Chapter 14

In Vivo Vascular Network Forming Assay

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Abstract

The capability of forming functional blood vessel networks is critical for the characterization of endothelial cells. In this chapter, we will review a modified in vivo vascular network forming assay by replacing traditional mouse tumor-derived Matrigel with a well-defined collagen–fibrin hydrogel. The assay is reliable and does not require special equipment, surgical procedure, or a skilled person to perform. Moreover, investigators can modify this method on-demand for testing different cell sources, perturbation of gene functions, growth factors, and pharmaceutical molecules, and for the development and investigation of strategies to enhance neovascularization of engineered human tissues and organs.

Key words Vascularization, Endothelial, Vessel formation, Tissue engineering

1 Introduction

The Matrigel plug angiogenesis assay was initially developed as a simple in vivo model to study the growth of new vessels [1]. This assay is widely used as a valuable technique for in vivo screening of pro- and antiangiogenic compounds [2]. Nonetheless, in this procedure, the Matrigel plug contains no added cell, and the assay solely reflects vascularization as the angiogenic ingrowth of surrounding host vessels into the implanted plug. Around 2007, we and others established an alternative in vivo vascular network forming assay by adding vascular cells into the Matrigel plug [3–7]. Cells were harvested from cell culture as single-cell suspensions and mixed in Matrigel for injection, mainly into the subcutaneous space of immunodeficient mice. In this assay, vascular cells rearrange and assemble into new blood vessels after implantation, a process sometimes known as vasculogenesis (i.e., the de novo formation of a primitive vascular network) [8]. Over the last decade, this assay has allowed testing the capability of various cell sources to form blood vessels in vivo [3, 5–7, 9, 10], and studies have consistently established that two major cell types are essential for robust vascularization: endothelial cells (ECs) and perivascular cells [3, 5].

Domenico Ribatti (ed.), *Vascular Morphogenesis: Methods and Protocols*, Methods in Molecular Biology, vol. 2206, https://doi.org/10.1007/978-1-0716-0916-3_14,

ECs are the cells that line the blood vessel lumens and are, of course, central to the process. Our group has tested EC sources from several species (namely, human, mouse, rabbit, and sheep) [3, 5, 9] and tissues of origin, including ECs from the umbilical cord or peripheral blood (i.e., endothelial colony-forming cells or ECFCs) [3], tissue-resident ECs (e.g., from adipose and dermal tissues) [9], umbilical vein (human umbilical vein endothelial cells or HUVECs) [3], and induced pluripotent stem cell (iPSC)-derived ECs [11]. We have consistently validated the ability of all these types of ECs to form perfused vessels using the in vivo vascular network forming assay [12].

The second cell type required for the formation of vascular networks is perivascular cells. Sources of perivascular cells include smooth muscle cells (SMCs) [3], mesenchymal stem cells (MSCs) [5, 7], and fibroblasts [13]. As with ECs, investigators have routinely tested multiple types of supporting perivascular cells using the in vivo vascular network forming assay [12], and there is consensus in that perivascular cells are critical to facilitating the engraftment of ECs [5].

The in vivo vascular network forming assay is very versatile, and its applications for both basic vascular biology and translational studies are numerous [12]. For example, using this assay, one could quickly characterize new sources of ECs such as those derived from embryonic stem cells, ECs isolated from pathological specimens, and ECs genetically modified via gene editing. Investigators could also histologically evaluate the characteristics of the newly formed blood vessels, including their size, morphology, microvascular density, whether the vessels are perfused or not. Also, applying routine immunofluorescence, we can identify the implanted ECs (e.g., using species-specific antibodies against EC markers) and perivascular cells (e.g., by staining for a-SMA), and distinguish them from the host cells [14]. We can also analyze and evaluate the functionality of the vessels, including the presence of proliferative cells (e.g., Ki67-positive cells) and apoptosis (TUNEL assay) [15–17]. The in vivo vascular network forming assay also allows testing pharmaceutical enhancers or inhibitors of blood vessel formation, which could be screened by simply adding the candidate molecules into the hydrogel mixture. Moreover, the entire process of vascular network formation occurs in a short period (typically, perfused vessels are formed within 7 days after implantation), thus facilitating the studies [17]. In summary, the in vivo vascular network forming assay is ideally suited for studies on the cellular and molecular mechanisms of vascular network formation and for developing strategies to vascularize tissues.

In this chapter, we provide an updated version of the in vivo vascular network forming assay. Of note, we describe an alternative hydrogel to Matrigel. Originally, this assay was developed with Matrigel due to its convenience to obtain and handle. However,

Matrigel is not fully defined chemically and thus has some drawbacks. Indeed, Matrigel is a mixture of extracellular matrix components isolated from Engelbreth-Holm–Swarm mouse sarcomas and contains various undefined proteins [2]. Thus, one should always be cautious when interpreting experiments on cellular activities in Matrigel. Moreover, there are significant limitations to Matrigel when considering translational studies, including its tumorigenic origin, diverse composition, and batch-to-batch variability in terms of mechanical and biochemical properties. Over the last decade, our group has characterized several alternative options to Matrigel (both synthetic and natural sources), and we have found that human vascular cells can form robust vascular networks in multiple types of hydrogels [4, 14–16, 18, 19]. Here, we describe a collagen–fibrin hydrogel mixture that is easy to use, produces very reproducible in vivo outcomes, and has a well-defined composition. Indeed, the assay we describe herein entails the injection of a collagen–fibrin hydrogel mix containing ECFCs and MSCs, which supports the formation of a robust vascular network within 7 days [19]. The assay is simple to perform, and it does not require any incision or surgical procedure, which reduces the potential influence of wound healing. Lastly, the assay is compatible with many immunodeficient mouse backgrounds, including athymic nude, SCID, and NSG mice.

2 Materials

Prepare all solutions using PBS or distilled water and analytical grade reagents. Prepare and store all reagents at 4 °C temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials.

2.1 Cell Culture of Human ECFCs and MSCs

1. We used human cord blood–derived ECFCs and adipose-derived MSCs as the standard cell sources. It is recommended to include ECFC/MSC as a positive control in all experiments. The isolation, characterization, and cultivation of ECFCs and MSCs are out of the scope of this chapter, but detailed procedures can be found in our previous publications [12]. Alternatively, we and others have also confirmed that HUVECs can be used instead of ECFCs [3]. Also, purified ECs can be purchased from several commercial sources like STEMCELL Technologies Inc or Lonza.
2. EC culture medium: Endothelial cell growth medium 2 (EGM2, except for hydrocortisone; PromoCell, Cat# C22111) supplemented with 10% FBS and 1× glutamine–penicillin–streptomycin.

3. MSC culture medium: Mesenchymal stem cell growth medium (MSCGM; ATCC, Cat# PCS-500-030) supplemented with 10% FBS and 1× glutamine–penicillin–streptomycin.

2.2 Collagen–Fibrin Hydrogel

1. Concentrated bovine collagen solution: We validated Cultrex® Bovine Collagen I (protein concentration, 5 mg/mL) from Trevigen. Similar products isolated from rat tail or porcine skin should also work, but the stock concentration has to be higher than 5 mg/mL. Keep collagen solution at 4 °C and handle it on ice.
2. Bovine fibrinogen solution: Prepare freshly 30 mg/mL of fibrinogen concentrated solution by dissolving bovine fibrinogen (Sigma-Aldrich, Cat# F8630) in prewarmed 0.9% sodium chloride saline. Keep the solution in a 37 °C water bath with occasional vortexing until dissolved.
3. Store components for collagen–fibrin hydrogels, including 1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, 10× M199 or DMEM media, fetal bovine serum (FBS), and 1 mg/mL of human fibronectin (hFN), at 4 °C and handle them on ice.
4. Final composition of collagen–fibrin hydrogel includes collagen (3 mg/mL), human fibronectin (30 μg/mL), FBS (10% v/v), HEPES (25 mM), and fibrinogen (3 mg/mL) at pH 7.4. Use Table 1 to calculate the amount of collagen–fibrin gel needed for the experiment and follow Subheading 3.1 to prepare the hydrogel solution.
5. Thrombin solution: Dissolve 50 U/mL of thrombin from bovine plasma (Sigma-Aldrich, Cat# T4648) in PBS. Aliquot and store at −20 °C.

2.3 Immunofluorescent Staining

1. Tris–EDTA antigen retrieval buffer: Add 0.6 g of Tris-base (Trizma® base from Sigma-Aldrich), 1 mL of 0.5 M EDTA, 250 μL of Tween 20 in 500 mL of ddH_2O. Mix and adjust pH with HCl to 9.0. Heat to 90–95 °C on a magnetic stir plate with gentle mixing.
2. 5% serum blocking buffer: Add 0.5 mL of normal horse serum (Vector Laboratories) in 9.5 mL of PBS. Mix and use within 6 h.

3 Methods

Prior to the experiment, make sure there are enough ECFCs and MSCs in culture; 0.8×10^6 ECFCs and 1.2×10^6 MSCs will be required for each implant and mouse. We suggest preparing 1.25 times more amount of cell–hydrogel mixture to compensate for the

Table 1
Composition of the collagen–fibrin hydrogel

Stock Conc.		Final Conc.
Collagen (5 mg/mL)	600 μL	3 mg/mL
10× M199	100 μL	1×
hFN (1 mg/mL)	30 μL	30 μg/mL
FBS	100 μL	10%
HEPES (1 M)	25 μL	25 mM
NaOH (1N)	~15 μL	pH 7.4 (by color)
Fibrinogen (30 mg/mL)	100 μL	3 mg/mL
ddH_2O	30 μL	
Total	1000 μL	

potential loss during handling and the dead volume of the syringe. For example, we would prepare 1-mL of a cell–hydrogel mixture containing 4×10^6 ECFCs and 6×10^6 MSCs but would only inject four mice, each with a 200-μL plug. Carry out all procedures in a tissue culture hood with sterile equipment.

3.1 Preparation of Collagen–Fibrin Hydrogel

1. Calculate the amount of collagen–fibrin gel needed for the experiment (Table 1). Keep all reagents on ice. Precool 1000 μL pipette tips at −20 °C for collagen solution handling.
2. Prepare the fibrinogen solution freshly by dissolving bovine fibrinogen (30 mg/mL) in 0.9% sodium chloride saline. Keep the vial in a 37 °C water batch until all fibrinogen is dissolved with occasional vortexing.
3. Under a tissue culture hood, carefully mix the reagents in a suitable vial on ice following this order: ddH_2O, 10× M199 medium, 1 M HEPES, and 5 mg/mL collagen solution. Use precooled pipette tips to transfer collagen solution and mix the hydrogel. Pay attention to the color change of pH indicator phenol red in the M199 medium. While adding 1 M HEPES, the color should turn to red for neutral pH (6.8–7.4). After mixing the collagen solution, the pH should decrease to ~pH 2 and show a yellow color. Then, slowly titrate the pH value back to neutral by adding 1N NaOH. Mix the solution thoroughly by gentle pipetting (avoiding bubbles) for accurate pH measurement. Transfer a tiny amount (~1 μL) of hydrogel solution to a pH strip (PH Test Strips, 0-14 PH, EMD Millipore cat# 9590) to confirm a neutral pH.

4. Once you obtain a neutral hydrogel solution, then add fibronectin, FBS, and fibrinogen to the solution and mix thoroughly. FBS and fibronectin are optional and can be replaced by the same amount of ddH_2O. Keep the hydrogel solution on ice and use it within 1 h (*see* **Note 1**).

3.2 Preparation of Cell–Hydrogel Mixture for Injection

1. Aspirate the medium of each cell culture plate and wash the ECFCs and MSCs with 10 mL of PBS. Remove PBS and add 5 mL of the trypsin–EDTA solution to each 100-mm plate. Gently shake the plates to evenly distribute the trypsin–EDTA solution. Incubate the plate for 4–5 min. Gently tap the plate to see the detached cells in suspension under an inverted microscope.
2. When cells completely detach from the plate, add 5 mL of DMEM/10% FBS, and collect the cell solution into a 15-mL conical tube. Take 10 μL of the solution to count the cells in a hemocytometer and work out the total number of ECFCs and MSCs harvested, respectively.
3. Transfer 4×10^6 ECFCs ($5 \times 0.8 \times 10^6$ cells) and 6×10^6 MSCs ($5 \times 1.2 \times 10^6$ cells) together into a single 50-mL conical tube. This is the total amount of cells required for four implants with some extra amount to account for losses during handling. Centrifuge the cells at 1200 rpm ($290 \times g$) and remove the supernatant (*see* **Note 2**).
4. Resuspend the cell pellet in 1 mL of ice-cold collagen–fibrin hydrogel solution. Mix the cells very gently to avoid any bubbles within the mixture. Keep the cell–hydrogel mixture on ice and inject it within 30 min (*see* **Note 3**).

3.3 Injection into Immunodeficient Nude Mice

1. All animal experiments are carried out with 6-week-old athymic nude mice. Mice are housed in compliance with Boston Children's Hospital guidelines, and all animal-related protocols are approved by the Institutional Animal Care and Use Committee (*see* **Note 4**).
2. Prior to the injection, anesthetize the mice by placing them into a gas chamber delivering isoflurane. Allow the mice to inhale the isoflurane for approximately 2 min until they are unresponsive to toe pinch and monitor their respiration by inspection (*see* **Note 5**).
3. Gently mix the cell–hydrogel mixture again and immediately load the mixture into 1-mL sterile syringes, and place 26-gauge needles with their caps on the tips of the syringes. Keep the loaded syringes horizontally on ice to prevent ununiform cell distribution (*see* **Note 6**).
4. Load a separate 1-mL sterile syringe with the thrombin solution and place a 30-gauge needle with its cap on the tip of the syringe.

5. Disinfect the injection area of nude mouse skin with ethanol pads. For each mouse, inject 50-μL of thrombin solution first and then, at the same location, 200-μL of the cell–hydrogel solution subcutaneously into the upper dorsal region. The fibrinogen portion in the hydrogel solution will be enzymatically converted by thrombin to fibrin within minutes to form a gel plug, which will become a small but appreciable bump under the skin (*see* **Note 7**).
6. After the injection, place the mice on a layer of gauze for comfort and warmth and observe them until they become ambulatory. Then, observe the mice daily for the first 3 days.

3.4 Harvesting the Implants

1. One week after the implantation, euthanize the mice by placing them in a gas chamber, delivering compressed CO_2 gas. The collagen–fibrin plugs should be appreciable under the skin (*see* **Note 8**).
2. Cut open the skin near the area of the original injection and surgically removed the collagen–fibrin plug. Digital photographs of the retrieved plugs with a scale are advised.
3. Place the harvested collage–fibrin plugs into histological cassettes and fix them in 10% neutral buffered formalin under a chemical hood overnight at room temperature.
4. After fixation, wash out the 10% neutral buffered formalin with ddH_2O several times and place the histological cassettes at 4 °C in 70% ethanol until histological evaluation.

3.5 Evaluation of Vascular Networks in Explanted Collagen–Fibrin Plugs

1. For histological evaluation, explanted collagen–fibrin plugs are embedded in paraffin and sectioned (7 μm-thick sections) using standard procedures.
2. Carry out Hematoxylin and Eosin (H&E) staining of the explant sections following standard protocols.
3. Quantify microvessel density (MVD) by evaluation of 10–30 randomly selected fields (0.1 mm^2 each; 40× objective lens) of H&E stained sections taken from the middle part of the implants. Microvessels can be identified as luminal structures containing red blood cells and counted. Calculate MVD as the average number of red blood cell-filled microvessels from the fields analyzed and expressed as vessels per square millimeter in the image (*see* **Notes 9** and **10**).

3.6 Evaluation of Human Lumens and Perivascular Coverage by Immunofluorescent Staining

1. Deparaffinize and rehydrate the paraffin-embedded sections by sequential immersion in xylene and then 100%, 90%, 80%, and 50% ethanol for 5 min in each step. Rinse the sections in PBS.
2. Heat the sections in Tris–EDTA antigen retrieval buffer (the default antigen retrieval buffer for most the antibodies we used unless mentioned) to 90–95 °C for 30 min. Rinse the sections in PBS.

3. Block the sections for 30 min in blocking solution (5% normal horse serum in PBS).
4. Incubate the sections with primary antibody solution (in 5% blocking serum) for 1 h at room temperature or 4 °C overnight. Validated primary antibodies include mouse anti-human CD31 (human EC-specific; Agilent Technologies Inc, Clone JC70A; 1:50 dilution); mouse anti-human vimentin (human EC and MSC-specific; Abcam, Clone V9; 1:100 dilution); mouse anti-α-smooth muscle actin (α-SMA; reactive with both human and mouse; Sigma-Aldrich; Clone 1A4; 1:100 dilution); and rabbit anti-α-smooth muscle actin (α-SMA; reactive with both human and mouse; Abcam; Clone ab5694; 1:100 dilution). Afterward, wash the sections with PBS twice (*see* **Note 11**).
5. Incubate the sections with fluorophore-conjugated secondary antibody solution (1:200 in 5% blocking serum) for 1 h at room temperature. Validated secondary antibodies include horse anti-mouse IgG conjugated with fluorescein (FI-2000) or Texas Red (TI-2000) and Texas Red goat anti-rabbit IgG (TI-1000; all from Vector Laboratories). Afterward, wash the sections with PBS twice. Protect the slides from light after adding secondary antibodies to avoid photobleaching.
6. Counterstain cell nuclei with DAPI solution at room temperature for 10 min. Wash the sections with PBS twice.
7. Wash the slides with ddH_2O once and mount them with ProLong antifade mounting medium (Thermo Fisher Scientific). Keep slides at 4 °C and protect them from light until imaging. Obtain high-resolution images with a confocal microscope (e.g., Leica TCS SP2 confocal system), using a 63× objective lens.

3.7 Evaluation of Cell Proliferation and Apoptosis in Vascular Networks

1. Evaluation of cell proliferation can be done by immunofluorescent staining of Ki67 on the paraffin-embedded sections. We have validated the rabbit anti-Ki67 antibody (reactive with both human and mouse; Abcam; Clone ab15580; 1:100 dilution) by the same staining protocol of Subheading 3.6. Double staining with human EC-specific CD31 may be required to distinguish proliferating human and mouse cells.
2. Evaluation of cell apoptosis can be done by using Click-iT™ Plus TUNEL Assay for In Situ Apoptosis Detection, Alexa Fluor™ 488 dye (Thermo Fisher Scientific), or other similar TUNEL assays. After performing the TUNEL assay according to the manufacturer's protocol, the sections can be stained by immunofluorescence with human EC-specific CD31 antibody using the same staining protocol of Subheading 3.6 to distinguish the origin of apoptotic cells.

4 Notes

1. The composition of collagen–fibrin hydrogel gel is adjustable. For example, FBS and fibronectin are optional and can be replaced by the same amount of ddH_2O. Additional reagents, including growth factors, cytokines, blocking antibodies, ECM proteins, concentrated conditioned medium, cell extract, or small pharmaceutical molecules, can be added into the hydrogel mixture to test theirs in vivo effects. Any protein or peptide should be added after adjusting the pH value to natural to prevent inactivation due to acidic denaturation.
2. The total numbers of cells and the ratio between ECs and MSCs can be adjusted according to experimental designs. We have validated the successful formation of vascular networks by implanting total cell numbers ranged from 1×10^6 to 6×10^6 per 200-μL plug, with EC-to-MSC ratios from 4:1 to 1:4.
3. The time between resuspension of the cells in collage-fibrin hydrogel and the subcutaneous injection into the mice should be kept to a minimum to maintain cell viability. We recommend the preparation of the hydrogel solution prior to harvesting the cells.
4. We have confirmed comparable levels of vascular network formation by injecting ECFCs and MSCs in different immunodeficient murine backgrounds, including athymic nude (nomenclature: Crl:NU(NCr)-*Foxn1*^{nu}), BALB/c nude (CAnN.Cg-*Foxn1*^{nu}/Crl), CD-1 nude (Crl:CD1-*Foxn1*^{nu}), NOD.SCID (NOD.CB17-*Prkdc*^{scid}/J), and NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice.
5. Other analgesics are compatible with this assay. However, due to the low invasiveness and the quick nature of this procedure, analgesics are usually not required. Consult with your animal housing facility to find the best practice for your experiment.
6. Injecting cells through needles smaller than 26-gauge may damage cell viability.
7. The purpose of the preinjected thrombin solution is to convert the fibrinogen into fibrin plug immediately after injecting it into the subcutaneous space. The quick formation of fibrin gel significantly improves cell retention and the proper volume of the hydrogel implant. The effect of thrombin is transient. Without adding thrombin, the hydrogel can still solidify by the formation of a collagen matrix at body temperature. However, the process may take 30 min or longer.
8. We routinely exam the formation of vascular networks after 7 days. The harvesting time points can be adjusted due to the purpose of the research. We have observed the earliest perfused

blood vessels at day 3 and reliable vascularization at day 5. The vascular networks are stable for at least 4 weeks. However, the Matrigel may be more suitable for long-term grafting due to the quick degradation of collagen–fibrin hydrogel.

9. The microvessel density formed by ECFCs and MSCs after 7 days should range from 80 to 250 vessels/mm^2. We recommend having at least four plugs per group and repeat 3 times to reduce the variation between individual mice.
10. Several studies using similar in vivo vascular network forming assays have shown the dysfunction of blood vessels by implanting pathological vascular cells. Some of the features are obvious while examining the H&E images and recapitulate their clinical pathology. For example, implanting cells isolated from the infantile hemangioma displayed unusually high microvessel density [20, 21]. Tie2-mutated HUVECs formed enlarged lumens that recapitulate the venous malformation lesions [22].
11. Human-specific ECs can also be stained by *Ulex europaeus* agglutinin I (UEA-I), a lectin that binds with high affinity to human (but not mouse) ECs. After performing antigen retrieval, incubate the sections with fluorescein- or rhodamine-conjugated UEA-I lectin (Vector Laboratories; 20 μg/mL in PBS containing 1 mM Calcium and 1 mM Magnesium ions) at room temperature for 30 min. Rinse the section with PBS twice. UEA-I lectin binding method is compatible with antibody-based staining and useful to avoid the cross-reactions of secondary antibodies.

Acknowledgments

This work was supported by the National Institutes of Health grants R01AR069038 and R01HL128452 to J. M.-M.

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