please add more details 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. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

We added more details throughout the protocol.

9. Please reference Figure 1 in relevant steps so the readers know where to add different solutions/media.

10. 1.1: How many wells are in the plate?

Added in the figure caption and in the text

11. 1.2: How many observation wells?

Added in the figure caption and in the text

12. 1.3: Is the plate kept in a sterile cell culture cabinet? For how long?

We added the following note:

“Note: the protocol can be paused here for up to 24 h. Leave the plate in the sterile culture cabinet at room temperature”.

13. 2.1: Please describe how to mix well, pipetting or vortexing?

2.1 changed to 2.2: Added ‘mix by pipetting’ in this step

14. 2.3: What volume of coating solution is needed? What is dPBS?

Added step 2.1 in which the readers prepares the FN-stock solution first, as this needs to be placed in a water bath.

“2.1 Prepare 2.5 mL of a 10 µg/mL fibronectin (FN) coating solution. Dilute 25 µL a 1 mg/mL fibronectin stock solution in 2.5 mL dulbecco’s PBS (dPBS, calcium and magnesium free). Place in the waterbath at 37 ℃ till use.”

15. 2.4: The top channel inlet and outlet of what? Please clarify. Referencing Figure 1 here may be helpful.

Added references to the corresponding well numbers shown in figure 1.

16. 2.5: What device?

Changed to ‘plate’, as we refer to the microfluidic microtiterplate in this case.

17. 3.8: Please specify the incubation temperature.

Added (37 ℃, 5% CO_2) to all the incubation steps done in the incubator

18. 3.9: What volume of medium is added? Please specify.

Added

19. 3.10: What are the temperature and rocking speed?

Added

20. 3.11: What is used to image?

Added the type of microscope and imaging settings

21. 4.5: How to refresh the growth factors?

Added the following sentence: “by aspirating all culture media from the wells and adding fresh medium including growth factors.”

22. 5.1: How to test the permeability?

Removed this step (as this is just a repetition of the title of this part), and start with the first step to perform this part of the experiment.

23. Please remove all commercial language from your manuscript and use generic terms instead: Repeater, Ncardia,

Changed

24. Representative Results: The paragraph text should refer to all of the figures. However for figures showing the experimental set-up, please reference them in the Protocol.

Done

25. Please reference all the figures in the manuscript text.

Done

26. References: Please do not abbreviate journal titles.

Changed references to Jove style format without abbreviations

27. Table of Materials: Please use SI abbreviations for all units (L, mL, µL). Please remove trademark (™) and registered (®) symbols. Please use the period symbol (.) for the decimal separator. Please sort the items in alphabetical order according to the name of material/equipment.

Done

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We are aware of this, and we do discuss the limitations of the products in the discussion section. We hope this is sufficient to give the reader and viewer a unbiased impression of the method and we would like to stress that the aim of this work is the fact that we see this work as a unique combination of using a standardized cell source in a standardized cell culture platform, without focusing on the commercial availability. To avoid any confusion, we changed the following sentences:

"Both the platform as well as the endothelial cells differentiated from human induced pluripotent stem cells are commercially available, which enables the adoption across research groups and accelerates its implementation within drug research."

To:

"The possibility to use iPSC-ECs in a standardized microfluidic cell culture platform will enable the adoption across research groups and accelerates its implementation within drug research."

Reviewers' comments:

Reviewer #1:

Standardized method to study 3D angiogenic sprouting of perfused hiPSC-derived endothelial microvessels in vitro

Van Duinen et al. present a protocol for high-throughput monitoring of angiogenesis from hiPSC-EC derived microvessels into a collagen 1 matrix triggered by a cocktail of angiogenic factors. The protocol is described in the appropriate depth to execute the experiments. Although, the described system is directed into a more physiological setting than other systems, it is still far away from processes occurring in the body. The system is mainly challenged by the bi-directional flow and the absence of other cell types e.g. pericytes in the culture. The authors mention these limitations in the discussion, but should therefore also phrase their statements in the summary more carefully. Moreover, the intervention with an inhibitor of angiogenesis as a proof of concept study would add more meaning to the presented protocol.

We appreciate the reviewer's very important comment about the limitations of this system. In comparison with other widely used angiogenic sprouting models in vitro (e.g. fibrin bead assay, tube formation assay), we conclude that this model offer increased physiological relevance by the integration of gradients and flow. Nonetheless, like any in vitro system, this is of course an simplification of processes that occur in vivo. We understand that the summary might give the impression that this system is used to study the effect of flow, while this is not the case. Thus, we changed "under physiological relevant culture conditions including the presence of gradients of growth factors and application of perfusion flow", to "gradient-driven angiogenic sprouting in 3D, including anastomosis and stabilization of the angiogenic sprouts", as this, in our opinion, better reflects the unique advantages of this assay compared to other in vitro assays.

Points which need to be addressed are the following:

Title:
Actually, the connection between hiPSC-derived endothelial cells that then give raise to microvessels is a bit unclear in the title and needs clarification.

We thank the reviewer for this suggestion and changed 'microvessels' to 'endothelial cells', thus the title now is: "Standardized and scalable assay to study perfused 3D angiogenic sprouting of iPSC-

derived endothelial cells in vitro”

Introduction
Page2, line 88: The authors have to add here, that the iPSC-derived EC also have drawbacks (reproducibility of differentiation from iPSC; iPSC-EC have an endothelial phenotype, but it is not known how comparable this is to native endothelial cells and to which type of).

We agree that this are the current questions that need to be answered with regards to using cells that are derived from iPSCs. We feel that only the rationale to use iPSC-ECs should be mentioned in the introduction, while the issues and drawbacks should be discussed in the discussion section of the paper. Thus, we added a paragraph of iPSC-EC to discuss these limitations. Although we think that this method will aid a better understanding of iPSC-ECs, the scope of this method is the generation of angiogenic sprouts from iPSC-ECs.

The section we added in the discussion:

“The possibility to differentiate ECs from iPSCs in nearly limitless quantities, these cells will be a valuable addition to study angiogenesis in a standardized platform using a standardized cell source. However, as the possibility to differentiate cells from iPSC is a relatively new discovery, it is still unclear to what extent these cells reflect primary ECs. For example, do iPSC-ECs still exhibit the plasticity that is typical for endothelial cells? And to what degree do iPSC-derived cells respond and interact to their cellular microenvironment? This platform could be used to answer some of those remaining questions in order to further validate the usage of iPSC-derived ECs in such *in vitro* models”

Protocol
2.4: Please label top channel inlet and outlet in the corresponding figure.

We have added the well nomenclature as described in Fig 1b throughout the protocol to help the readers well we refer to. (Well ‘A1, C1, etc. etc.)

3.4: Please explain what means 2E7 cells/mL.

We changed this to 2×10^7 (scientific notation)

3.6: Please label top channel inlet and outlet in the corresponding figure.

We have added this to Fig 1b

3.9: TI and TO are introduced, but are not shown in the figure. Is it the same as top channel inlet? Please clarify.

We understand that TI-TO could cause confusion (as Top in this case refers to a channel that is adjacent, not above the gel channel), thus we removed the TI-TO references and changed this to refer to the well positions. (Well ‘A1, C1, etc. etc.)

3.10: The rocker platform is set to which speed? Please add.

Added to the description

4.1: Please introduce the abbreviations PMA and S1P.

Included

5.4: The additional information can be put in a normal sentence without brackets.

Done

6.3: Please introduce the abbreviation HBSS.

Done

Representative Results
First of all, it would be necessary to show the reproducibility of the method also by measurements of cord number, number of sprouts, and thickness of the sprouts throughout several experiments on one plate.

We agree with the referee that the reproducibility of this assay is important. However, we feel that quantification of the sprouting is outside of the scope of this manuscript, and we have described this in our previously published manuscript for the readers interested in the quantification. Furthermore, we demonstrate a low variability between different wells within the plate (Fig 2b).

What it actually the final thickness of the gel? How 3D is the setup? As no side views or stacks are presented, an estimation of the final height is not possible. Please add this information. Moreover, it is not really explained how the Phaseguides work, what is quite important to understand the system. You can find the information on the homepage of the company but for better understanding this information needs to be added. It is also not clear, if the system is open on top or if there is a cover in the area of the channels? Please add the information.

We understand the reviewers concerns regarding the lack of information about the geometry and design, and agree that phaseguides are an important and unique part of the system. We included more details about the height and system in Figure 1b and would like to include an 3D animation in our final video to clearly illustrate the geometry of the device. Also, we have added a figure 2a to illustrate how phaseguides assist in gel stabilization/patterning and show examples of correct and incorrect gel loading

Page 6, line 208: How fast is the reduction of sprouts? How does the vessel system look like at a later time point? Please provide pictures!

We included more pictures and timepoints to illustrate the retraction of the vessels in Figure 3.

Figure 1
page 6, line 211: Must read hiPSC-EC-derived microvessels
page 6, line 212: Must read: …..cell culture device is shown displaying…..
b: The picture with the wells is a bit confusing. Should this show that the microfluidic device is just in the bottom of the plate? Please clarify!

Indeed this part of the illustration is to show that the microfluidics is integrated underneath the 384-wells plate and we added this to the caption for Fig 1a: “The bottom of the microfluidic cell culture device is shown displaying the 40 microfluidic units that are integrated underneath the 384-well plate”

c: subfigure 3: Shouldn't the ECs cover the whole vessel as in subfigure 4? Or should this demonstrate the growth phase of the ECs? Please clarify.

This indeed demonstrates the growth phase of ECs, as they attach to the bottom first, before they grow at the sides/top of the channel to form a complete microvessel. We added an explanation of this growth phase in the representative results

Figure 2
a: Add scale bar information even when it is depicted in the picture. In the picture, it is hard to read.
b: Page 6, line 222:"….first lumen are visible…." How can you see this from 2D pictures? Please explain. In addition, it would be better to add a frame to each of the 15 pictures for better orientation.

We have added a border to the montage of 15 images to help the reader in discriminating the different images. In our experience, lumen formation is observed as two close straight parallel lines (cell borders). Without a lumen, the cell bodies are visible as a single straight lines. We have added arrows to show an example of these lumen.

c: Scale bar information missing. Please add.

Added

Figure 3
a: PMA missing in the angiogenic cocktail? Please check.

We are aware that the growth factor combination is different than the data in the previously shown pictures. PMA is included in the protocol as this promotes the lumen formation of primary ECs, but we have found that this is not required for the sprouting of iPSC-EC. However, our previous work with primary ECs did require PMA for robust sprouting, so for compatibility reasons, we included PMA in the protocol. We have updated the figure legend to avoid confusion.

b, d: I assume that i and ii represent 0 and 10 min. Please add this information.

We have included information about these time points in the figure

d: Scale bar not visible. Please add.

We have included the scale bar in the figure

As the images are acquired by confocal microscopy it should be possible to provide stacks to demonstrate the presence of TRITC-albumin in the lumen, also with a co-staining for ECs. Please provide this information.

Yes, it is indeed possible to acquire Z-stacks, but we preferred not to do this due to phototoxicity issues. Thus, the images shown are wide-field images. We also changed the protocol accordingly, as any widefield fluorescent microscope with automated stage is sufficient for imaging.

The authors mentioned that the vessel system starts to retract in prolonged cultures. Is 7 days (c) such a long culture already? Please explain.

In this case, prolonged refers to additional culture time after the onset of angiogenesis. While 4 days is sufficient to study angiogenesis, the culture time extended to study the effect of perfusion on the capillary network. We rephrased this to extended culture.

Critical steps
Cell Seeding

Page 3 line 287: How long can the culture of ECs be extended in the microfluidic device without stimulus? Please add.

The amount of growth culture media present in the wells in sufficient to culture the cells for up to 3 days without media refreshment. Generally, 2 days is already sufficient for a confluent monolayer against the gel, but thus the culture time can be extended for another 24 h if not. We specified this 24 h period in the text.

Reviewer #2:

Using the commercially available MIMETAS product and combining iPSC cells from NCardia, the authors developed a high-throughput angiogenic sprouting experimental platform. Since this paper is very similar in scope and content to a recently published paper in Angiogenesis, it will need major revisions to makes sure it is significantly different from previously published work.

We thank the reviewer for his/her helpful considerations and comments about our manuscript. We are aware that this paper is similar to our previously published work. However, as the scope of Jove is detailed videos of already published methods, we disagree with the reviewer that we need to change the manuscript in order to make this significantly different from previously published work.

* It would be helpful to have the total amount of time needed to obtain the vessels including loading the gel-waiting and loading iPSCs.

We have included a time schedule in figure 1

* The vessels seem rather large with diameters close to or larger than 100 um, which do not seem to qualify as microvessels.

In this case, the term 'microvessels' refers to sub-millimeter vasculature. The smaller microvessels that form by angiogenic sprouting, we refer to as 'capillaries' or 'angiogenic sprouts'.

* The factors used, S1P and PMA are potent angiogenesis inducing factors and are not very physiological-need explanation for using these at the concentrations indicated. The effect of each factors separately at different concentrations would be helpful for the readers who want to replicate the experiment and so they can expect different morphology-yield-diameter of the vessel and etc.

We agree that PMA is not very physiologically relevant, and as mentioned before. We used this platform to optimize the growth factor combinations, and concluded that in the case of iPSC-EC sprouting, PMA is not required per-se. However, our previous work did require this, so for compatibility reasons, we included PMA in the protocol. We did not go into detail in the effect of different growth factors, as this is, in our opinion, beyond the scope of this manuscript.

S1P, on the other hand is a physiological relevant angiogenic inducing factor. In vivo studies of angiogenic sprouting in the mouse cornea showed that S1P inhibition prevents angiogenesis. Also, S1P is produced and present in all sorts of angiogenic sprouting, including wound healing where S1P is released by platelets.

* A confocal micrograph that show the lumen in 3D would be helpful.

We have shown this in our previously published work that the capillaries form lumen. We have added a cross-sectional view of a single capillary (Figure 5g)

* What is the limit of imaging the vessels and cells in this device? The channels are 200 um thick and it could be imaged at low magnification only.

As the bottom glass has a thickness of ±170 µm, we regularly use 10x and 20x lenses, which have a sufficient working distance for imaging these cultures, and these lenses have an sufficient field of view to image the complete gel region with sprouts using 2 or 4 sites. The thickness of the glass also enables the use of 60x lenses, but the very limited working distance has limited use in these devices.

* When working with microfluidic devices, bubbles are big problems for practical implementation, the author has mentioned at the end of the article how it could be removed manually. More detailed explanations and or experimental suggestions to remove them would be very helpful.

Indeed, bubble formation is an issue in many microfluidic devices, but this can be prevented with a right operating procedure. We have added an illustration in how to correctly fill the wells to prevent bubble formation and an illustration of the filling order that reduces the risk of air bubble trapping.

Also, we modified the protocol for cell seeding to prevent evaporation when seeding cells to also prevent evaporation of the low volumes of cell medium. The old protocol used 2 µL of cell suspension, which showed clear signs of evaporation after 1 hr. However, with the use of the passive pumping method, there is always a surplus of ±25 µL of medium present, which prevents evaporation issues.

* Could you visualize how long the gradient of angiogenic factor was maintained? More data is needed.

Yes, using fluorescently labeled dextrans this is possible. We have added this to figure 3 to demonstrate the gradient stability over time.

* Necessary to consider whether cell seeding is properly uniform. Is there any variations in seeding uniformity depending on the users and positions?

There are variations in cell density, especially without using passive pumping method to seed the cells. Using passive pumping solves this issue, as the cells seeding is more reliable and less dependent on pipette position and displacement volume. We do not see a clear effect on the position in the plate itself (e.g. no edge effects in the plate)

* Rocker protocol does not specify the orientation of the plate placement (it would be helpful to describe the rocker settings, i.e. cycle angle, time, etc).

We have added this information in the protocol

* The vascular sprouts in figure 2b seems to have similar length but having different density. Please explain reason for this and provide some strategies to generate constant morphology of vascular sprouts

Density differences are mainly caused by two factors: 1. either regression just started to occur as some sprouts have anastomosed with the opposite channel or 2. Seeding densities are different in the start, leading to different sprout densities later on. This is solved by using the passive pumping method for cell seeding. We added these explanations in the “Critical steps and troubleshooting” parts of the manuscript.

* In figure 3(c), did the ECs escape from the collagen matrix to bottom channel? Could you provide day by day images since ECs reach to the opposite end of collagen to make permeable vessel?

Yes, ECs did escape from the collagen. We have added more day by day images to clearly illustrate this.

* Figure 2b, additional explanation is needed
* Figure 2c, adjust image size
* Figure 3b, difficult to tell. Additional information need (e.g., time scale…)

Added

* Typos:
line 129, 133 (etc…) Pen/Step ◊ Pen/Strep for penicillin streptomycin
line 60 pre-clinal ◊ pre-clinical
line 32 amendable ◊ amenable

Fixed