Microvasculature in Microfluidics: matching complexity with compatibility

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CHAPTER

5

A standardized and scalable angiogenesis assay using iPSC-derived endothelial cells

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Abstract

Pre-clinical drug research towards vascular diseases requires *in vitro* models of vasculature which 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 and scalability. Thus, there is a need for physiologically relevant *in vitro* models which can be integrated in the existing high-throughput screening infrastructure.

We demonstrate a robust platform to culture 40 3D microvessels under perfusion and against a 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 angiogenesis. Importantly, this assay is compatible with primary endothelial cells (ECs) as well as ECs derived from human induced pluripotent stem cells (iPSC-ECs). Finally, as the platform is suitable for high-content imaging, it is possible to automatically quantify the sprout permeability and sprout length.

In conclusion, the method presented here is suitable to study angiogenesis in a physiological relevant cellular microenvironment, including perfusion and gradients. The platform is compatible with automated microscopes, and has the required robustness and scalability to be integrated within the drug screening infrastructure. Both the platform as well as the endothelial cells differentiated from iPSC are commercially available, which enables the adoption across research groups and accelerates its implementation within drug research.

Introduction

It is now well recognized that angiogenesis, the growth of new blood vessels from pre-existing vasculature, plays a fundamental role in both health and disease[1]. For the discovery of new drugs targets of the microvasculature, research relies heavily on *in vitro* models, due to their unparalleled level of experimental control. However, it is still common practice to study endothelial cells (ECs) in culture systems that have limited physiological relevance[2]. To meet the demands of pre-clinical vascular drug research, improved *in vitro* models of vasculature are required that are amenable to high-throughput screening, with a scalable and robust cell source in a physiologically relevant 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 like tissue-culture plastics, endothelial cells can be embedded in three-dimensional scaffolds, such as fibrin and collagen gels[3]. 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 in the homeostasis of vasculature. Also, the possibility to spatially control fluids within microfluidics allows the formation of biomolecular gradients[4-7]. Such gradients play an important role *in vivo* during the formation and patterning during angiogenesis.

However, while many microfluidic platforms show unparalleled physiological relevancy over traditional 2D and 3D cell culture methods, they often lack the required throughput and scalability[8]. Also, many of these platforms are not commercially available and require the end-users to microfabricate their devices prior to use[9]. This not only requires manufacturing apparatus and technical knowledge, it also limits

the level of quality control and negatively affects the reproducibility[10].

To date, primary human endothelial cells remain the most widely used cell source to model angiogenesis *in vitro*[11]. 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, like other primary cells, endothelial cells generally lose relevant properties when cultured *in vitro*[12,13]. Furthermore, such cells intrinsically suffer from genomic differences and batch-to-batch variations.

Endothelial cells derived from human induced pluripotent stem cells (iPSC) are a promising alternative: they resemble primary cells, but with a more stable genotype which is also amenable to precise genetic editing. Furthermore, as 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[14].

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. This ensures robust operation and increases the scalability of the platform. 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. Although the protocol is compatible with many different sources of (primary) endothelial cells[15,16], we focused on using commercially available human iPSC-derived ECs in order increase the standardization of this assay.

Protocol

Device preparation

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

1.2 Take of the lid and add 50 µL of water or PBS in every of the 40 observation wells (Fig 1b well 'B2') using a multichannel or repeater pipette.

Note: the protocol can be paused here. Leave the plate in the sterile culture cabinet at room temperature.

Prepare gel and coating

1.3 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.

1.4 Prepare a 100 µL collagen-1 solution: add 10 µL HEPES (1M) to 10 µL $NaHCO_3$ (37 g/L) and mix by pipetting. Place the tube on ice and add 80 µL 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.

1.5 Add 1.5 µL of collagen-1 solution (4 mg/mL) to every gel inlet (Fig 1b, well 'B1'). Make sure the droplet of gel should is placed in the middle of the well in order for the gel to enter the channel (see Fig 2a).

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

1.6 Place the microfluidic plate in an incubator (37 ℃, 5% CO_2) for 10 minutes to polymerize the collagen-1.

Note: timing of the polymerization is crucial: due the low volumes used in mi-

crofluidics, evaporation can already be observed after 15 minutes of incubation, which results in gel collapse or shrinkage.

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

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

1.9 Put the plate in the incubator (37 ℃, 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.

Cell seeding/microvessel culture

1.10 Add 5 mL serum and 2.5 mL Pen/Strep (P/S) to 500 mL 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'.

1.11 Prepare vascular growth medium: Add 3 µL of 50 µg/mL vascular endothelial growth factor (VEGF) and 2 µL of a 20 µg/mL basic fibroblast growth factors (bFGF) to 5 mL basal medium + 1% serum + 0.5% P/S.

1.12 Thaw the iPSC-EC, transfer to a 15 mL tube and dilute in 10 mL basal medium.

1.13 Count the cells, a single vial contains 1 million cells with >90% viability.

1.14 Centrifuge at 100 RCF for 5 minutes

1.15 Aspirate supernatant without disturbing the cell pellet and resuspend in basal medium to a yield a concentration of 2×10^7 cells/mL.

1.16 Aspirate FN-coating solution (well 'A1')

1.17 Add 25 µL of basal medium in the inlet wells (Fig 1b, well 'A1').

1.18 Add a 1 µL droplet of cell suspension on every top perfusion inlet (Fig 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 the microscope whether the seeding is homogeneous. If not, add another 1 µL in the outlet and wait till the droplet flattens.

1.19 Incubate the microfluidic well-plate for 1 h at 37 ℃, 5% CO_2. After this, the cells should have adhered. If not, wait another 30 minutes.

1.20 Remove the basal medium from the top perfusion outlet wells ('A1')

1.21 Add warm vessel culture medium in the top perfusion inlet and outlet (Fig 1b, well 'A1' and 'A3')

1.22 Place the plate on the rocker platform (set on 7° angle, 8 min rocking interval)

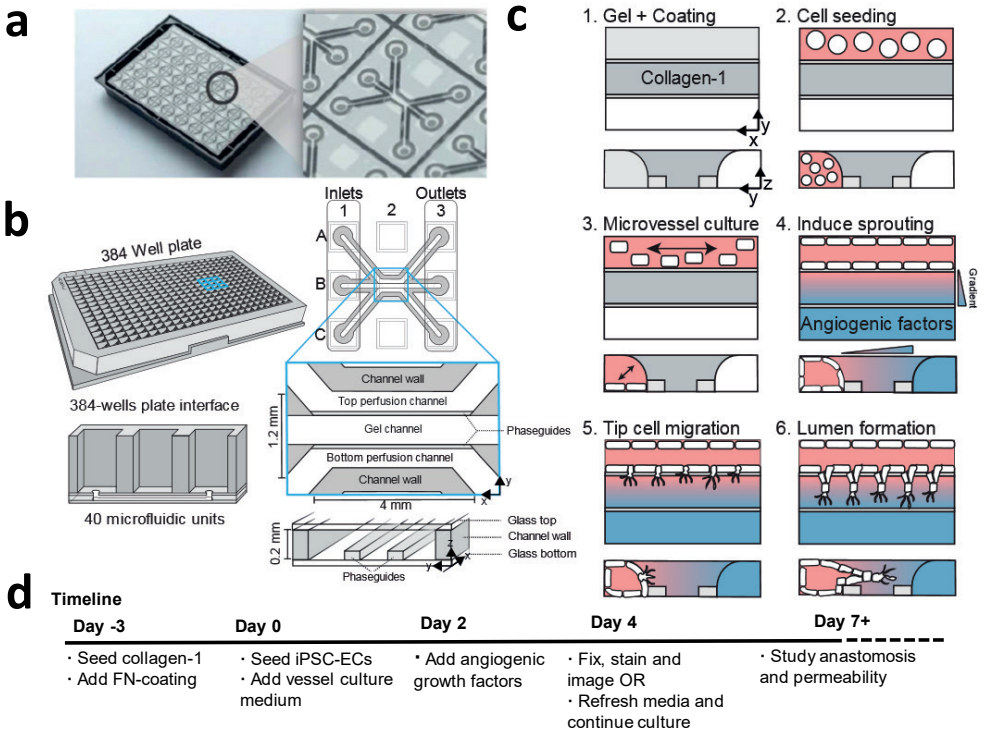

Figure 1 | Microfluidic cell culture protocol for iPSC-derived microvessels

(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 wells that are used as inlets and 3 wells as outlet. 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. Figure has been modified from*[15].

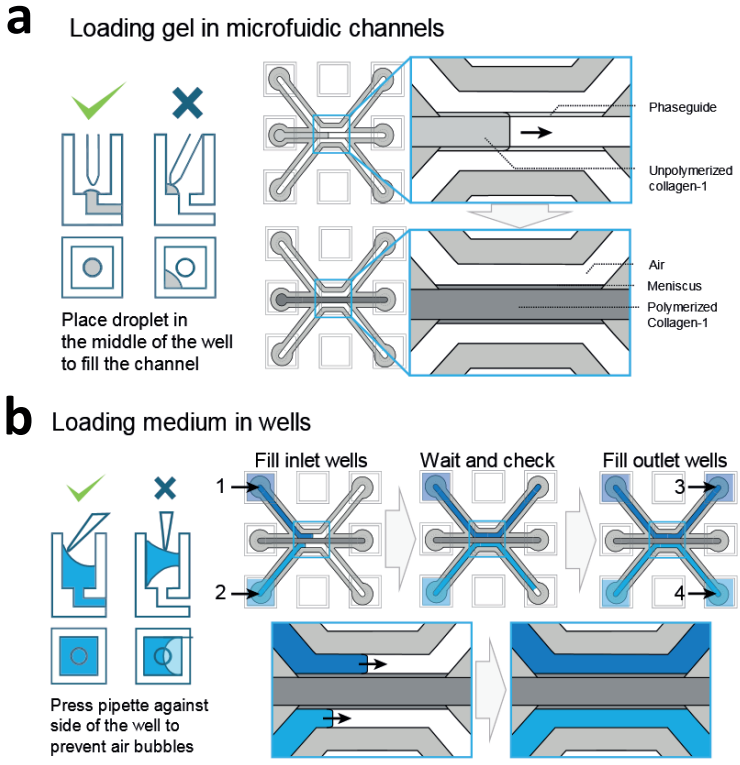

Figure 2 | Loading procedures for gel and medium

(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 order 1-4 to prevent air-bubble trapping within the microfluidic channels.*

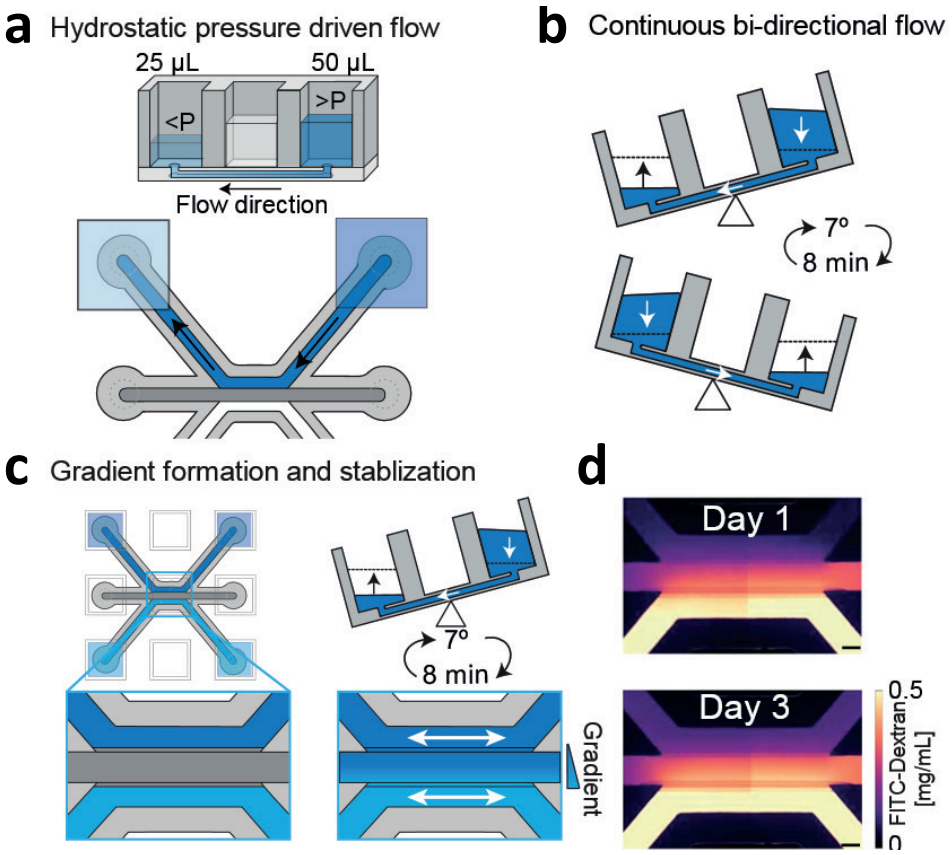

Figure 3 | Continuous hydrostatic pressure driven flow and gradient stabilization

(a) *Hydrostatic pressure differences between wells result in passive levelling and flow within the microfluidic channels* **(b)** *When the device is placed on a rocker platform set at 7° and 8 min cycle time results in continuous, bidirectional perfusion* **(c)** *Gradients are formed by introducing two different concentrations within the wells, which are continuously refreshed by passive leveling.* **(d)** *Gradient visualization using FITC-Dextran. Bi-directional flow stabilizes the gradient up till 3 days. Modified rom Scale bar = 200 µm. Figure has been modified from* [15].

in the incubator.

1.23 Image the plate using a brightfield microscope with automated stage at day 1 and 2 post-seeding to confirm cell viability.

1.24 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 hours.

Study angiogenic sprouting

1.25 Prepare 4.5 mL angiogenic sprouting medium by supplementing basal medium with 4.5 µL VEGF (1:1000, 50 µg/mL stock), 4.5 µL phorbol 12-myristate-13-acetate (PMA) (1:1000, 20 µg/mL stock) and 2.25 µL sphingosine-1-phosphate (S1P) (1:2000, 1 mM stock).

1.26 Prepare 8.5 mL vessel culture medium (basal medium supplemented with 30 ng/mL VEGF and 20 ng/mL bFGF)

1.27 Aspirate medium and add 50 µL fresh vessel culture medium in the top perfusion inlet and outlet wells and gel inlet and outlet wells (Well 'A1, A3, B1 and B3'),

1.28 Add 50 µL angiogenic sprout mixture to every bottom perfusion channel inlet and outlet well (Fig 1b, well 'C1' and 'C3').

1.29 Place the device back on the rocker platform to form a gradient of angiogenic growth factors.

1.30 And image after 1 day and 2 days using a brightfield microscope with automated stage.

1.31 The microvessels can be fixed, stained and imaged in order to quantify sprouting length and morphology (go to step 7).

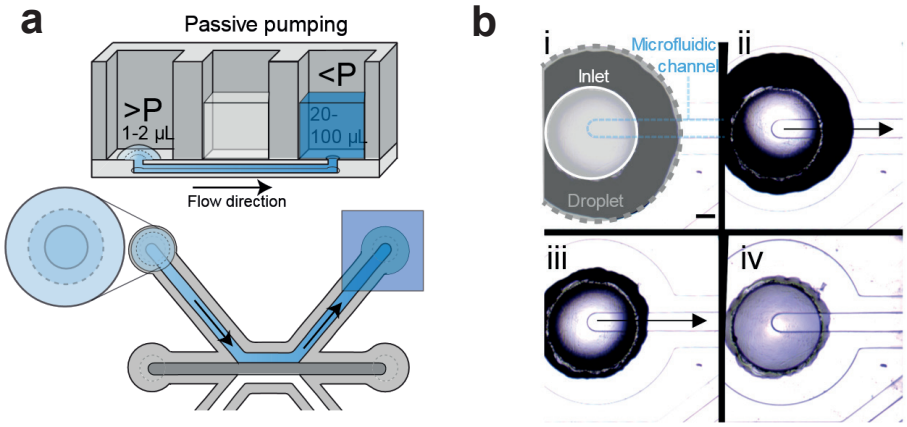

Figure 4| Passive pumping method for cell seeding

(a) *Passive pumping is driven by pressure differences which 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 (i, the droplet on top of the inlet shrinks (ii:, 1 sec after addition, iii: 2 sec after addition), which results in a flow towards the opposite outlet. This continuous till the droplets meniscus is pinned by the inlet. Scale bar = 400 µm.*

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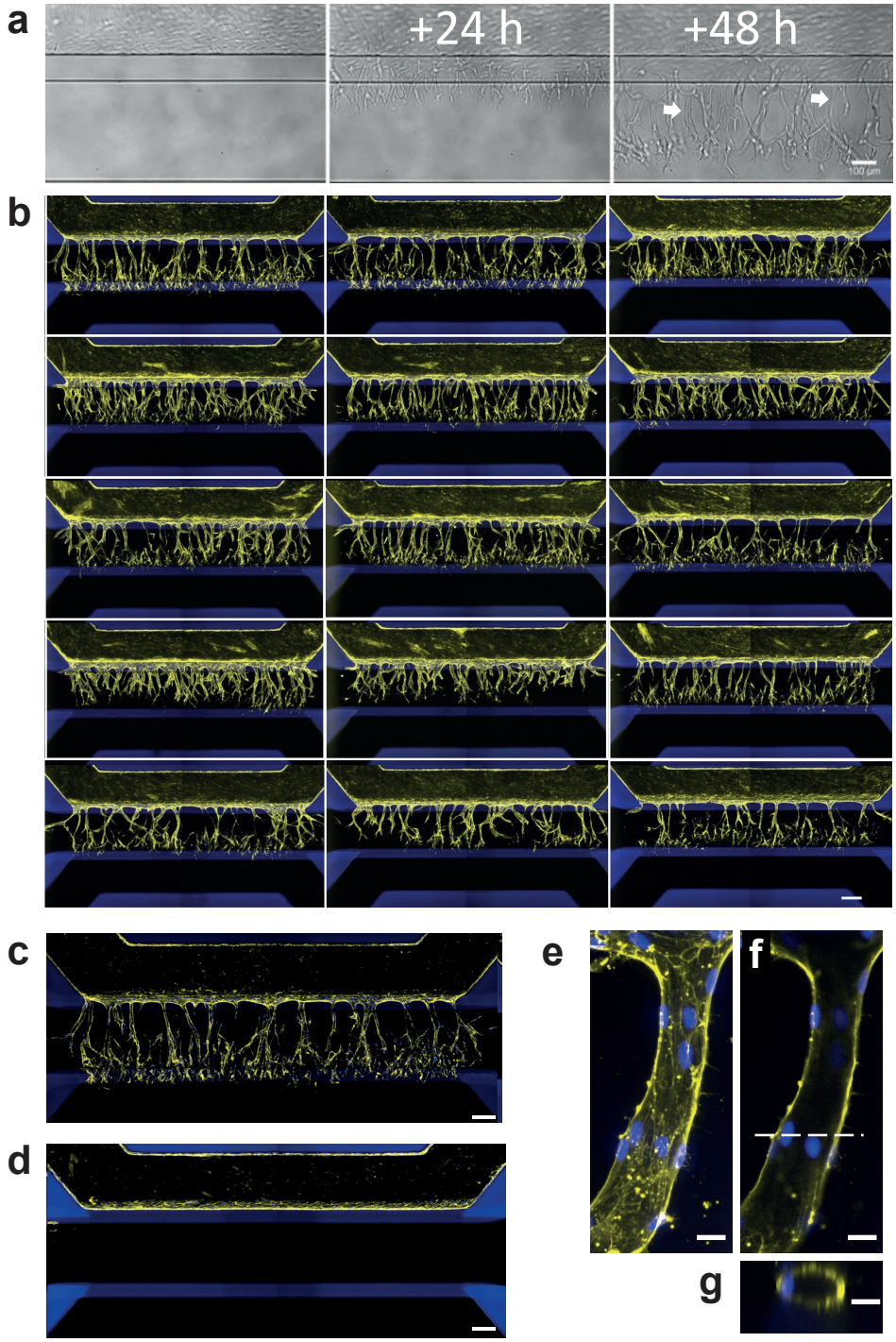
►Figure 5 | Robust 3D sprouting of iPSC-EC microvessels

(a) *Sprouting of iPSC-EC over time. Microvessels were grown for 48 hours (left) and then stimulated with an angiogenic cocktail containing 50 ng/mL VEGF, 500 nM S1P and 2 ng/mL PMA. 1 day after exposure, the first tip-cells are visible that invade the collagen-1 scaffold (middle. 2 days after exposure (right), the first lumen are visible (arrows) 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)** *Max projection of a single capillary within the gel* **(f)** *Same as e, but focused on middle.* **(g)** *orthogonal view of the vessel displayed above***.** *Scale bars (a-d 200 µm, e-g: 20 µm)*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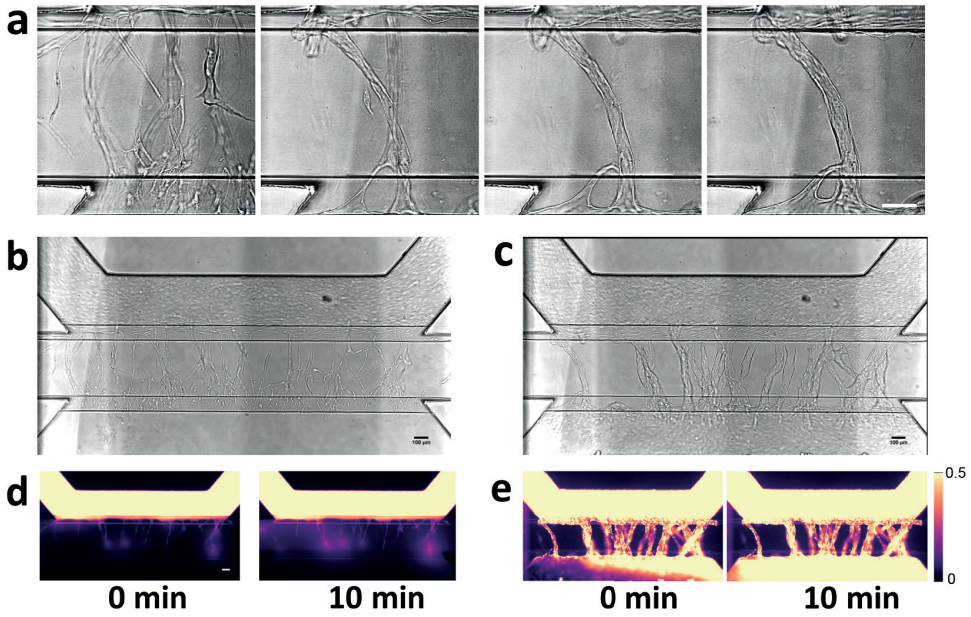

Figure 6 | Visualization of angiogenic sprout permeability before and after anastomosis

(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 TRITC-albumin solution. Fluorescent images obtained at 0 and 10 min.* **(d-e)** *Same as in b+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*

Study anastomosis and sprout stabilization

1.32 Refresh both the angiogenic sprouting medium and vessel culture medium every 2-3 days

1.33 4 days after addition of angiogenic growth factors, the sprouts start to anastomose with the bottom channel, resulting in perfusion of the angiogenic sprouts. Permeability can be quantified at multiple time-points (see step 6)

Visualization of angiogenic sprout permeability

1.34 Add 1 µL of TRITC-albumin (0.5 mg/mL) to the top perfusion inlet and mix using a 50 µL pipette.

1.35 Transfer the plate to a fluorescent microscope with automated stage and incubator set at 37 ℃. .

1.36 Acquire a time-lapse for 10 minutes, with images taken every minute.

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

1.38 Remove the TRITC-albumin by aspiration both the vessel culture medium and the angiogenic sprouting medium and replace both the vessel culture medium and angiogenic sprouting medium in the corresponding wells (see step 4.3-4.4).

1.39 Place the device back into the incubator to continue angiogenic sprouting.

Fixation, staining and imaging

1.40 Aspirate all culture media from all the wells.

Note: residual medium or liquids in the microfluidic channels does not influence the fixation: due its low volume (1-2 µL)

1.41 Add 25 µL of 4% paraformaldehyde (PFA) in PBS to all the perfusion inlet and outlet wells.

1.42 Incubate for 10 minutes at room temperature. Place the device under a slight angle to induce flow (e.g. by placing one side of the plate on a lid).

1.43 Aspirate the PFA from the wells.

1.44 Wash twice with 50 µL hanks balanced salt solution (HBSS) in all the perfusion inlets and outlets

1.45 Aspirate the HBSS from the wells.

1.46 Permeabilize by adding 50 µL 0.2% Triton-X100 to all the perfusion inlets and outlets

1.47 Aspirate the 0.2% Triton-X100 from the wells

1.48 Wash the perfusion channels twice by adding with 50 µL of HBSS to all perfusion inlets and outlet wells

1.49 Aspirate the HBSS from the wells.

1.50 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 every perfusion inlet and outlet well ('A1';'A3';'C1';C3'). Place the plate under a slight angle and incubate at room temperature for at least 30 minutes.

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

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

Results

The microfluidic 3D cell culture platform consists of 40 perfused microfluidic units (Fig 1a+b), which is used to study angiogenic sprouting of perfused microvessels against a patterned collagen-1 gel (Fig 1c). These microvessels are continuous perfused and exposed to a gradient of angiogenic growth factors (Fig 3a-d).

Seeding the iPSC-EC using the passive pumping method should result in homogenous seeding densities (Fig 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 (Fig 5a-g). Clear tip cell formation and invasion into the collagen-1 gel and was visible 24h after addition of the angiogenic gradient, while stalk cells including lumen formation was visible after 48h (Fig 5a). The angiogenic sprouts can be either studied 2 days after gradient exposure, or cultured for +5 days after gradient exposure to study anastomosis and sprout stabilization (see timeline, Fig 1d).

After fixation and staining, the capillary network can be clearly visualized more clearly using Phalloidin to stain F-actin and Hoechst 33342 to stain the nucleus (Fig 5b-c). These sprouts can be quantified (e.g. shape and length[15]). Without addition of growth factors, no invasion into the collagen-1 gel should be observed (Fig 5d). Confocal imaging was used to determine the sprout diameter and to confirm lumen formation(Fig 5e-g).

The sprouts continue to grow towards the direction of the gradient and reach the opposite perfusion channel after 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 (Fig 6a). Lumen formation was assessed by perfusion of the vascular network with fluorescently labeled macromolecules (e.g. TRITC-albumin or FITC-dextran). Perfusing the microvessels with 0.5 mg/mL TRITC-albumin before and after anastomosis revealed a clear difference in sprout permeability after 10 min (Fig 6b-e), which suggests that the capillaries stabilize and mature after anastomosis.

Discussion and conclusion

This method describes the culture of 40 perfusable endothelial microvessels within a robust and scalable microfluidic platform. Compared to traditional 2D and 3D cell culture methods, this methods 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 the 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.

The lack of a pump is a limitation for continuous imaging, as this is restricted to sequential time intervals. Furthermore, the perfusion in this platform consist 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. Though we do not observe negative effects of the bidirectional flow in the angiogenic sprouting, flow is an important biomechanical stimulus in vascular biology 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.

Another major advantage of this method is the commercial availability, 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.

The most straight-forward future direction for this assay will be the integration of other cells types that play an important role during angiogenesis, such as pericytes and macrophages. To study for example 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 a extracellular matrix (e.g. we have shown the culture of neurons and various epithelial structures such as proximal tubules and small intestines). Another interesting direction is the usage of synthetic hydrogels. Not only does this further increase the standardization of the assay, it also allows the tuning of for example the stiffness and cell-matrix interactions.

In conclusion, the method presented here is suitable to study angiogenesis in a physiological relevant cellular microenvironment, including perfusion and gradients. The platform is compatible with automated microscopes, and has the required robustness and scalability to be integrated within the drug screening infrastructure. Both the platform as well as the endothelial cells differentiated from iPSC are commercially available, which enables the adoption across research groups and accelerates its implementation within drug research.

Critical steps

Collagen 1 gel

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 or by flipping the plate upside down. While filling, the collagen gel should remain in the center channel, without flowing into the adjacent perfusion channels.

We found 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 minutes of polymerization at 37 degrees, the gel should be homogenous and clear. If collagen 1 is not stored properly (e.g. due to fluctuating temperatures in the fridge), the collagen will polymerize in- homogenously 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.

Cell seeding

When the cells are seeded, the fibronectin coating solution is only removed from the wells, leaving the microfluidic channels filled with coating solution. Aspiration of the coating from the microfluidic channels could cause gel disruption or gel aspiration.

The cell suspension need to replace/displace the FN-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.

Angiogenic sprouting

But, 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.

Adding or replacing medium

Immediately filling outlet wells without waiting for the media to flow from the inlet well through the microfluidic channels will trap air bubbles within the microfluidic channels. Thus, addition of medium should always be done in the order indicated in the protocol to prevent dehydration and trapping air bubbles (see Fig 2b). These air bubbles will restrict or even stop the flow, even when the device is placed on a rocker platform.

Air bubbles can also occur when medium has been removed from the wells: if the wells are empty, evaporation of liquid in the channel can occur in about half an hour. When medium is added again, the evaporated medium in the microfluidic channels will cause air bubbles to be trapped within the 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.

Within the wells, air bubbles can be trapped by incorrect filling of the wells. Pressing the pipette tip against the side wall of the wells will increase the success of completely filling the well. If an air-bubble if trapped within the wells, these can be removed by gently inserting a sterile pipette tip till the glass bottom.

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Disclosures

P. Vulto and T. Hankemeier are shareholder of Mimetas BV. V. Borgdorff and A. Reijerkerk are employees of Ncardia BV. V. van Duinen, W. Stam and A.J. van Zonneveld have no disclosures.

Troubleshooting

Problem	Cause	Solution
ECM		
Collagen-1 does not enter or fill the channel completely	*Collagen-1 droplet is not placed on top of the inlet*	*Carefully place the droplet on top of the inlet from the gel channel*
	Volume of collagen-1 is too low	*Use 1.5 µL of the gel to fill the channel completely*
	Collagen-1 is too viscous	*Use another batch of collagen-1*
Collagen-1 flow into perfusion channels	*Collagen-1 is pipetted directly into the channels*	*Carefully place the droplet on top of the inlet from the gel channel*
Collagen-1 is not clear/fiber formation	*Collagen-1 is not stored properly*	*Store collagen-1 at 4 °C (do not freeze)*
	$NaHCO_3$ and HEPES are not mixed before adding collagen-1	*Carefully mix the $NaHCO_3$ and HEPES by pipetting before adding the collagen-1*
Cell seeding and sprouting		
Droplet does not shrink using passive pumping method	*Droplet adheres to side of the well*	*Aspirate droplet and add new droplet on top of the inlet*
		Make sure the outlet well if filled with at least 20 µL of medium
No sprouting is observed	*Growth factors not added or aliquots not stored properly*	*Prepare fresh angiogenic sprouting medium*
	Air bubble blocks perfusion/ gradient formation	*Remove air bubbles using a P20 or P200 pipette*
	Volume differences between wells	*Volumes in all wells need to be equal in order to form a linear gradient*
Cells not viable	*Plate not placed on rocker platform/rocker platform turned off*	*Make sure rocker platform is on and has the right cycle time/angle (8 min/7°)*
	No perfusion possible due presence of air bubbles	*Remove air bubbles using a P20 or P200 pipette*
No lumens are formed, cells migrate as single cells	*Angiogenic sprouting mixture was added before a monolayer was formed*	*Wait an additional 24h before adding the angiogenic growth factors*
Major variation in sprouting density	*Differences in cell densities after seeding*	*Check if cell density is homogenous and comparable between microfluidic units. Add another droplet of cell suspension if necessary.*

Table 1: troubleshooting common errors

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