IFlowPlate™ – A customized 384-well plate for the culture of perfusable vascularized colon organoids

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Abstract:

Despite the complexity and structural sophistication that 3D organoid models provide, their lack of vascularization and perfusion limit the capability of these models to recapitulate organ physiology effectively. We have engineered a microfluidic platform, named IFlowPlate™, using which we can culture up to 128 independently perfused and vascularized colon organoids *in vitro*. Unlike traditional microfluidic devices, our vascularized organoid-on-chip device with an "open-well" design does not require any external pumping systems and allows tissue extraction for downstream analyses, such as histochemistry or even in vivo transplantation. By optimizing both the ECM and the culture media formulation, we have co-cultured patient-derived colon organoids successfully within a self-assembled vascular network and found the colon organoids grow significantly better in our platform under constant perfusion vs. conventional static condition. Furthermore, we demonstrated a colon inflammation model with an innate immune function where circulating monocytes can be recruited from the vasculature, differentiate into macrophage and

infiltrate the colon organoids in response to TNF-α inflammatory cytokine stimulation. With the ability to grow vascularized colon organoids under intra-vascular perfusion, our IFlowPlate™ platform could unlock new possibilities for screening potential therapeutic targets or modeling relevant diseases.

Introduction

In an effort to explore modeling systems that seek to recapitulate bodily functions and create systems as physiologically relevant as possible to address the limitation of existing paradigms in drug development and biological research, many recent studies have focused on the use of organoids. Organoids are a 3D cell culture of self-organized differentiating cells. What makes them such a remarkable modeling system is their ability to recapitulate in vivo morphology and cell organization on a smaller scale, while also displaying genetic fingerprints very similar to that of the original tissue[1]. These cells are usually grown on natural extracellular matrix (ECM) to provide the structural support for cell attachment and organization[2]. Organoids can be derived from PSCs (ESCs or iPSCs), or adult primary tissues. Organoids derived from ESCs retain embryonic development characteristics and provide a good way of studying organ development and the development of genetic disorders. By providing the appropriate biochemical and physical cues, it is now possible to culture many different types of organoids, including kidneys[3], intestines[4], brain[5], and prostate[6], etc.

The main advantage of an organoid is structural sophistication, though this approach is limited by the lack of vascularization and perfusion[7,8]. Organoids are usually cultured inside static multi-well plates. Even though vasculature is prevalent in the body, many organoid model systems entirely

omit the vascular network. For the ones that do contain vasculature, the vascular networks are not intra-vascularly perfused[9]. Recently, researchers have developed an elegant 3D printed bioreactor for vascularizing kidney organoids. However, their device introduced flow through superfusion (flow over the organoids) and not intra-vascular perfusion which limits the ability of the device to probe the intra-vascular space of the vascularized organoids[10]. Previous studies have also shown that intravascular perfusion is critical for vessel stabilization and lack of perfusion could lead to vessel regression[11,12]. Therefore, integration of both vasculature and intravascular perfusion is crucial for generating functional vascular networks for supporting epithelial tissue. While several strategies for vascularizing organoids via animal implantation have been attempted and yielded excellent results[13-16], these lack scalability and adaptability required for in vitro applications. Important biological events, such as intercellular cross-talk, immune cell migration, and biomolecular transport, occur at the tissue vascular and epithelium interface. Without a way to access this biological interface, some biological processes, such as immune cell recruitment during tissue inflammation, cannot be easily recapitulated and studied. To overcome this challenge, we developed a platform to vascularize organoids by growing the organoids within a perfusable vascular bed self-assembled from endothelial cells. This allows intra-vascular perfusion of vascularized organoids for the first time. We then further scaled this platform to a customized 384-well plate (here referred to as IFlowPlate^{TM}) on which we can readily fabricate, culture, perfuse, and test up to 128 independent vascularized organoid cultures at a time. Unlike many closed microfluidic-based systems, the open-well design allows the tissue to be easily retrieved for downstream analysis, such as gene expression and histopathological analysis, or even in vivo transplantation. Lastly, we exploited the unique perfusion capabilities of our platform to model colon inflammation, which allowed us to observe the different steps involved in the inflammatory

process such as recruitment of circulating monocytes and infiltration of the colon organoids by differentiated macrophages in response to epithelial tissue injury.

Results

Device fabrication

To create a perfusable culture platform, we customized a standard 384-well plate by adding micro-channels with a cross-section of 300 μm in width and 120 μm in height at the bottom of the plate to connect three adjacent wells together. The three wells connected together become one independent unit **(Figure 1 a-b)**. The well in the middle served as the culture chamber where a natural hydrogel mixed with cells and organoids was cast to the bottom. The other two wells served as the inlet and outlet media reservoir. During tissue culture, the entire plate was placed on a programmable rocker that tilted the plate at a 30-degree angle to produce a pressure head that drives media perfusion from the inlet well through the middle well to the outlet well. To sustain media perfusion for long periods, the perfusion direction was altered by programming the rocker to change tilt direction every 15 min. This configuration, without the use of any tubing or syringe pump, was able to maintain constant media perfusion in all 128 independent units on the 384-well plate simultaneously. Gel casting, cell seeding, culture media changes, or drug tests can all be performed with simple pipetting techniques or even robotic handling systems. The device was also designed to contain minimal amounts of drug absorbing glues or PDMS materials to prevent unspecific absorption of small hydrophobic molecules. The base of the plate was made of an optically transparent polystyrene sheet of less than 1 mm in thickness to allow automatic imaging in standard plate readers and image cytometers. Next, to support vascularized organoids culture using this platform, we optimized the extracellular matrix (ECM) and media formulation **(Figure**

1c). Specifically, we used patient-derived healthy colon organoids in this study, but the same approach could potentially be applied to other organoid systems.

ECM optimization for vascular network formation and colon organoid culture

Previously it has been shown that endothelial cells embedded in fibrin gel can self-assemble into a perfusable vascular network inside closed microfluidic channels[17]. Here, we showed this self-assembly capability is not limited to a microfluidic environment **(Figure 2a)**. Human primary endothelial cells mixed with human fibroblasts can self-assemble into a perfusable microvascular network inside a customized well-plate in three days **(Figure 2b)**. We found the self-assembled vascular network can cover the entire well. At a cell seeding density of 5 million cells/mL, the endothelial cells were sufficiently close to each other to form an interconnected vascular network. This process can take place in fibrin gel with a fibrinogen concentration of 5 and 10 mg/mL **(Figure 2c, top)**. While it is well known that endothelial cells self-assemble quite well in fibrin matrices, most of the organoid cultures use Matrigel®. Matrigel® contains large quantities of laminin and collagen IV which are the building blocks of the basement membrane that supports the organoid epithelium. Nevertheless, in Matrigel® alone, we found that the endothelial cells were unable to self-assemble into a vascular network **(Figure 2c, bottom)**. This is likely because endothelial cells alone cannot easily remodel the laminin and collagen IV in Matrigel®. Therefore, to support endothelial assembly without removing the Matrigel® completely, we added 10% (v/v) Matrigel® in fibrin gel and demonstrated that vascular assembly from endothelial cells could take place in this gel formulation (**Figure 2c, bottom)**.

Thrombin concentration was also crucial for the self-assembly of endothelial cells in fibrin matrices. Lower concentration of thrombin (1.5 U/ml) allowed more cells to settle at the bottom of the well, thereby facilitating the formation of the vascular network **(Supplementary Figure 1)**. The network can connect to the inlet and outlet channels, and media perfusion was established through the entire network on day 5, as shown by the perfusion of 70kDa fluorescently labeled dextran (**Figure 2b**). The perfusability of the network was highly consistent between different wells (**Supplementary Figure 2**). The entire vascular network formed a tight vascular barrier that can contain large proteins. Although vascular perfusion appears to be faster in vessels near the well edges due to lower flow resistance, vascular permeabilities are similar in all regions, indicating that vessels were not leaky and perfusates did not leak or pool in the center well (**Figure 2d-e**). Due to the self-assembly nature of this system, the shear stress within the vascular network can vary from 0.02 to 1.2 dynes/cm^2 consistent with previous reports in similar devices[18] (**Supplementary Figure 3, Supplementary Video 1**). However, this level of heterogeneity is expected and resembles native tissues. The vessels formed intercellular junctions, secreted Von Willebrand Factors (vWF) important to thrombogenicity, and deposited laminin-rich basement membranes (**Figure 2f-g**). Specifically, we found the formation of vWF fiber along the flow direction consistent with previous reports of flow-driven assembly of vWF fiber[19]. This self-assembled perfusable microvascular bed is positioned entirely inside a well with an open top that will allow the addition of other tissue samples both on top of and within the gel matrix as well as the extraction of the tissues from the well.

To validate the feasibility of integrating engineered microvasculature with organoids, here we used patient-specific colon resident stem cells to grow colorectal organoids *in vitro* (**Figure 3a**). To

culture the colorectal organoids in this study, we embedded the organoids inside the gel matrix to maximize the contact surface between the organoids and the surrounding vasculatures. However, this required the optimization of the matrix for both organoids and the vasculature. Primary colon tissue contains functional adult stem cells that reside in the base of the crypt, and thus, biopsied resident intestine stem cells can undergo differentiation in Matrigel® to recapitulate the cellular diversity of the colon epithelium[20] (**Supplementary Figure 4)**. As expected, the colorectal organoids were unable to grow in fibrin alone as laminin and collagen IV are completely absent in fibrin gel. This is consistent with previous findings[21]. However, we found that a mixture of fibrin and 10% Matrigel® can support the formation of colon organoids (**Figure 3b)**. The colon organoid continued to grow for at least eight days in and went through the crypt budding process with multiple budding structures forming. Localized staining of F-actin on the luminal surface of the organoid indicated a polarized epithelium (**Figure 3c**). We also quantified the cross-sectional area of the colon organoids in different hydrogel matrices and found that the combination of fibrin and Matrigel® yielded significantly larger organoids **(Figure 3d)**. This is likely because fibrin gel tends to be more porous than Matrigel® which could have enhanced mass transport in the gel to enable faster organoid growth.

Formation of perfusable vascularized colon organoids

We determined that both vasculature and colon organoids can grow in a matrix formulation that combined fibrin and Matrigel®. This laid the groundwork for establishing the perfusable vascularized colon organoids by co-culture of organoids with a pre-established microvascular bed. We next optimized the media formulation that can sustain the co-culture of colon organoid and vasculature **(Figure 4 a,b)**. Three media formulation that combines the endothelial growth media

(ECGM2) and colon media at different ratios were tested (**Figure 4b**). We found that even though all three media conditions were able to support organoid formation, vascular networks formed in media 2-3 were quite narrow (**Figure 4c**). We used AngioTool[22] and Image J software to quantify the vascularization of the colon organoids in all three media conditions **(Figure 4 d-h).** While the organoid area between the three media conditions showed no significant differences, we found that the vessel diameter and vessel area were significantly higher in media 1 compared to the other two conditions. The average vessel length (distance between two junctions) of media 1 was significantly better than media 2 but showed no significant difference with media 3. We also quantified junctional density (junctions/area), which showed no significant differences between the three media conditions. Given that the large diameter of vascular networks formed in media 1 would allow for better perfusion of the organoids and higher percentage of vessel area was observed in this condition, we decided that media formulation 1 would be ideal for growing vascularized colon organoids in IFlowPlate^{TM}.

Using the established matrix and media formulation, we were able to culture colon organoids with a self-assembled microvascular network for at least 13 days **(Figure 5a)**. Every single organoid cultured in the well was surrounded by blood vessels (**Figure 5a**). To demonstrate intravascular perfusion of the engineered vascularized colon organoids, we perfused the vascular network with red fluorescent particles that clearly labeled the compartmentalized vascular lumen, interstitial space, and organoid lumen (**Figure 5b)**. Confocal images showed that the colon organoids were surrounded by GFP- blood vessels in close proximity (**Figure 5c**). Both organoids and the microvasculature contain hollow lumens that can be visualized from tissue cross-sections. Furthermore, we also observed polarization of the colon epithelium with clear F-actin localization

on the apical side of the colon epithelium. We also tracked the simultaneous self-assembly of both vasculature and organoids and found the vasculature to structurally stabilize at around day 5 while the organoids grew continuously (**Figure 5d**). Interestingly, we noticed that the growth of organoids appears to have accelerated after perfusion was established on day 5. Therefore, the earliest time point for using the models would be around day 5, when the vasculature, vascular perfusion, and the organoids were well established.

The "open-top" design of our platform allowed us to remove the entire vascularized colon tissue out of the well and perform histological analysis **(Figure 5e)**. Using this feature, we directly compared our vascularized organoids with human colon tissues and non-vascularized organoids (**Figure 5e**). Specifically, villin staining showed the organoids were polarized and expressing micro-villi. Ki67 showed the presence of proliferating progenitor cells that fueled the growth of the organoids. More interestingly, compared to the non-vascularized organoids, the vascularized organoids were surrounded with perfusable blood vessels in very close proximities similar to the native colon tissues. We measured the distance between the organoid and the nearby vessels and compared it against native colon tissues. We saw nearly indistinguishable differences (**Figure 5f**, **Supplementary Figure 5**). Moreover, 19% and 28% of vessels counted were in direct contact (a distance equal to 0) with the epithelium in human colon tissue and vascularized organoids, respectively. To further understand the importance of the vascularized co-culture for organoid growth, we quantified the percentage of recovered organoids on Day 9 under different experimental conditions, including the conventional static culture in Matrigel®. We observed that the colon organoid formation was significantly more robust in IFlowPlate™ in the presence of both vascular network and perfusion **(Figure 5 g-h)**. Specifically, we found that there is significant

cross-talk between the vasculature and the organoids in IFlowPlate™ as organoid growth completely stopped without the presence of vasculature while using our optimized matrix and media formulation. This highlights the importance of vasculature and perfusion in supporting organoid growth and development.

Vascularized human colon organoid model of tissue inflammation with innate immunity

To demonstrate the importance of vasculature, we applied a scenario of colon inflammation as an example, which involves the complex interaction between circulating monocytes, endothelium, and colon epithelium. Release of TNF-α inflammatory cytokine during colon inflammation triggered the expression of ICAM-1 surface receptor on the vascular endothelium. ICAM-1 receptor then facilitated the attachment of circulating monocytes on the vascular endothelium. Adhered monocyte then went through transendothelial migration, followed by differentiation into macrophages and lastly, infiltration into the colon epithelium (**Figure 6a**). This entire process was captured and visualized in our model. With static organoid culture, even though it's possible to embed monocytes in a matrix around the colon organoids, the concentration of monocytes will have to be controlled manually in an arbitrary way where the intravascular recruitment of monocytes which plays a crucial role in the amplification of inflammatory response is missing. In addition, without transendothelial migration, monocytes will have to be artificially activated to differentiate into macrophages with M-CSF, which is not physiological. It is well known that the endothelium niche plays a vital role in the activation and phenotypic transformation of macrophages[23,24].

We demonstrated that the extent of monocyte recruitment is correlated to the extent of inflammation and TNF-α release by simply perfusing circulating monocytes through the vasculature emulating the exact process that happens in the body without the need for artificially activating the monocytes with M-CSF (**Figure 6b-e**). With TNF-α stimulation, most perfused monocytes were quickly captured by the microvasculature, while most monocytes passed through the network in non-stimulated vasculature. After one day incubation, we found the monocyte-differentiated macrophages were able to infiltrate nearly 80% of the colon organoids with TNF-α stimulation (**Figure 6f**). Specifically, we found the macrophages have a strong tendency to aggregate around the cell debris produced by colon organoids, which correctly correspond with the scavenger function of macrophages. Given the critical role of the vasculature in the recruitment and activation of circulating monocytes and the amplification of inflammatory response, this is a biological process that cannot be accurately replicated with static organoid culture alone. Therefore, we think this makes the platform a useful tool to expand the application of organoids to new biological processes. Here we are using the colon inflammatory process as an example. But we think this platform will find more utilities than just this one scenario as there are many more biological processes that involve vasculature and the interplay between vasculature and epithelium.

Discussion

Some existing approaches combine organoids with organ-on-a-chip systems by dissociating organoids and then seeding the heterogeneous cell population into a membrane-based organ chip[10]. Even though vascular perfusion can be established in this format, the membrane physically restricts 3D biological remodeling that organoid culture offers. Therefore, there is a need to

vascularize and perfuse organoids as is without cell dissociation or fragmentation to preserve the organ-level architecture and the remodeling capability of the organoids. The challenge we seek to address here is the incorporation of perfusable vasculature that could guide the development of organoids without using physical structures to artificially define and restrict biological structure and remodeling. We found the microvasculature to be in close proximity to the organoids and physically intertwine with the organoids. Active flow circulation through the vasculature around the organoids could potentially enhance mass transport across the organoid epithelium and improve organoid function. It's also important to note that although the self-assembled vascular network does not have uniform structures and flow rates, native vessels do not either. This level of heterogeneity we see makes the biological model more physiological and certainly more interesting to study. Furthermore, the vessel structures are not static and can also vary from day to day. The self-assembled vascular network is constantly making structural adjustments in response to flow and to nearby organoids, which is a valuable physiological feature that we cannot acquire if we force uniformity on the system.

The platform also allows the organoids to be placed both inside and on top of the gel. However, in this work, we choose to embed the organoids inside the gel because we would like to introduce more contact areas between the organoids and the matrix to provide the vessels with more opportunities to intertwine with the organoids. In addition, supporting cells like fibroblasts were also incorporated in the gel. This embedding strategy is especially important to smaller epithelial organoids that don't come with its own matrix. Without a supporting matrix, it will be difficult for the vasculature to grow upwards into the organoids if most parts of the organoids are exposed in suspension. For tissue explants or larger organoids, surface vascularization might be sufficient as

the vessels will be able to grow into the organoids in the presence of significant tissue mass and matrix within the organoids. A potential advantage of surface vascularization is that the organoids will likely be less sensitive to the choice of the matrix, hence perhaps allowing the vascular environment and the organoids to be decoupled.

The scalable, robust, and cost-effective manufacturing of this platform will be a significant advantage to the future commercialization of this technology. The minimalistic design of this platform simplifies the manufacturing process. In fact, with the industrial injection molding method, the manufacturing process will just require a simple modification to the design of the injection mold of a standard 384-well plate to include an array of short straight channels. The rest of the manufacturing process will remain the same. For this reason, there is no need to invent new manufacturing methods, which will significantly reduce the barrier and costs of translation.

Limitations

With a perfusable vasculature in place, it remains to be seen if the size of these organoids could continue to increase and thus overcome the oxygen diffusion limitation for tissue growth. Future studies are also needed to confirm the feasibility of applying the same techniques to other types of organoids. Organ-specific endothelial cells could also be used for the vascular assembly to provide a more organ-specific microenvironment around the colon organoids[25]. In our future studies, we intend to analyze the change in organoid gene expression in response to the vascular co-culture. However, conventional transcriptome analysis of the bulk cell population will fall short for this complex culture environment that involves multiple types of cells in close proximity to each other. Single-cell sequencing might be needed to dissect the heterogeneous cell population. If it is critical

to provide unidirectional recirculating flow in the biological model, the fluid circuit inside the plate can potentially be adapted based on recently published strategy[26]. Alternatively, the plate cap could be modified to include an array of microfluidic pumps to recirculate the media back to the inlets[27]. The shear stress we achieved in the microvasculature was still significantly below the physiological range. If shear stress is an important parameter to study, the height of the well plate could be increased to apply a higher pressure gradient. Furthermore, the incorporation of pericytes and smooth muscles has been shown to decrease vessel diameter, which will help increase shear stress inside the microvessels. Considering the large footprint of the 384-well plate, connecting multiple types of organoids between different wells through the perfusable vasculature is potentially feasible. It is also potentially possible to scale this method up to a 96-, or 24- well plate system. Given the right cell density, it appears the endothelial cells will have no problem connecting with each other and transport fluid across a large surface area.

Conclusions

Organoids will likely become an indispensable tool for biological research and drug discovery. We will continue to see an expanding application of organoids. For instance, organoids can also be used to study genetic mutations where mutations can be introduced by CRISPR/Cas9 systems, so precise control of gene expression allows for a detailed study of genetic manipulations. In addition to the application of biological modeling, another promising application is in regenerative medicine. Patient-derived organoids can be grown in the lab and transplanted back into the patient to correct disorders. For this application, the ability to mature and grow the organoid in vitro and then collecting the tissue for implantation becomes crucial. This is one of the key capabilities that IFlowPlate^{TM} could provide.

Materials and Methods

Fabrication of the microfluidic device

Using standard photolithography technique, a SU8 master mold patterned with an array of inlet and outlet channels in the format of a standard 384-well plate was first fabricated. Then, polydimethylsiloxane (PDMS), purchased from Ellsworth Adhesives (Cat# 4019862), was prepared at a ratio of 1:30 and poured onto the SU8 master mold. The PDMS was degassed for 40 minutes and then cured at 47 ºC overnight. The PDMS mold was then de-molded and soaked in 5% (w/v) pluronic acid (Sigma Aldrich, Cat# P2443-250G) for 30 minutes. After 30 minutes, the PDMS mold is then washed in distilled water and capped onto a plasma-treated optical quality polystyrene sheet (11.5×7.5 cm) (Jerry's Artarama, Cat# V16013). Melted poly (ethylene glycol) dimethyl ether (PEGDM) (Sigma Aldrich, Cat# 445908-50G) was then injected into the inlet and outlet channels and allowed to fill the channels at 70 ºC. Next, the PDMS mold was cooled to room temperature, which allows the PEGDM to re-solidify. The PDMS mold was then peeled off from the polystyrene sheet, and the PEGDM features in the pattern of an array of inlet and outlet channels were then transferred onto the polystyrene sheet. The polystyrene sheet containing the PEGDM structures was then glued onto a bottomless 384-well plate using a highly viscous PDMS glue at a ratio of 1:10 (Ellsworth Adhesives, Cat# 2137054) at room temperature overnight. The well plate (IFlowPlate^{TM}) was then packaged and sterilized with gamma-ray sterilization.

Endothelial and stromal cell culture

Green fluorescence protein-tagged human umbilical vein endothelial cells (GFP-HUVEC), and primary human lung fibroblasts were both purchased from Cedarlane labs (Cat# CAP-0001GFP,

PCS-201-013). GFP-HUVECs were cultured in Endothelial Cell Growth Media (ECGM2, Cat# C-22011) as instructed by the supplier (Cedarlane labs). Primary human lung fibroblasts were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin solution (100X) and 1% HEPES (1M). Cells used for all the experiments are between passage 2-5. Prior to cell seeding, all cells were strained through 40μm cell strainers to get a single cell suspension.

Organoid culture and expansion

The colorectal organoids were acquired from the University Health Network (UHN) Princess Margaret Living Biobank in Toronto Canada. The use of patient-derived organoids was approved by Hamilton Integrated Research Ethics Board under the project number, 5982-T. The colon organoids were cultured in Intesticult™ human organoid growth media purchased from Stemcell Technologies (Cat# 06010) according to the manufacturer's protocol. Specifically, frozen vials of organoids were thawed and embedded in growth-factor reduced Matrigel® (Corning, Cat# CACB356231). Each vial of organoid was mixed with 150 μL of Matrigel® . The organoids were cultured in a regular 24-well plate with each well containing 50 μL of Matrigel®-Organoid mixture in the center of the well (Matrigel® dome). Organoids were cultured for a week until they fully recover and later passaged. Organoids were dissociated and expanded with two different methods. For the mechanical dissociation method, the Matrigel® containing the organoids were dislodged from the wells and collected in a tube. The Matrigel® was then broken into small fragments by repeated pipetting using a fire-polished Pasteur pipette. To this mixture, fresh Matrigel® was added and plated into a new 24-well plate. For the TrypLE method, the Matrigel® was first degraded by incubating the organoids with 1 mL of Cell Recovery Solution (Corning, Cat# CACB354253) per

each well on ice for 1 hour. To this mixture, 5mL of cold Advanced DMEM/F12 media (Gibco, Cat# 12634-010) was added and centrifuged at 200G for 4 minutes. The supernatant containing the Matrigel® fragments were discarded, and the organoids were incubated in a water bath (37 °C) for 10 minutes with the 1mL of TrypLE™ express enzyme (Gibco, Cat# 12605-010). This mixture is then centrifuged again at 200G for 4 minutes and fresh Matrigel® was added after discarding the supernatant. For both methods, organoids from each well were expanded into three wells or a splitting ratio of 1:3. The organoids were cultured for a week and the media were changed every two days. The organoids expanded using both methods were cryopreserved by breaking the hydrogel into small fragments and collected using the Recovery™ cell culture freezing media (Gibco, Cat# 12648-010). For all experiments in the IFlowPlate™, organoids were thawed from the frozen vials and applied directly to the IFlowPlate™. The colorectal organoids used for all the experiments were between passage 13-17.

Hydrogel formulation

The hydrogel matrices for IFlowPlate™ cell seeding were prepared by mixing 10mg/mL fibrinogen with 10% (v/v) Matrigel® . The hydrogel mixtures were aliquoted into 125µL aliquots. To this 125µL gel aliquot, 25µl of thrombin (1.5 U/mL) was added and mixed before casting. 25 µL of this final mixture was then cast into each well. For the ECM optimization experiments, 5 mg/mL fibrin, 10mg/mL fibrin and pure Matrigel® were also used. Fibrinogen and thrombin were purchased from Sigma Aldrich (Cat# F3879-1G, T6884-100UN) and stock solutions were prepared as per manufacturer's instructions and stored at -20 °C. For experiments with static conditions, we suspended colon organoids in pure Matrigel® and cultured them in colon media on a regular 384-well plate with no perfusion.

Cell seeding and device operation

Sterilized IFlowPlate^{TM} was first incubated with sterile distilled water to dissolve the PEGDM inside the plate and prime the plate overnight at 37 ºC. After overnight incubation, the plate was then centrifuged at 40G for 30 seconds to remove any air bubbles inside the plate. The wells were then rewashed with sterile water to remove any residual PEGDM. HUVEC (5 million cells/mL), Fibroblasts (1 million cells/mL), and colon organoids (10-12 organoids/well) were suspended in the hydrogel mixture according to the hydrogel formulation. 25 μL of this gel mixture was then added to the corresponding tissue wells. The plate was gently tapped to allow the gel to fall to the bottom of the plate. The plate was then incubated at 37 ºC for 30 minutes to allow gelation. To prevent the gel from entering the inlet and outlet channels, 25μL of fibrin gel (10 mg/ml fibrinogen with 10 U/ml Thrombin) can be applied to the inlet and outlet well prior to casting gel in the center well. After casting in the center well, the gels in the inlet and outlet well can be aspirated and removed. Endothelial cells (0.6 million cells/ml) were also seeded in the inlet and outlet wells. The plate was maintained under the static condition to allow the cells to attach overnight. Culture media were changed in all wells the following day and the plate was placed on a programmable rocker that tilts at a 30° angle. The tilt direction was programmed to change every 15 min to maintain perfusion. The culture media were supplemented with 1% (v/v) Aprotinin (Sigma Aldrich, Cat# 616370-100MG-M) to prevent fibrin degradation. The culture media were changed every other day. For optimizing vascular network formation and organoid culture, we tested ECGM2 media, colon organoid media and their mixture 1:1 or 1:9 (v/v) ratio accordingly. A mixture of ECGM2 and colon organoid media at a ratio of 1:1 (Media 1) was found to be the optimal media condition for the culture of vascularized colon organoids.

Immunofluorescent staining and histology

The entire immunostaining procedure was done in IFlowPlate^{TM} under perfusion on a programmable rocker. Cultured tissues in IFlowPlate^{TM} were first washed with 1X PBS to remove residual media. The tissue was then fixed overnight under perfusion in 4 ^{o}C with 10% Formalin solution. The next day, the fixative was removed, and the tissue was washed again with 1X PBS three times and blocked for 2 hours under perfusion at room temperature with 5% normal goat-serum (Sigma Aldrich, Cat#NS02L-1ML) containing 0.1% Triton-X. The tissue was then stained with primary antibodies, Anti-CD31 (Abcam, Cat# ab28364), Anti-vWF (Abcam, Cat# ab6994), Anti-Laminin (Abcam, Cat# ab11575) overnight at 4 ^{o}C under perfusion. The following day, tissues were washed with PBS. The anti-rabbit secondary antibody (Abcam, Cat# ab150077) or F-actin conjugate antibody (Cedarlane Labs, Cat#20553-300) was added along with DAPI (Sigma Aldrich, Cat#D9542-5MG) and incubated at room temperature for 2 hours under perfusion. After incubation, the samples were washed in PBS overnight and imaged using a confocal microscope (Nikon SR-SIM) or image cytometer (Biotek Instruments). All the antibodies were diluted at 1:100 ratio in PBS with 2% (v/v) FBS. For histology, the cultured tissues were fixed in 10% formalin solution for 48 hours. The tissue was then removed from the well using a tweezer and was placed in histology cassettes. The cassettes were then immersed in 70% ethanol until ready for paraffin wax embedding. The embedded tissues were then sectioned and stained with hematoxylin and eosin, E-Cadherin (Abcam, Cat# ab1416), CD31 (Abcam, Cat# ab28364), Villin (Abcam, Cat# ab130751) and Ki67 (Abcam, Cat#16667). The human colon tissue sections used for histological analyses were a generous gift from the John Mayberry Histology Facility at McMaster University. For imaging vascular network using Transmission Electron Microscopy (TEM), the tissue was

fixed for 1 hour in 1% osmium tetroxide in 0.1M PBS. The fixed samples were then immersed in a series of ethanol dilutions (50%, 70%, 70%, 95%, 95%, 100%, and 100%) to dehydrate the sample. The dehydrated sample was then embedded in 100% Spur's resin and was allowed to polymerize overnight. The embedded tissue was then sectioned and stained with uranyl acetate and lead citrate before imaging.

Tissue quantification

We quantified the increase in the size of the colon organoids in different matrices from six wells per condition. For each well, five independent growing organoids were counted and averaged. The organoid area on Day 8 was then normalized against Day 1 values for each condition. The average diameter of the vascular networks was also quantified similarly where the vascular diameters at ten different positions were measured per well. At least three different wells were used per experimental group. Both the area of organoids and the vascular network diameters were quantified using Image J. To quantify the vessel area, junction density, average vessel length of the vascularized colon organoids in different media conditions (n= 3-5), we used AngioTool software. We measured the organoid growth and the vessel junction density over time (11 days) to quantify the assembly process of vascularized organoids in our platform using Image J and AngioTool (n= 6 wells). We quantified the percentage of organoids recovered in our IFlowPlate^{TM} vs. static condition with and without vascular network by quantifying the organoids and cellular clusters in at least three different wells per condition. Using Image J, we also quantified the distance between the colon epithelium and the nearest vessel in both human colon tissue (n= 5 samples and 69 measurements) and vascularized colon organoids (n= 3 wells and 69 measurements).

Vessel and organoid perfusion studies

To study the perfusion through our self-assembled vascular networks, we perfused the networks with 70kDa TRITC-labelled dextran (Sigma Aldrich, Cat# T1162-100MG). In this experiment, we added 90 μL of TRITC-labelled dextran (500 μg/mL) in PBS to the inlet well and 60 μL of PBS to the tissue well. The perfusion of the dextran molecules through the vascular network was then imaged using an image cytometer (BioTek Instruments Inc.). Time-lapse images of perfusion of 15 minute time intervals were captured. From the time-lapse images, diffusive permeability, P_d at the edge and center of wells (n=5) were calculated using previously reported equation[28,29].

$$P_d = \frac{1}{I_i - I_b}\left(\frac{I_f - I_i}{\Delta t}\right) \times \frac{d}{4}$$

Here, I_i and I_f represents the average intensities at final and initial timepoint while I_b represents the average background intensities. Δt is the time interval between the images and d is the average diameter of the vessel in the chosen ROI.

To calculate the shear stress of the vascular network, we perfused the network with red fluorescent particles (1μm in diameter). The microscope was tilted at 30-degree angle to mimic the programmable rocker. Videos of perfusion were taken at 13.13 frames/sec and the exposure time was set at 700 μs. For calculating the shear stress at different locations, the instantaneous velocity and the vessel diameter were used. The instantaneous velocity was calculated by tracing a particle's positions in two adjacent frames and the vessel diameter was measured in Image J. Shear stress was calculated using the formula[30], $\tau = \frac{4Q \times \eta}{\pi r^3}$. We calculated the shear stress at 18 different regions in 6 wells. To demonstrate perfusion in vascularized organoids, we

perfused the vascular networks with 90µl of red fluorescent particles in the inlet and 60 µl of PBS in the outlet. The delivery of particles to the organoids through the vascular network was then imaged using an image cytometer.

Modeling colon inflammation in IFlowPlate™:

To model colon inflammation in our platform, we first stimulated the tissue by supplementing culture media 1 with 50 ng/ml of TNF-α incubated at 37 °C for 12 hours (n=3). For the no treatment group (n=3), media was not supplemented with TNF-α. After incubation, the tissues were washed and THP-1 monocyte cells (Cedarlane Labs, Cat#TIB-202) at 0.3 million cells/mL concentration labeled using red cell tracker (Thermo Fisher Cat#C34552) were perfused from the inlet wells. We added 90 µL of cell suspension to the inlet and 60 µL of culture media to the tissue well to allow gravity-driven perfusion. The platform was perfused at 37 °C for 30 minutes. Next we washed the networks with culture media to remove unattached monocytes and the platform was incubated at 37 °C overnight under perfusion. The monocyte attachment and organoid infiltration at Day 0 and Day 1 were imaged using the image cytometer. From these images, the monocyte attachment and percentage of organoid infiltration were quantified using Image J. To quantify ICAM-1 expression, we stained the treated and non-treated vascularized organoid tissues with Anti-ICAM-1 (Abcam, Cat#ab2213) and used Image J to quantify the percentage of stained area in the entire tissue well.

Sample size and statistical analysis

Normality and equality of variance were tested using SigmaPlot software. One-way ANOVA or one-way ANOVA on ranks in conjunction with either Holm-Sidak or Dunn's or Tukey's method at $p < 0.05$ and a power greater than 0.90 was used to determine the statistical significance. For all

quantitative analysis, at least three independent samples per condition were utilized. Data in all graph were plotted as mean with SD

Acknowledgments

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Author contribution

S.R. performed the experiments, analyzed the results, and prepared the manuscript. D.S.Y.L contributed to vascular perfusion study. L.A, A.L, A.S helped with plate fabrication. F.Z fabricated the IFlowPlate^{TM} master mold. B.Z. envisioned the concept, supervised the work and prepared the manuscript.

Competing financial interests

S.R., D.S.Y.L., and B.Z. are inventors of a provisional patent filed based on this work.

Supporting Information

Supporting Information is available from the Wiley Online Library or the author.

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Figures

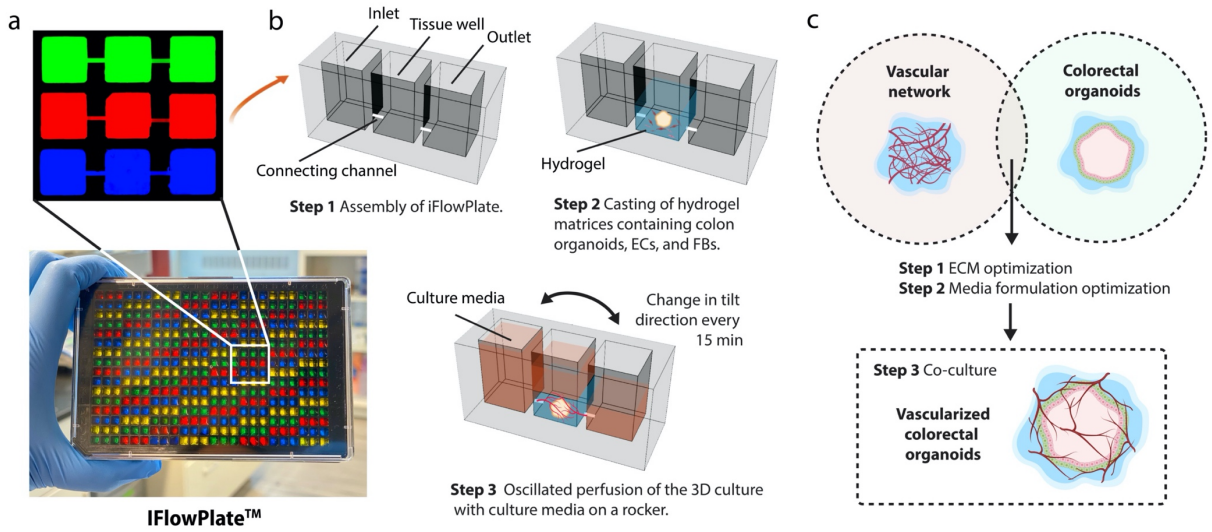

Figure 1. IFlowPlate™ operation and setup. **a,** Image of an IFlowPlate™ filled with different colored dyes to depict the 128-independent units. The microfluidic device is fabricated by introducing microchannels to a customizable 384-well plate. Three wells (inlet, tissue well and outlet) together make one perfusable unit. These perfusable microchannels are shown with color dyes for better visualization. **b-c,** Illustration of our microfluidic device and experimental set up for vascularization of colon organoids cultured in IFlowPlate™. The matrices containing colon organoids, endothelial cells and fibroblasts are cast on to the bottom of the tissue well. After gelation, media is perfused into the vascular network by placing the plate on a programmable rocker. We also tested different hydrogel matrices and media formulations to find the optimum condition for growing these engineered vascularized colon organoids.

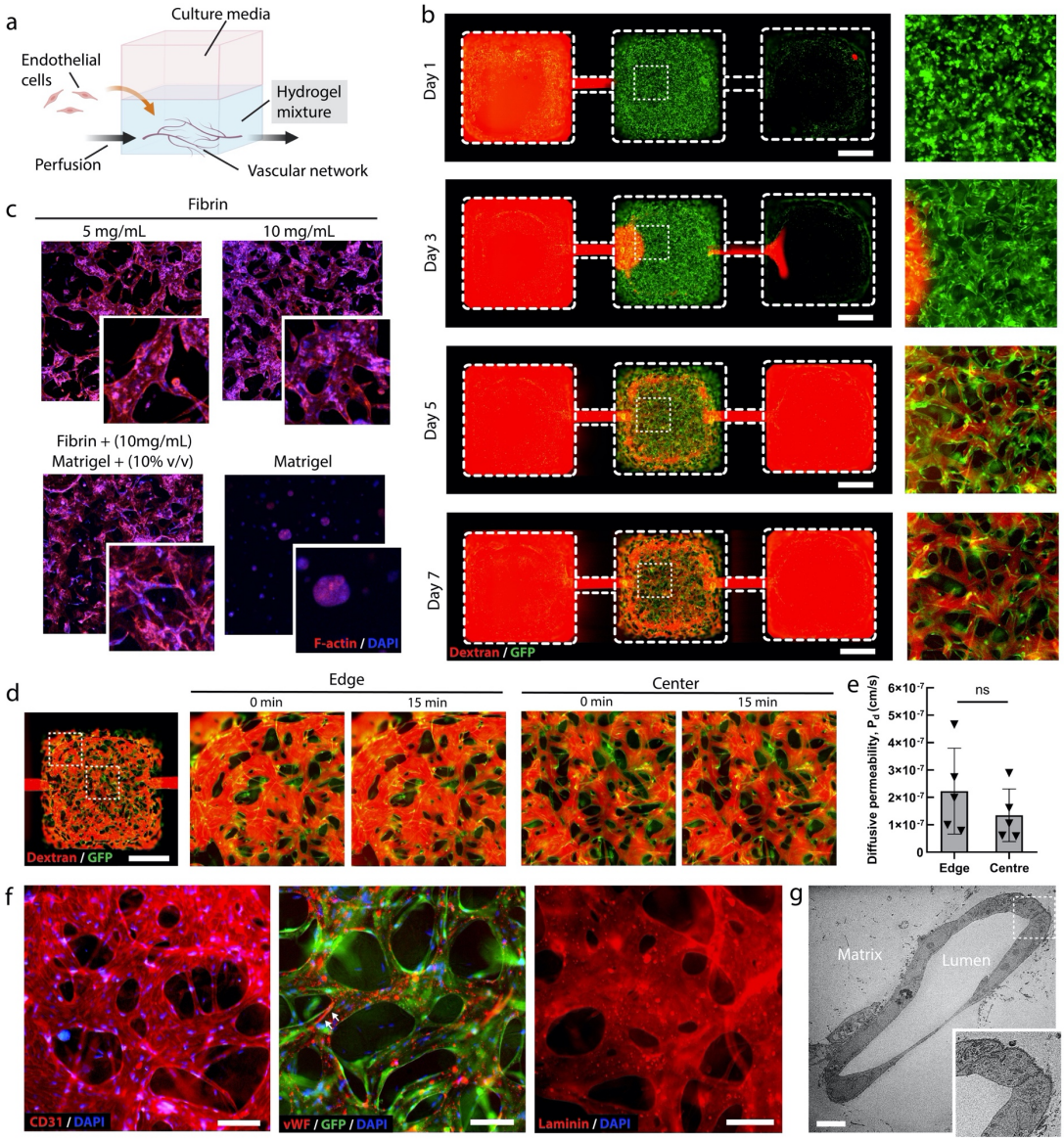

Figure 2. Culture of perfusable microvascular bed in IFlowPlate^{TM} and optimization of hydrogel matrices. **a**, Illustration of the experimental setup. **b**, Fluorescent images of GFP-HUVECs (green) assembled into a microvascular network and perfused with fluorescent 70kDa dextran (red) over time in the presence of fibroblasts. Images are stitched from multiple images. Dotted white lines outline the edge of the wells and the microchannel. Scale bar, 1mm. **c**, Confocal fluorescent images of self-assembled microvasculature network in matrices of different formulation on IFlowPlate^{TM}. Cells were stained for F-actin (red) and DAPI (blue). **d**, Time-lapse of perfused (70kDa dextran, red) vessels used for permeability quantification on Day 6. Images are stitched from multiple images. Scale bar, 1mm. **e**. Quantification of vessel diffusion permeability in different regions of the network. n=5. ns indicates not statistically significant. **f**, Fluorescent images of vessels stained for CD31 (red), vWF (red), Laminin (red), GPF, and DAPI. White arrows indicate vWF fiber. Scale bar, 100µm. **g**, Transmission Electron Microscope image of the cross-section of a single vessel. Scale bar, 5µm.

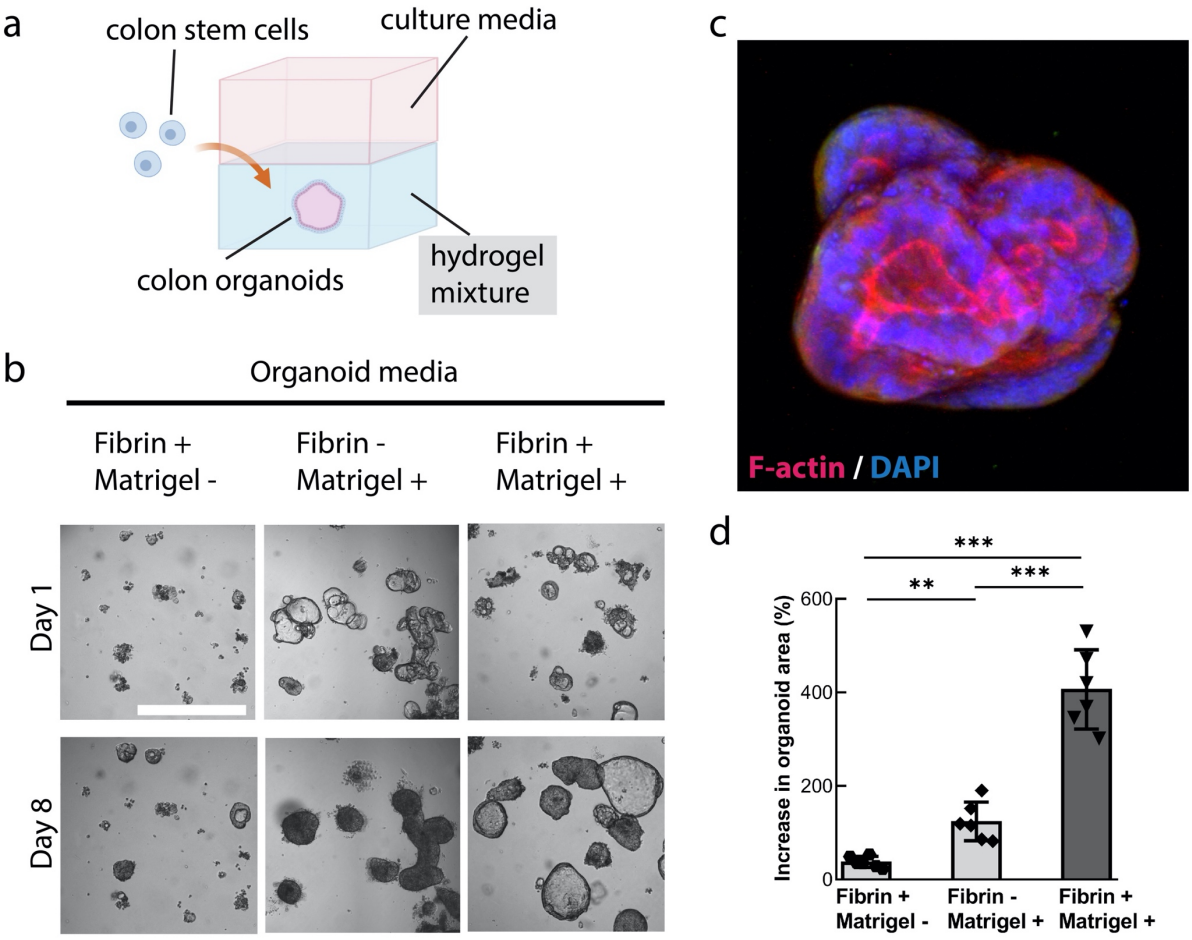

Figure 3. Optimization of hydrogel matrices for culturing human colon organoids.
a, Illustration of the experimental setup. **b**, Brightfield images of colon organoids growing in matrices of different formulation over time in a 384-well plate. Scale bar, 500 μm. **c,** Confocal fluorescent image of colon organoids grown in fibrin gel with 10% (v/v) Matrigel® for eight days on static condition. The organoid was stained for F-actin (red) and DAPI (blue). **d**, Quantification of organoid areas in different hydrogel matrices was done using Image J (n= 6, a total of 30 organoids analyzed per group). Statistical significance was determined using one-way ANOVA with Holm-Sidak method. *P<0.05 **P<0.01 ***P<0.001

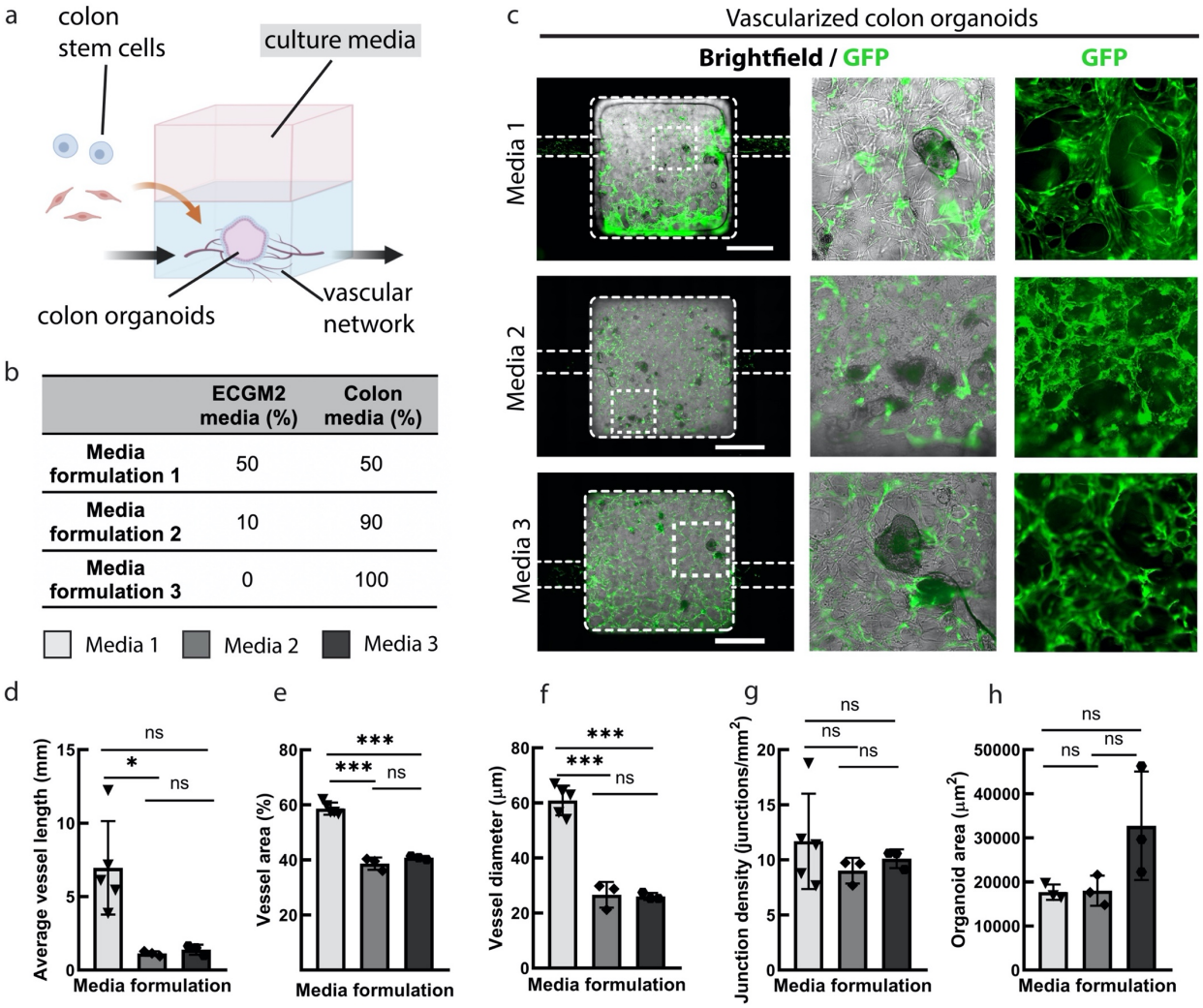

Figure 4. Media formulation optimization of vascularized human colon organoids on IFlowPlate™. **a**, Illustration of the experimental setup. **b**, Table listing the different media conditions tested for optimizing the growth of vascularized organoids. **c,** Brightfield-GFP overlay images of vascularized colon organoids in different media formulations. Zoomed in fluorescent images of the self-assembled microvascular network (green) and vascularized colon organoid (Brightfield-GFP overlay) are shown again on the right to visualize the assembled tissue better. Images are stitched from multiple images. Dotted white lines outline the edge of the wells and the microchannel. Scale bar, 1mm. **d-g,** Quantification of characteristic features of the assembled vascular network in different media formulations using AngioTool and Image J (Day 4, n=3-5). **h,** Quantification of organoid areas in different media formulations. **d,g,** Statistical significance was determined using one-way ANOVA on ranks with Dunn's method. **e,f,h,** Statistical significance was determined using one-way ANOVA with Holm-Sidak method. *P<0.05 **P<0.01 ***P<0.00. ns indicates no significant

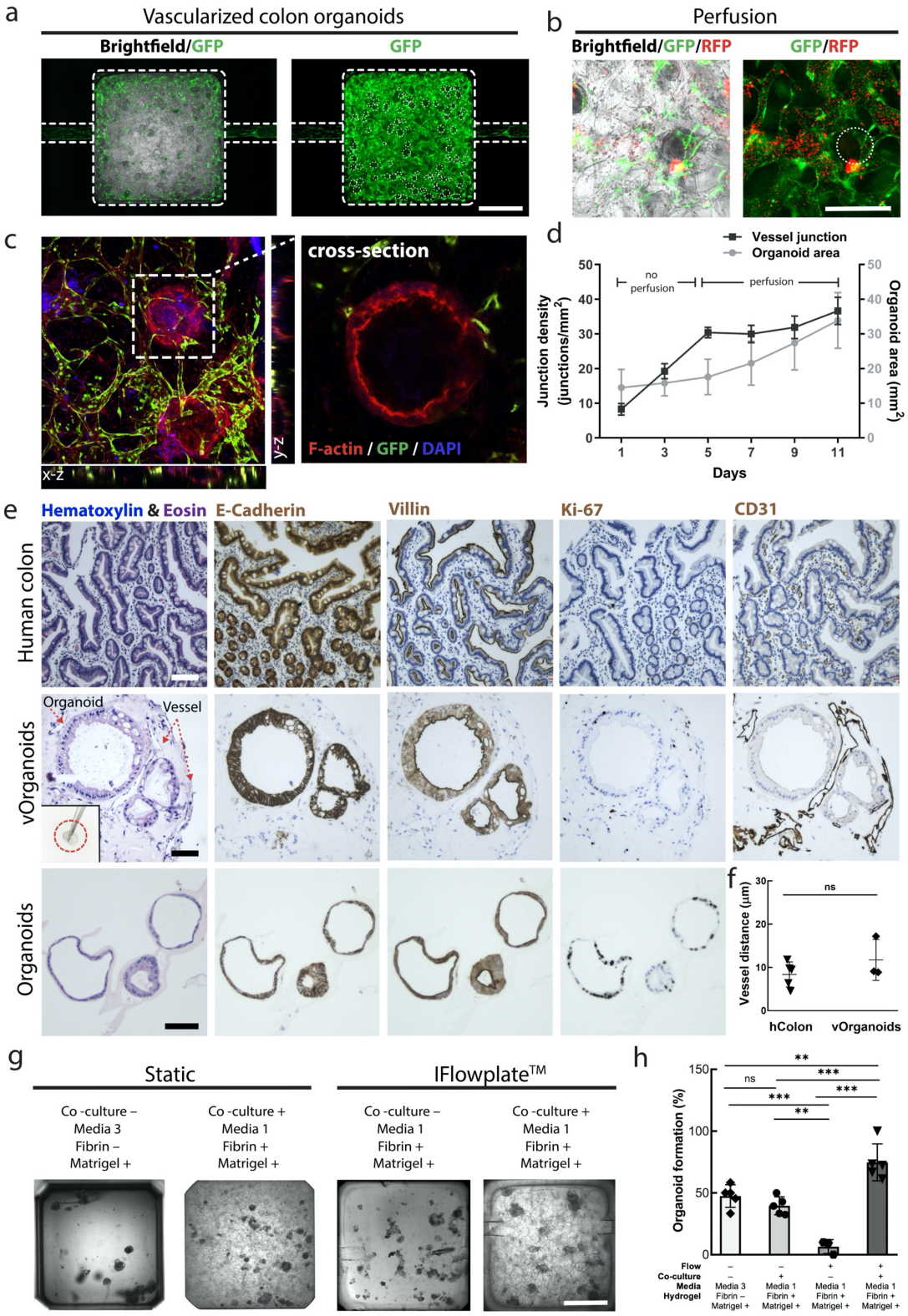
a
Vascularized colon organoids
Brightfield/GFP
GFP
b
Perfusion
Brightfield/GFP/RFP
GFP/RFP
c
cross-section
F-actin / GFP / DAPI
y-z
x-z
d
Vessel junction
Organoid area
no perfusion
perfusion
Junction density (junctions/mm²)
Organoid area (mm²)
Days
e
Hematoxylin & Eosin
E-Cadherin
Villin
Ki-67
CD31
Human colon
vOrganoids
Organoids
Organoid
Vessel
f
Vessel distance (µm)
ns
hColon
vOrganoids
g
Static
IFlowplate™
Co -culture –
Media 3
Fibrin –
Matrigel +
Co -culture +
Media 1
Fibrin +
Matrigel +
h
Organoid formation (%)
ns
Flow
Co-culture
Media
Hydrogel

Figure 5. Culture of vascularized human colon organoids on IFlowPlate™. **a**, Brightfield images of vascularized colon organoid overlaid with fluorescent images of self-assembled microvascular network from GFP endothelial cells (green) in media 1 on Day 13. Fluorescent images of the self-assembled microvascular network (green) are shown again on the right. Images are stitched from multiple images. Dotted white circles outline the colon organoids. Dotted white lines outline the edge of the wells and the microchannel Scale bar, 1mm. **b**, GFP-brightfield overlay images of vascularized colon organoid perfused with RFP-particle (1μm) in IFlowPlate™. Fluorescent images of the perfused vascular network are shown on the side for better visualization. White dotted circle labels the organoid. Scale bar, 250μm. **c**, Confocal fluorescent z-stack image of vascularized organoids stained for F-actin (red), DAPI (blue), and GFP-endothelial cells (green). The inset shows the cross-section of a colon organoid. **d**, Quantification of the dynamic assembly process of vascularized organoids over 11 days. n=6. **e**, Vascularized colon organoids (the entire tissue including the vasculatures) on day 11 were picked out of the well using a tweezer (shown bottom left corner) and then sectioned for histology. Histological section of normal human colon tissues, vascularized colon organoids and non-vascularized colon organoids stained for hematoxylin and eosin (Nuclei stains blue; ECM and cytoplasm stain pink), E-cadherin, CD31, Villin, and Ki67 (dark brown). Scale bar, 100 μm. **f**, Quantification of the distance between the vessels and the colon epithelium in both vascularized colon organoids (vOrganoids) and human colon tissues (hColon). n=3-5 independent samples with 69 measurements. 19% and 28% of vessels counted were in direct contact (a distance equal to 0) with the epithelium in human colon tissue and vascularized organoids, respectively. **g**, Brightfield images of colon organoids at Day 9 grown in IFlowPlate™ and static 384-well plate with and without endothelial co-culture in a combination of fibrin gel and Matrigel®. **h,** Quantification of the percentage of colon organoid formation in the four conditions (n = 3-5). Statistical significance was determined using one-way ANOVA followed by the Holm-Sidak method. *P<0.05 **P<0.01 ***P<0.001

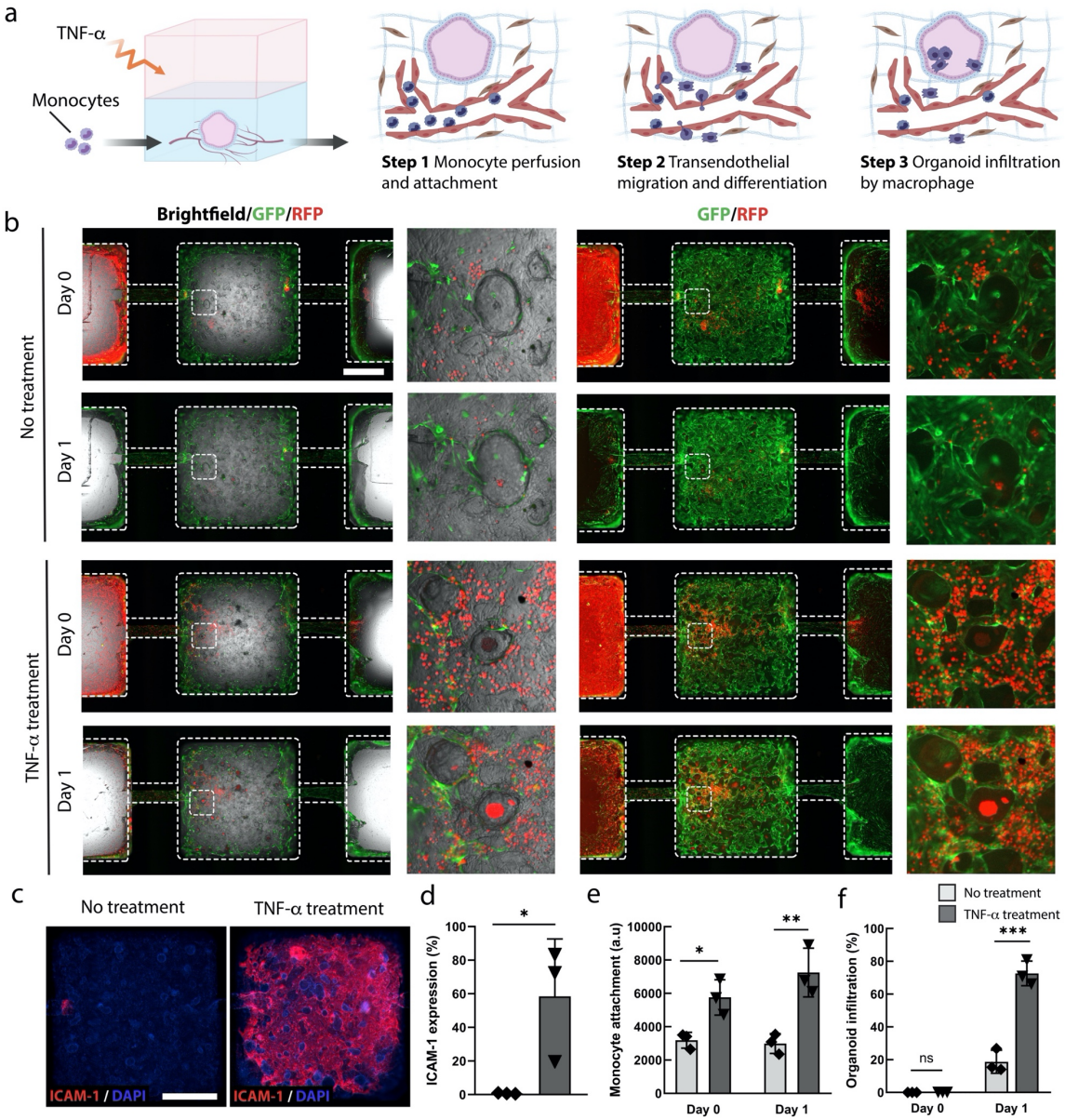

Figure 6. Vascularized human colon organoid model of tissue inflammation with innate immunity. **a**, Illustration of experimental setup and the process of monocyte infiltration into colon organoids in response to inflammatory cytokines. **b**, Brightfield and fluorescent images of vascularized colon organoid immediately and one day after monocyte perfusion with and without TNF-α stimulation (50 ng/ml, 12 h). Monocytes are labeled in red. Vasculatures are labeled in green. Dotted white lines outline the edge of the wells and the microchannel Scale bar, 1mm. **c**, Fluorescent images of blood vessels (green) treated with or without TNF-α and stained for ICAM-1 (red) and DAPI (blue) Scale bar, 1mm. **d**, Quantification of ICAM-1 staining. n=3. **e**, Quantification of monocytes attachment with and without TNF-α stimulation. n=3. **f**, Quantification of monocyte infiltration into colon organoids with and without TNF-α stimulation. Statistical significance was determined using one-way ANOVA followed by the Holm-Sidak method. *P<0.05 **P<0.01 ***P<0.001.

Table of contents

A customized 384-well plate platform, named IFlowPlate™, can be used to vascularize and perfuse patient-derived colorectal organoids for biological studies. In recent years, there have been significant interests in organoid systems for biological modeling. However, organoids usually lack vascularization and are cultured under static conditions. In this work, the matrix and culture formulation were optimized to enable the vascularization of colorectal organoids under perfusion. The platform was also used to model colon inflammation with an innate immune response. This is the first system that has demonstrated organoid vascularization with intra-vascular perfusion.

Keywords: Organoids, vasculature, colon, organ-on-a-chip, hydrogel, microfluidics

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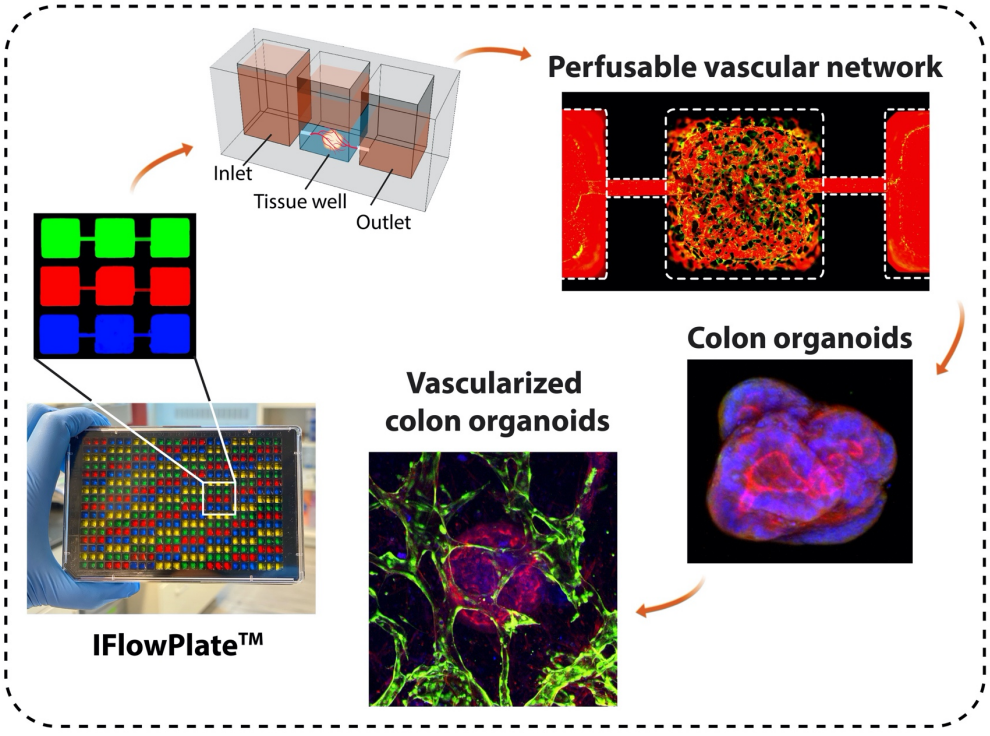
Title: IFlowPlate™ – A customized 384-well plate for the culture of perfusable vascularized colon organoids



Supplementary Materials

IFlowPlate™ – A customized 384-well plate for the culture of perfusable vascularized colon organoids

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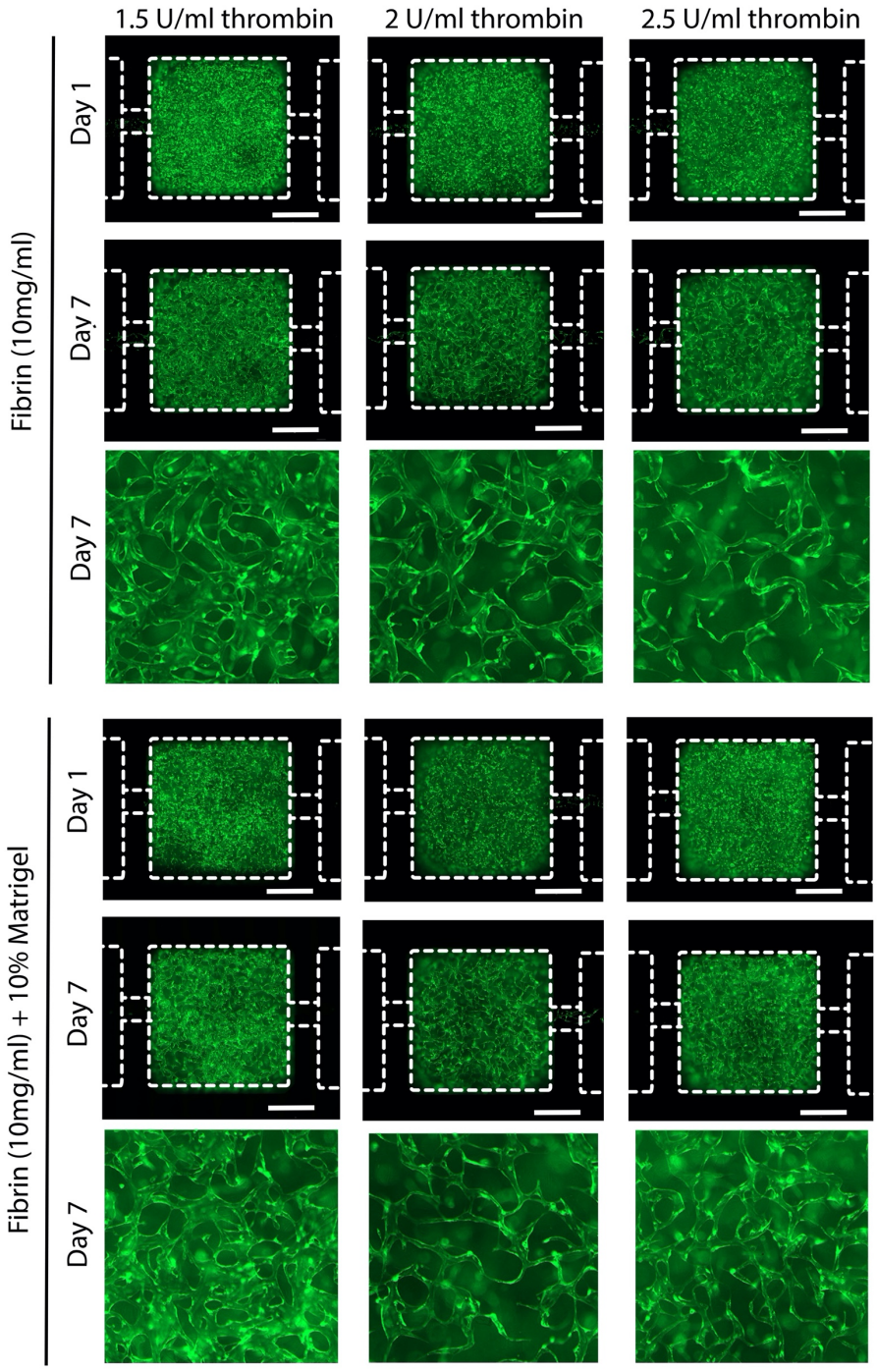
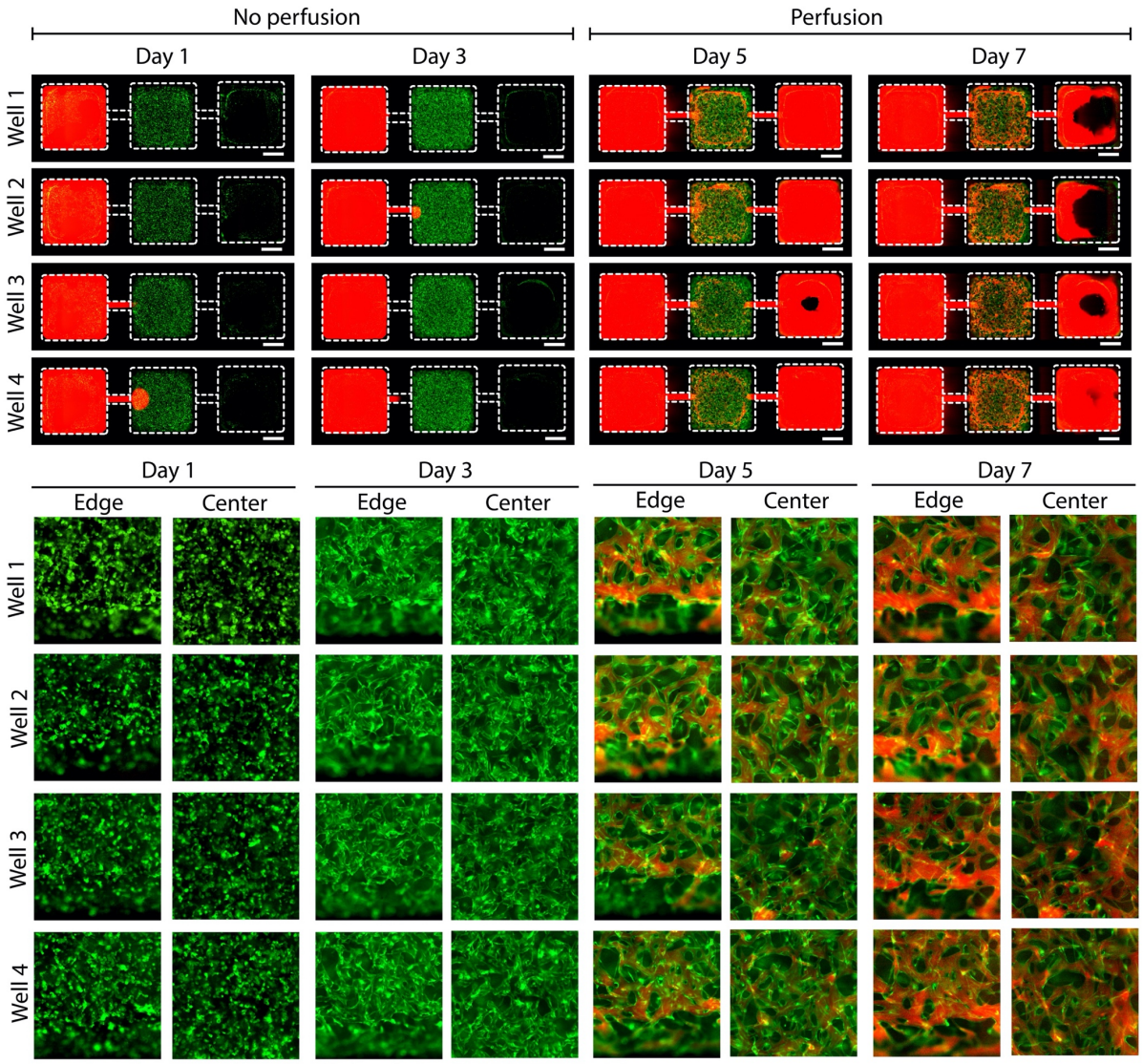
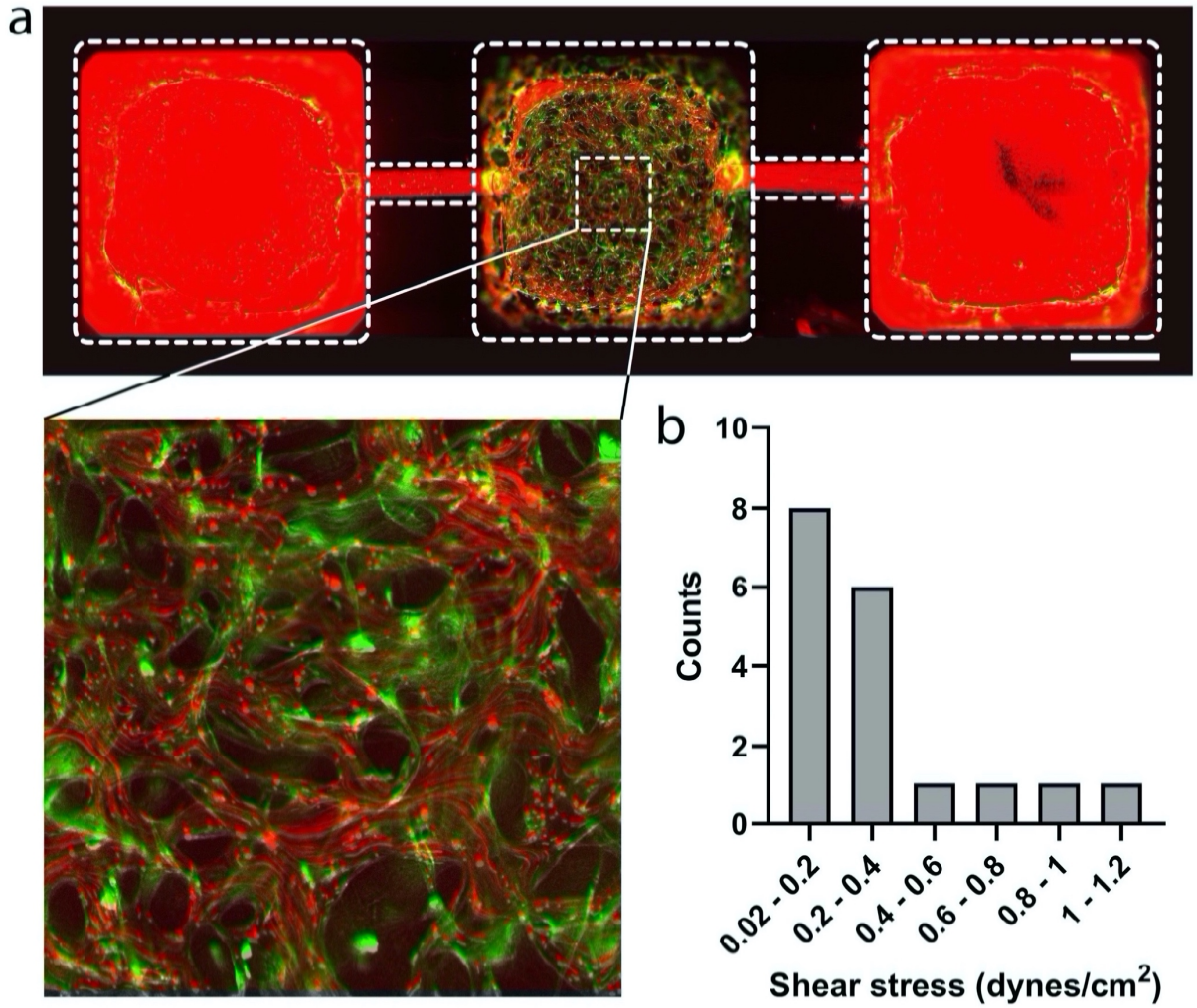


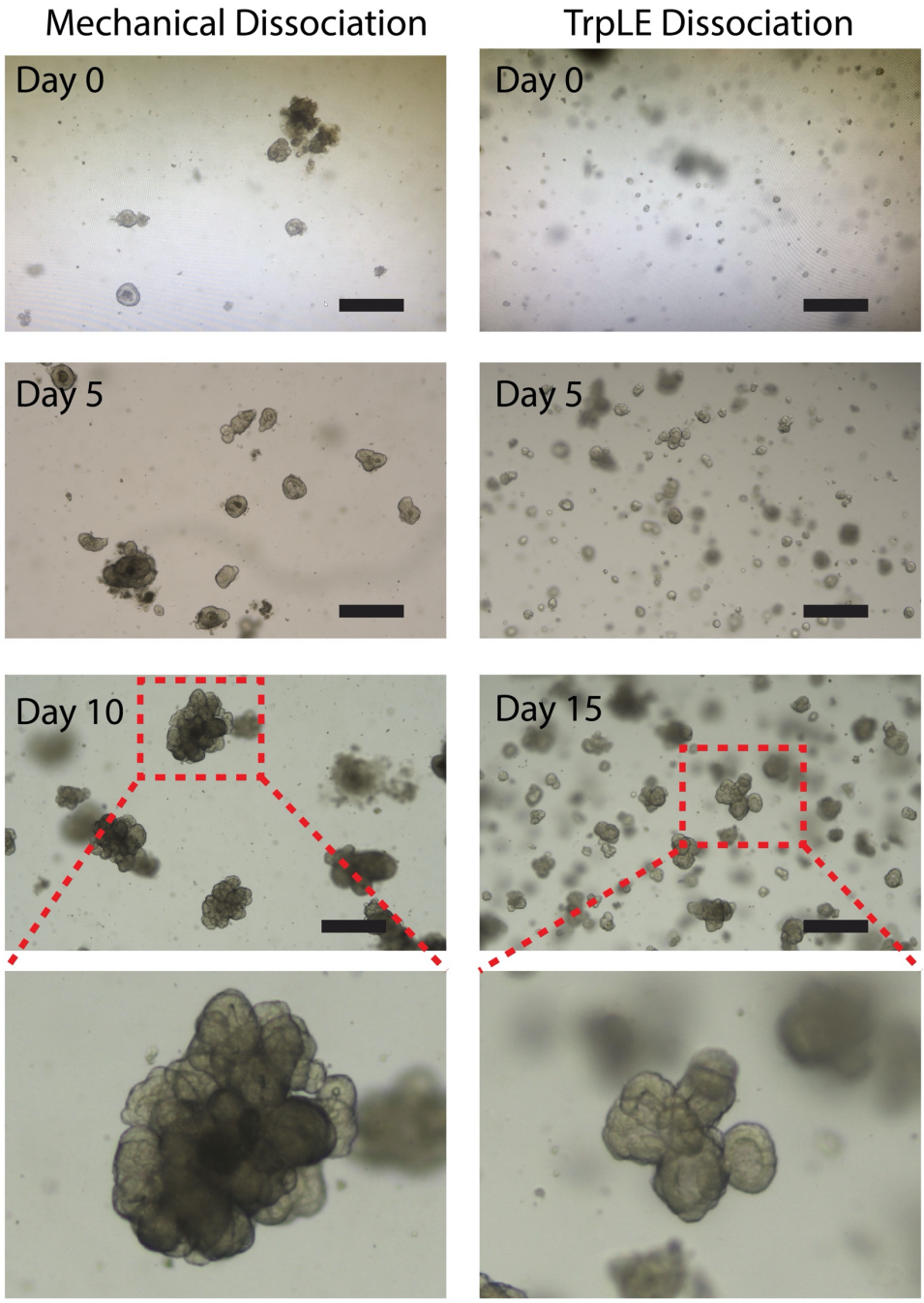
Supplementary Figure 1. Optimization of thrombin concentration. Fluorescent images of self-assembled vasculature with different concentrations of thrombin in Fibrin and Fibrin/Matrigel formulation for microvasculature (green) assembly in IFlowPlate^{TM}. Lower thrombin concentration allowed the endothelial and fibroblast cells to settle at the bottom and facilitated the formation of continuous vascular networks. Scale bar, 1mm.



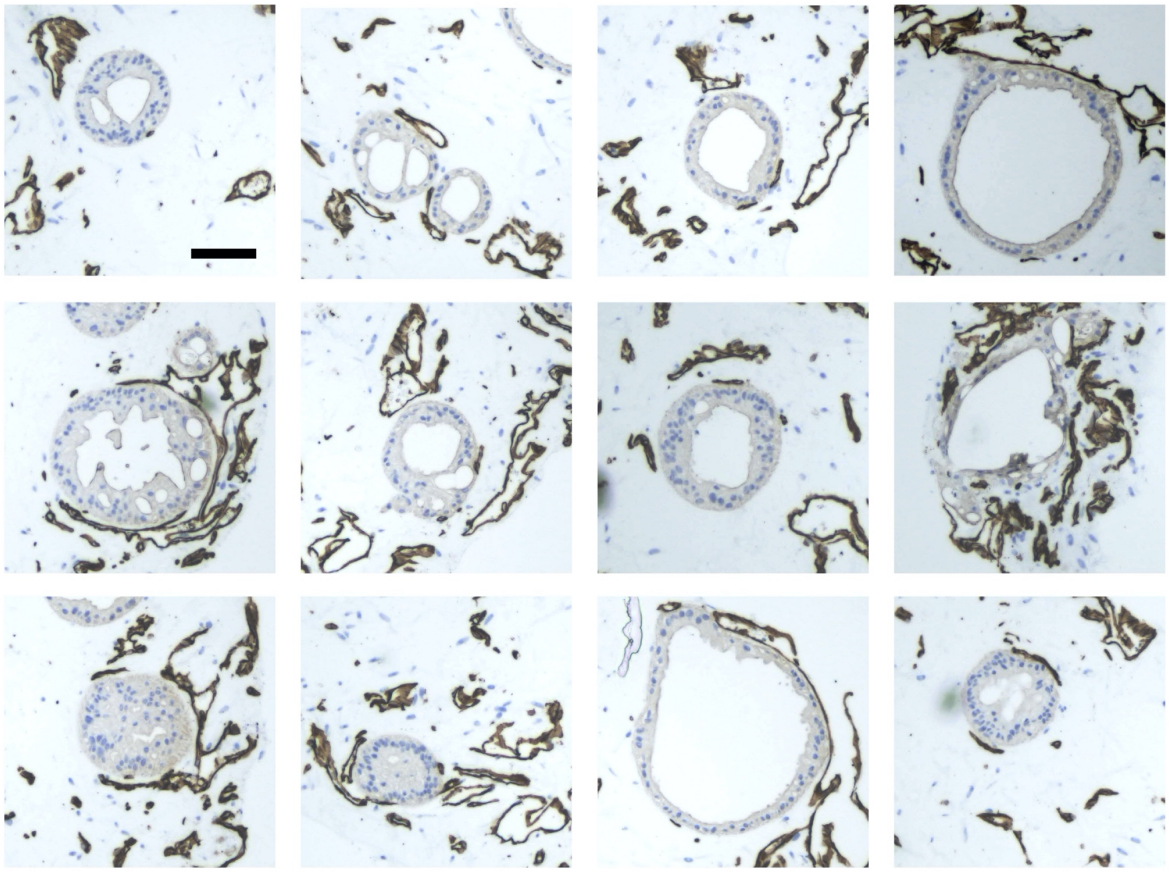
Supplementary Figure 2. Consistency in vascular perfusion. Fluorescent images of vasculatures (GFP green) perfused with 70kDa dextran (red) over time in multiple wells of an IFlowPlate™. Scale bar, 1mm.



Supplementary Figure 3. Shear stress distribution in the vascular network on IFlowPlate™. **a**, Fluorescent image of the vascular network perfused with red fluorescent particles (1um in diameter). **b**, Quantification of shear stress and shear stress distribution by tracking particle perfusion in the vascular networks. The histogram was produced by analyzing 18 regions from 6 different wells.



Supplementary Figure 4. Organoid passaging. Brightfield images of dissociation, passaging, and growth of human colon organoids in pure Matrigel™. Scale bar, 100 µm



Supplementary Figure 5. Association of vasculature and colon organoids. a, Histological section of vascularized colon organoids stained for CD31(dark brown). Scale bar, 100 μm.

Supplementary Video 1. A vascular network perfused with red fluorescent particles (1 μm) in IFlowPlate™. Exposure time 500 ms, Frame rate, 1 frame/sec.