Video Article

An *Ex Vivo* Method for Time-Lapse Imaging of Cultured Rat Mesenteric Microvascular Networks

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Abstract

Angiogenesis, defined as the growth of new blood vessels from pre-existing vessels, involves endothelial cells, pericytes, smooth muscle cells, immune cells, and the coordination with lymphatic vessels and nerves. The multi-cell, multi-system interactions necessitate the investigation of angiogenesis in a physiologically relevant environment. Thus, while the use of *in vitro* cell-culture models have provided mechanistic insights, a common critique is that they do not recapitulate the complexity associated with a microvascular network. The objective of this protocol is to demonstrate the ability to make time-lapse comparisons of intact microvascular networks before and after angiogenesis stimulation in cultured rat mesentery tissues. Cultured tissues contain microvascular networks that maintain their hierarchy. Immunohistochemical labeling confirms the presence of endothelial cells, smooth muscle cells, pericytes, blood vessels and lymphatic vessels. In addition, labeling tissues with BSI-lectin enables time-lapse comparison of local network regions before and after serum or growth factor stimulation characterized by increased capillary sprouting and vessel density. In comparison to common cell culture models, this method provides a tool for endothelial cell lineage studies and tissue specific angiogenic drug evaluation in physiologically relevant microvascular networks.

Video Link

The video component of this article can be found at https://www.jove.com/video/55183/

Introduction

Microvascular network growth and remodeling are common denominators for tissue function, wound healing, and multiple pathologies and a key process is angiogenesis, defined as the growth of new blood vessels from existing ones[1,2]. For tissue engineering new vessels or designing angiogenic based therapies, understanding the importance of the cellular dynamics involved in angiogenesis is critical. However, this process is complex. It can vary at specific locations within a microvascular network and involves multiple cell types (*i.e.* endothelial cells, smooth muscle cells, pericytes, macrophages, stem cells) and multiple systems (lymphatic networks and neural networks). Although *in vitro* models have contributed tremendously to examining the relationship between different cells involved in angiogenesis[3], their physiological relevance can be undermined due to their limited complexity and the fact that they do not closely reflect an *in vivo* scenario. To overcome these limitations, three-dimensional culture systems[3], *ex vivo* tissue models[4], microfluidic systems[5,6], and computational models[7] have been developed and introduced in recent years. However, there is still a need for a model with time-lapse capability to investigate angiogenesis in intact microvascular networks *ex vivo*. The establishment of new time-lapse models for angiogenesis studies with that level of complexity will provide an invaluable tool to understand the underlying mechanisms regulating angiogenesis and to improve therapies.

A potential model that enables the *ex vivo* investigation of angiogenesis across an intact microvascular network is the rat mesentery culture model[8]. In recent work, we have demonstrated that blood and lymphatic microvascular networks remain viable after culture. More importantly, the rat mesentery culture model can be used to investigate functional pericyte-endothelial cell interactions, blood and lymphatic endothelial cell connections, and time-lapse imaging. The objective of this paper is to provide our protocol for the time-lapse imaging method. Our representative results document the multiple cell types that remain viable after the stimulation of angiogenesis with serum and offer examples of using this method for quantifying tissue specific angiogenic responses as well as endothelial cell tracking studies.

Protocol

All animal experiments and procedures were approved by the Tulane University's Institutional Animal Care and Use Committee (IACUC).

1. Surgical Procedure Setup

1. Autoclave instruments, surgical supplies, and culture supplies prior to surgery. Surgical supplies for each rat include: 1 drape, 1 drape with pre-cut hole (0.5 in x 1.5 in) in the center, gauze pads, and 1 absorbent underpad. Surgical instruments include: 1 scalpel with a number 10

blade, 2 pairs of tweezers, and a pair of fine scissors. Culture supplies include: 1 drape, 1 pair of tweezers, and prepared 6-well plate inserts with polycarbonate filters.

2. Sterilize a plexiglass platform, a surgical stage and a surgical benchtop space with 70% ethanol. Keep the surgical stage in a sterile bowl until use.
    1. Create a surgical stage by drilling an approximately 2 in by 1 in hole in the center of a 100 mm culture dish. Next, use sandpaper to smooth any sharp edges and add a layer of silicone glue to the hole's edges to create a raised surface for the tissues.
    2. Alternatively, design the surgical stage using CAD software and make by 3-D printing (**Figure 1**).
3. Place a sterile absorbent underpad down and lay a plexiglass platform on top of it. Place the drape, without a pre-cut hole, over a heated pad next to the absorbent underpad.
4. Pre-warm sterile phosphate-buffered saline (PBS), media and saline to 37 °C. Place media and PBS in separate culture dishes atop the heating pad and place saline in a 50 mL conical tube next to the surgical setup.
5. Make sure all packages are opened prior to the beginning of the surgery to ensure sterile handling of all materials. A complete list of the common tools used in this procedure are listed in the **Table of Specific Surgical Materials and Tools**.

2. Mesentery Tissue Harvesting

1. Use adult male Wistar rats (350 ± 25 g; 6 - 8 weeks of age). Other strains and ages of rats can be substituted.
2. Anesthetize the rat *via* an intramuscular injection of ketamine (80 mg/kg body weight) and xylazine (8 mg/kg body weight). Confirm the rat is under anesthesia by pinching between the toes to check for a reflex response; there should be none. Pre-emptive analgesia for this terminal procedure is not necessary.
3. Shave the abdominal region and remove remaining hair using hair removal cream. Wipe abdominal skin twice with 70% isopropyl alcohol followed by povidone-iodine. For the wipes the surgeon should start at the center of the surgical site and move to the outside of the prepared area in a circular manner as to not overlap areas that have been previously scrubbed with the same piece of sterile gauze or sterile cotton swab. Then transfer animal to the sterile surgical setup and place atop the plexiglass platform.
4. Using a scalpel blade, make a 0.75 in - 1.25 in incision in the gut starting 1 in below the sternum. Be careful not to puncture the bowel or mesentery (1 layer of skin, 1 layer of connective tissue, and 1 layer of muscle).
5. Place a drape with a pre-cut hole over the incision and place a sterile surgical stage atop the drape. Ensure the opening aligns with the incision. Use sterile cotton-tipped applicators to locate and pull out the ileum through the surgical stage opening.
6. Pull 6 - 8 mesenteric windows through the stage using cotton-tipped applicators, and be careful not to touch the windows (**Figure 1**). Tissues are typically harvested from the ileum region of the small intestine starting near the cecum. Keep exposed tissues moist with warmed sterile saline as needed using a sterile syringe to drip the solution.
7. Euthanize the rat *via* intracardiac injection of pentobarbital sodium (0.2 mL per rat). Before removing mesenteric windows, ensure the rat is euthanized by palpating the heart; there should be no pulse.
8. Remove desired mesentery tissues by using tweezers to grab the fat pad and fine scissors to cut the window. Leave a border of fat (2 mm) around the window. Wash tissues once in warmed sterile PBS and once in media.
9. Return exteriorized ileum to the abdominal cavity and dispose of animal according to institutional guidelines.

3. Mesentery Tissue Culture for Time-Lapse Studies

1. Transfer autoclaved culture supplies (see section 1.1) and tissues to a sterile laminar flow hood.
2. Use tweezers to transfer each tissue atop a polycarbonate filter membrane. Grab tissues by the fat pad to avoid damaging the vasculature.
3. Quickly spread the tissue using the fat pad, being careful not to touch the window. Invert the insert with the tissue into the bottom of a 6-well plate and cover with 3 mL of media (**Figure 1**). Typical media used for this procedure includes Minimum Essential Media (MEM) with 1% Penicillin Streptomycin (PenStrep) and 10% Fetal Bovine Serum (FBS). Media can be supplemented with other serums and/or growth factors to stimulate angiogenesis.
4. Repeat steps 3.2 - 3.3 for each tissue and culture in standard incubator conditions (5% CO_2, 37 °C) for up to 5 days.

4. Time-Lapse Imaging of Mesentery Tissue

1. On the day of imaging, supplement the media in each well with conjugated BSI-Lectin and incubate under standard culture conditions for 30 min. Wash tissues twice with lectin-free media. BSI-Lectin stain will remain visible on the mesentery tissue for up to 3 days in culture.
2. Transfer the plate to a microscope stage. Identify blood and lymphatic vessels based on their morphology and network structure.
3. Locate a desired network region on each tissue and take images. Take note of the imaging location to ensure the same region will be captured for subsequent images. If using a motorized stage, document the coordinates.
4. Return tissues to the incubator and continue to culture until desired end point. Repeat steps 4.1 - 4.3 as needed depending on desired experimental time points.

5. Tissue Immunolabeling

1. **BSI-Lectin Labeling**
    1. Incubate tissues for 30 min at 37 °C with 1:40 FITC-conjugated lectin in media (2.5 mL antibody solution per well in 6-well plate) followed by two rinses with media. For rinses, add media and then immediately replace.
2. **Live/Dead Labeling**
    1. Incubate tissues for 10 min at 37 °C with 1:500 2 mM ethidium homodimer-1 and 1:500 1 mM calcein AM in media (2.5 mL antibody solution per well in 6-well plate) followed by two rinses with media.

3. **BSI-Lectin/NG2 Labeling**
    1. Spread tissues on a microscope slide (1 - 2 tissues/slide) and allow to dry. Remove excess fat with a scalpel by pressing down firmly to excise the fat.
    2. Fix tissues in cold methanol for 30 min at -20 °C. Wash tissues with PBS (3 x 10 min).
    3. For primary antibody labeling incubate tissues for 1 h at room temperature with 1:100 rabbit polyclonal NG2 antibody and 5% normal goat serum (NGS). Wash tissues with PBS (3 x 10 min).
    4. For secondary antibody labeling incubate tissues for 1 h at room temperature with 1:100 goat anti-rabbit CY2-conjugated antibody (GAR-CY2) and 5% NGS. Wash tissues with PBS (3 x 10 min).
    5. Incubate tissues for 30 min at room temperature with 1:40 FITC-conjugated lectin in PBS followed by two rinses with PBS. For rinses, add PBS and then immediately replace.
    6. To mount the slides, cover tissues with 50:50 PBS and glycerol solution and place coverslip on top. Seal the slide edges using nail polish.

4. **LYVE-1/PECAM Labeling**
    1. Spread tissues on a microscope slide (1 - 2 tissues/slide) and allow to dry. Remove excess fat with a scalpel by pressing down firmly to excise the fat.
    2. Fix tissues in cold methanol for 30 min at -20 °C. Wash tissues with PBS + 0.1% saponin (3 x 10 min).
    3. For primary antibody labeling incubate tissues for 1 h at room temperature with 1:200 mouse monoclonal biotinylated CD31 antibody and 1:100 rabbit polyclonal LYVE-1 antibody in PBS + 0.1% saponin + 2% bovine serum albumin (BSA) + 5% NGS. Wash tissues with PBS + 0.1% saponin (3 x 10 min).
    4. For secondary antibody labeling, incubate tissues for 1 h at room temperature with 1:500 CY3-conjugated streptavidin antibody and 1:100 GAR-CY2 in PBS + 0.1% saponin + 2% BSA + 5% NGS. Wash tissues with PBS + 0.1% saponin (3 x 10 min).
    5. To mount slides, cover tissues with 50:50 PBS and glycerol solution and place a coverslip on top. Seal the slide edges using nail polish.

5. **BrdU/BSI-Lectin Labeling**
    1. Add 1 mg/mL BrdU to media and replace tissue media with BrdU solution. Incubate for 2 h at 37 °C.
    2. Spread tissues on a microscope slide (1 - 2 tissues/slide) and allow to dry. Remove excess fat with a scalpel by pressing down firmly to excise the fat.
    3. Fix tissues in cold methanol for 30 min at -20 °C. Wash tissues with PBS (3 x 10 min).
    4. Denature tissue DNA in 2 M HCl for 1 h at 37 °C. Wash tissues in PBS + 0.1% saponin (3 x 10 min).
    5. For primary antibody labeling, incubate tissues for 1 h at room temperature with 1:100 monoclonal mouse anti-BrdU in PBS + 0.1% saponin + 2% BSA + 5% NGS. Wash tissues with PBS + 0.1% saponin (3 x 10 min).
    6. For secondary antibody labeling, incubate tissues for 1 h at room temperature with 1:100 goat anti-mouse Cy3-conjugated antibody (GAM-Cy3) in PBS + 0.1% saponin + 2% BSA + 5% NGS. Wash tissues with PBS + 0.1% saponin (3 x 10 min).
    7. Incubate tissues for 30 min at room temperature with 1:40 FITC-conjugated lectin in PBS followed by two rinses with PBS.
    8. To mount slides, cover tissues with 50:50 PBS and glycerol solution and place coverslip on top. Seal the slide edges using nail polish.

6. **BSI-Lectin/CD11b labeling**
    1. Spread tissues on a microscope slide (1 - 2 tissues/slide) and allow to dry. Remove excess fat with a scalpel by pressing down firmly to excise the fat.
    2. Fix tissues in cold methanol for 30 min at -20 °C. Wash tissues with PBS + 0.1% saponin (3 x 10 min).
    3. For primary antibody labeling incubate tissues for 1 h at room temperature with 1:100 mouse anti-rat CD11b in PBS + 0.1% saponin + 2% BSA + 5% NGS. Wash tissues with PBS + 0.1% saponin (3 x 10 min).
    4. For secondary antibody labeling incubate tissues for 1 h at room temperature with 1:100 GAM-Cy3 in PBS + 0.1% saponin + 2% BSA + 5% NGS. Wash tissues with PBS + 0.1% saponin (3 x 10 min).
    5. Incubate tissues for 30 min at room temperature with 1:40 FITC-conjugated lectin in PBS followed by two rinses with PBS.
    6. To mount slides, cover tissues with 50:50 PBS and glycerol solution and place coverslip on top. Seal the slide edges using nail polish.

Representative Results

After 3 days in culture, tissues were labeled with a live/dead viability/cytotoxicity kit to demonstrate the viability of the microvasculature in the rat mesentery culture model (**Figure 2A**). The majority of cells present in the mesentery remained viable in the culture where endothelial cells were identified based on their location in microvascular segments. Endothelial cell proliferation was also confirmed by lectin/BrdU labeling (**Figure 2D**). Smooth muscle cell and pericyte presence along vessels was confirmed with NG2 labeling (**Figure 2B**). Labeling for LYVE1 and PECAM identified branching lymphatic and blood microvascular networks and confirmed the maintained lymphatic *versus* blood endothelial cell phenotype (**Figure 2C**).

The time-lapse feature of this model was utilized by labeling the microvascular networks with BSI-lectin at different time points and imaging the same region within the network over time; this capability is particularly valuable for investigating tissue specific angiogenic responses. The supplementation of media with 10% serum caused a robust angiogenic response after 3 days of stimulation. Additionally, new vessel segments and capillary sprouts were identified by day 5 of stimulation (**Figure 3**). The time-lapse imaging method allowed for the quantitative comparison of network regions before and after stimulation (**Figure 4**). For this representative study, which corroborates our previous results[9], the number of vessels per vascular area and the number of capillary sprouts per vascular area were quantified from one 4X image per tissue. Blood vessel segments were defined as lectin-positive blood endothelial cell segments present between two branch points and capillary sprouts were defined as blind ended segments originating from a host vessel. Time-lapse comparison of network regions also enabled tracking of endothelial cell segments (**Figure 5**) and identification of blood/lymphatic vessel mis-patterning (**Figure 6**). Labeling of cultured tissues for lectin and CD11b additionally confirmed the presence of interstitial resident macrophages (**Figure 7**) in remodeling networks.

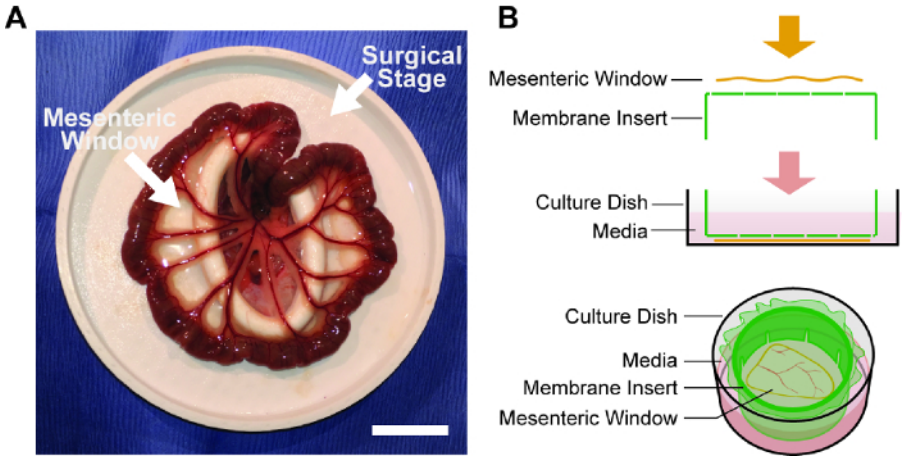

Figure 1. Mesenteric windows were located by pulling out the small intestine through a surgical stage. The surgical stage was designed and made by 3-D printing. The elliptical hole in the center is approximately 2 in by 1 in **(A)**. The mesenteric windows were then spread out on top of a membrane insert, and the insert was inverted and put into a well **(B)**. Scale bar = 2 cm. Please click here to view a larger version of this figure.

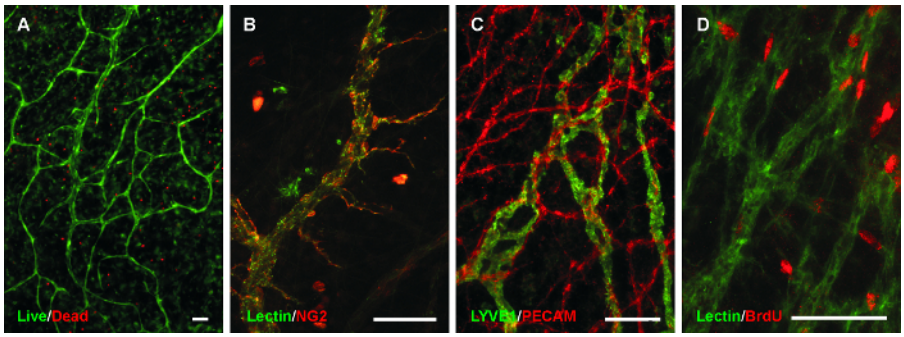

Figure 2. Blood vessels remain viable in the rat mesentery culture model. Live/dead assay performed after culture showed a high ratio of live cells (green) to dead cells (red) specifically along the blood vessels **(A)**. Mesentery tissues were labeled with lectin and anti-NG2, to identify pericytes (red) alongside vessels (green) and to confirm that different types of cells are present in the post-culture tissues **(B)**. Tissues were also labeled against PECAM/LYVE-1 to identify blood (red) vessels from lymphatic (green) vessels **(C)**. To investigate if microvascular cells undergo proliferation in culture, mesentery tissues were labeled with lectin/anti-BrdU. On capillary segments labeled with lectin (green), multiple cells were confirmed to be proliferative (red) **(D)**. Scale bars = 100 µm. Please click here to view a larger version of this figure.

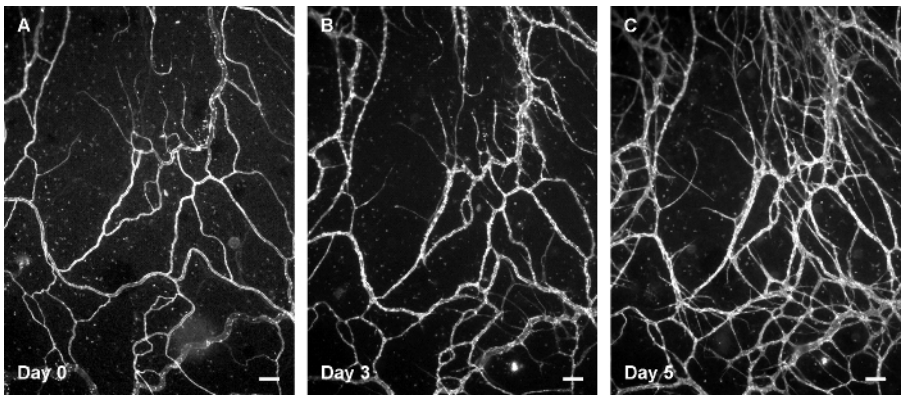

Figure 3. Time-lapse imaging of the rat mesentery enables observing microvascular remodeling over the course of the culture. A robust angiogenic response was observed after 3 **(B)** and 5 days **(C)** of culture with 10% serum stimulation. Scale bars = 100 µm. Please click here to view a larger version of this figure.

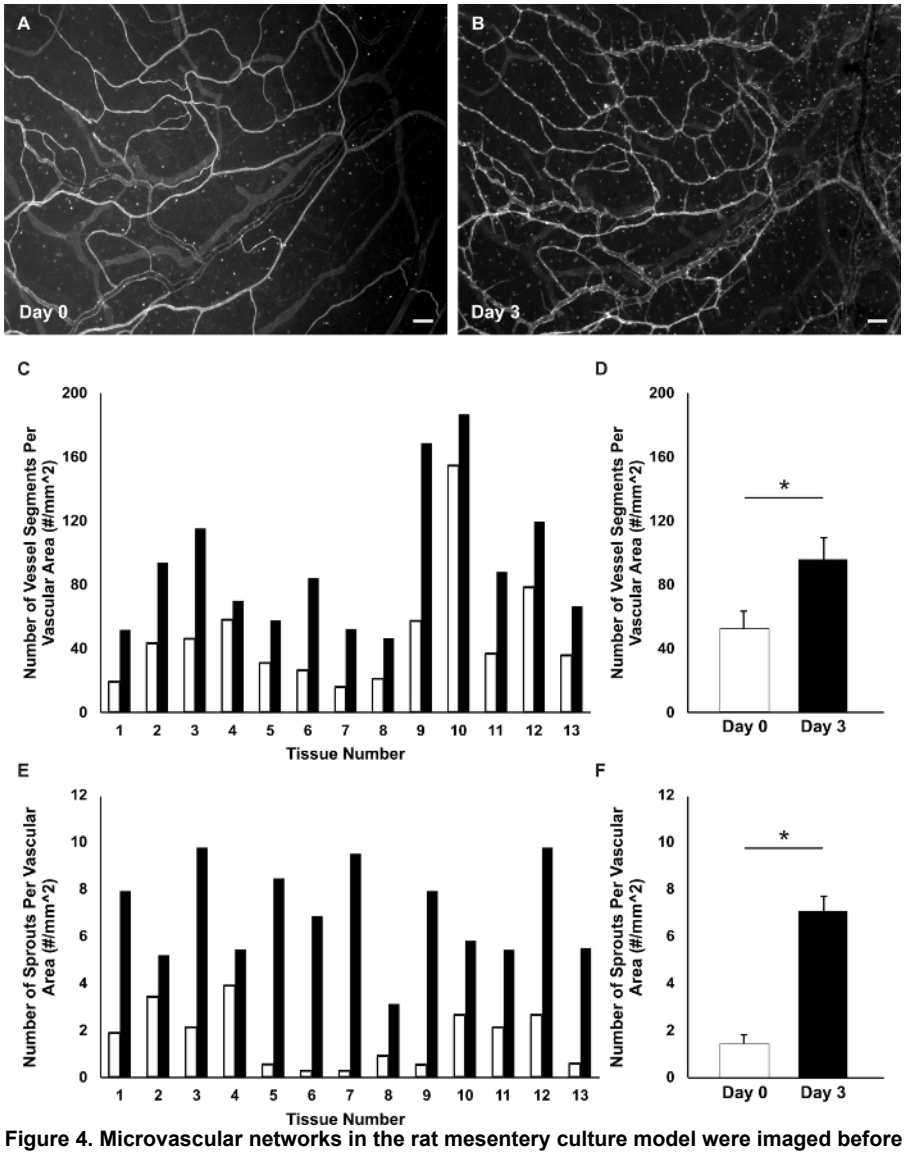

Figure 4. Microvascular networks in the rat mesentery culture model were imaged before and after angiogenesis. Comparison of the same network labeled with lectin on day 0 and day 3 (**A, B**) post-stimulation with 10 % serum identifies new vessels. Lectin also labels a population of unidentified interstitial cells. Quantification of vessel density (**C, D**) and the number of capillary sprouts per vascular area (**E, F**) confirmed an increase in both metrics for each tissue. **C, E**) Before (day 0) and after (day 3) comparisons per tissue. **D, F**) Comparison between day 0 and day 3 averages using a paired Student's t-test confirmed a significant difference in both the average number of vessel segments ($p < 0.0001$) and the average number of sprouts ($p < 0.00001$) per vascular area. White bars represent day 0, and black bars represent day 3. Values are averages ± SEM. For this representative analysis, 13 tissues were harvested from 2 rats. Scale bars = 100 µm. Please click here to view a larger version of this figure.

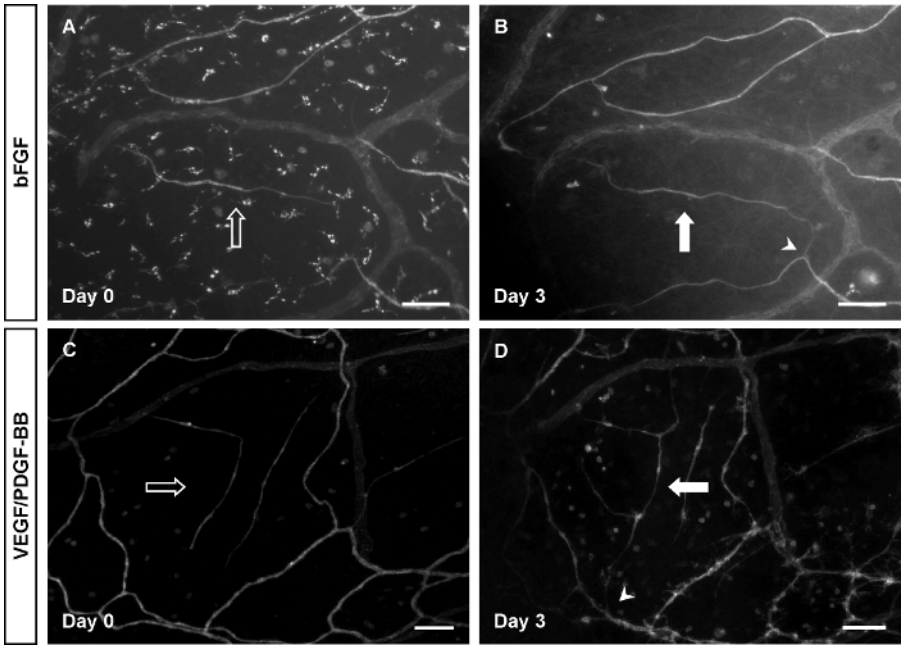

Figure 5. The rat mesentery culture model can be used for investigating vascular island fate and incorporation into nearby networks. Using time-lapse imaging, vascular islands, defined as disconnected endothelial segments, were identified on day 0 and their connection to the nearby network was confirmed by day 3 post-angiogenic stimulation. Mesentery tissues were stimulated with bFGF (**A, B**) and VEGF/PDFG-BB (**C, D**). Hollow arrows show disconnected segments on day 0 and solid arrows represent island connection to the network. Arrowheads indicate the location of connections between a vascular island and the nearby network. Scale bars = 100 µm. Please click here to view a larger version of this figure.

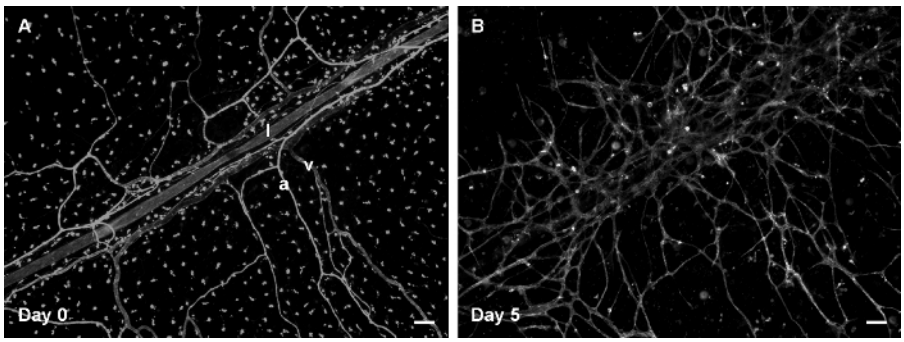

Figure 6. Time-lapse images demonstrate the ability to observe lymphatic and blood vessel patterning. Lymphatic (l) vessels can be distinguished from arterioles (a) and venules (v) based on labeling morphology on day 0 **(A)**. On day 5 post-stimulation with 10% serum, lymphatic morphology is lost and vessels appear to have integrated with the nearby angiogenic blood vessels **(B)**. Scale bars = 100 µm. Please click here to view a larger version of this figure.

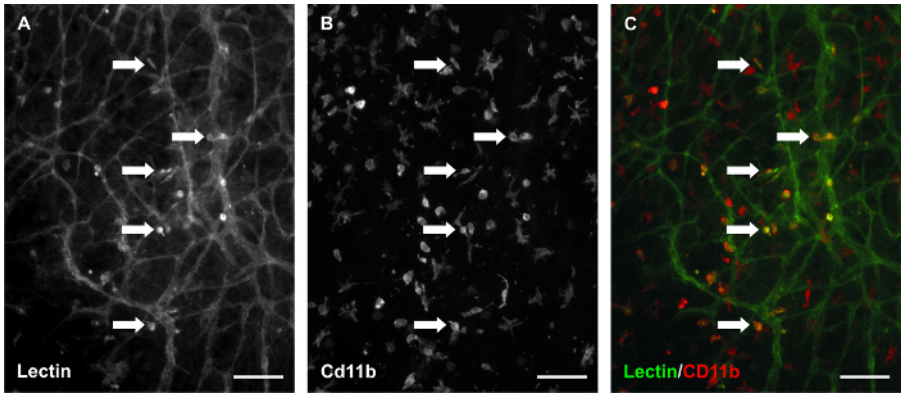

Figure 7. Macrophages remain present in cultured rat mesenteric tissues. Lectin/CD11b co-labeling of tissues cultured for 3 days with 10% serum suggest that lectin positive interstitial cells are a subset of macrophages. **(A)** A representative image of BSI-lectin labeling. **(B)** Cd11b labeling in the same field of view. **(C)** The merged image. The arrows identify examples of co-labeling. Scale bars = 100 µm. Please click here to view a larger version of this figure.

Discussion

This protocol documents a method for using the rat mesentery culture model as an *ex vivo* tool for time-lapse imaging of microvascular network growth. Previous work in our laboratory has established the use of our model for 1) angiogenesis[8], 2) lymphangiogenesis[8], 3) pericyte-endothelial cell interactions[8], and 4) anti-angiogenic drug testing[9]. The ability for imaging cultured rat mesentery tissues at multiple time points offers a quantitative assay for evaluating tissue-specific growth responses and the tracking of cell-cell interactions during various angiogenic stimuli. The increased proliferation of endothelial cells during angiogenesis and the presence of pericytes are consistent with our previous work[8] and validate the dynamic interactions between multiple cell types during angiogenesis in cultured rat mesenteric tissues.

Compared to commonly used tissue culture models and *in vitro* cell culture systems, the rat mesentery culture model is unique because growth occurs within an intact, real microvascular network. Consider in contrast the aortic ring assay, which was established to study angiogenic sprouting from aortic segments in a collagen gel[10]. While sprouting in the aortic ring involves multiple cell types, capillary sprouts grow out of the excised segments of the aorta, which is very different from the *in vivo* scenario. The brain slice model is another *ex vivo* model, but it is void of lymphatic vessels. Moreover, the brain slice model has not been shown to be capable of time-lapse imaging before and after angiogenic stimulation[11]. Another *ex vivo* model that has been recently introduced is the retina culture model. The advantage of the retina model is that angiogenesis occurs from intact microvascular networks within the tissue[12,13]. For these models, GFP-transgenic mice strains can be used to be able to observe capillary sprouting over time, but unfortunately, the mouse mesentery is avascular[14], eliminating the GFP-transgenic mice mesentery substitution for rat mesentery, as utilized in our model. Furthermore, we show that a simple lectin labeling of rat mesentery tissues in culture is sufficient to determine network growth at different time points and in comparison to the other *ex vivo* models, our model allows for simultaneous observation of both blood and lymphatic endothelial cells.

BSI-lectin was used in this paper to visualize microvascular networks and detect angiogenic responses. Lectin is a protein structure that binds to glycoproteins on endothelial cells and was selected for this protocol due to its short incubation time compared to endothelial antibody markers. Lectin is less expensive than antibodies and it does not require fixing; it can also be easily mixed in the culture media and replaced with fresh media after the incubation period ends. While future studies are needed to elucidate the potential effects of the lectin labeling technique on the angiogenic process, our representative results (**Figure 4**) demonstrate that robust angiogenesis can be induced and previous work[9] demonstrates that angiogenesis in lectin labeled networks can be inhibited *via* targeting Vascular Endothelial Growth Factor (VEGF). Antibody markers can potentially be used as an alternative labeling approach when there is a need for more specific markers, or when there is a need to investigate other cell types that are present in the microvascular networks such as smooth muscle cells, pericytes, and nerves. Another potential method for visualizing cells would be gene transfection.

The advantage of using the time-lapse rat mesentery model has been highlighted in the representative results for this protocol. The comparison of images before and after treatment reduces issues of variability that influence non-paired statistical analysis. The explant specific responses varied from 20% to 233% increase in vessel density and from 40% to 3,500% increase in sprout density. The specific causes for this variation remain unknown, but measuring growth in the same tissue over time present the ability to confirm tissue specific responses.

Comparative analysis of images at different time points during microvascular growth also allows for tracking endothelial cells. For example, our lab has identified vascular islands as endothelial cell segments in the vicinity of microvascular networks that are disconnected from nearby networks[15,16]. To confirm that these islands connect to the nearby network in response to angiogenic stimuli, the rat mesentery culture model was used. As shown in **Figure 5,** vascular islands were tracked after tissue stimulation with basic Fibroblast Growth Factor (bFGF) or VEGF plus Platelet-Derived Growth Factor (PDGF). We have also shown similar results post serum stimulation (data not shown here). After the stimulation of angiogenesis, the originally disconnected vascular islands can be found connected to nearby networks.

Other potential applications of the rat mesentery culture model could leverage the ability to investigate the relationships between lymphatic and blood vessels and their respective endothelial cells and the tracking of interstitial cell fate. Time-lapse images of the same microvascular networks before and after stimulation with 10% serum in this model provided examples of potential lymphatic-to-blood vessel integration (**Figure 6**). Before stimulation, lymphatic and blood vessels were distinguished based on vessel morphology. After stimulation, lymphatic versus blood vessel identity became less clear. The potential for lymphatic/blood endothelial cell interactions is supported by the observation of PECAM +/LYVE-1- blood endothelial cells connecting with PECAM+/LYVE-1+ lymphatic endothelial cells (data not shown here). These observations

support the use of the rat mesentery culture model for investigating lymphatic/blood endothelial cell plasticity. **Figure 6A** also highlights the lectin labeling of apparent interstitial cells. While this labeling is inconsistent and heterogeneous from tissue to tissue, it does emphasize the presence of endogenous tissue resident cells. CD11b labeling of cultured tissues (**Figure 7**) suggests that these lectin-positive interstitial cells could be a sub-set of macrophages. Given the emerging interest in macrophage involvement in angiogenesis[20], an additional strength of the model could be its use to track macrophage dynamics over time.

Much like other *ex vivo* models, a current limitation of studying angiogenesis in the rat mesentery culture model is the lack of blood flow. Shear stress caused by blood flow has been shown to play a role in endothelial cell morphology and proliferation as well as angiogenesis[17,18,19]. For the representative results presented in **Figure 4**, the absence of shear stress alone may have been sufficient to induce an angiogenic response in the cultured networks. However, we know that based on our initial publication characterizing the rat mesentery culture model[8], that media supplementation causes increased angiogenesis versus media alone. Future studies incorporating flow within the cultured microvascular networks are undoubtedly needed to more closely mimic the *in vivo* scenario. Potential approaches for incorporating flow might include cannulation of network feeding arterioles or even cannulation of further upstream arteries within the fat border of mesenteric windows. However, despite the lack of flow, the viability of multiple cell types, the maintenance of blood and lymphatic microvascular networks, and cell proliferation during angiogenesis supports the rat mesentery culture model's relative increased level of complexity compared to cell based *in vitro* models.

In conclusion, this protocol describes a simple, reproducible *ex vivo* method for imaging angiogenic responses in intact microvascular networks. Such a method offers an alternative to cell based *in vitro* models for evaluating angiogenic cell dynamics at specific locations within a network environment. The method also offers a novel tool for investigating angiogenesis, lymphangiogenesis and blood/lymphatic mis-patterning simultaneously.

Disclosures

The authors declare that they have no competing financial interests.

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