# Chapter 14

# A Modified Aortic Ring Assay to Assess Angiogenic Potential In Vitro

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## Abstract

Angiogenesis, an integral part of many physiological and pathological processes, is a tightly regulated multistep process. Angiogenesis assays are used to clarify the molecular mechanisms and screen for pharmacological inhibitors. However, most in vitro angiogenesis models measure only one aspect of this process, whereas in vivo assays are complex and difficult to interpret. The ex vivo aortic ring model allows the study of many key features of angiogenesis, such as endothelial activation, branching, and remodeling as well as later steps such as pericyte acquisition. This model can be modified to include genetic manipulation and can be used to assess the pro- or anti-angiogenic effects of compounds in a relatively controlled system.

**Key words** Angiogenesis, Endothelium, Mouse aorta, Organ culture, Pericyte recruitment, Sprouting

## 1 Introduction

Angiogenesis, the sprouting of new blood vessels from pre-existing ones, is an essential process of normal development, tissue remodeling, wound healing, as well as the reproductive cycle and pregnancy. At the same time, angiogenesis is an integral part of pathological processes, such as tumor growth and metastasis, proliferative diabetic retinopathy, rheumatoid arthritis, and psoriasis [1]. Angiogenesis is a complex event comprised of several distinct steps, including endothelial activation, basement membrane disruption, and invasion of the extracellular matrix by endothelial sprouts which develop from the parent vessel followed by their elongation, branching, and structural remodeling [2]. These processes are tightly regulated by a fine equilibrium of pro- and anti- angiogenic modulators, such as vascular endothelial growth factor (VEGF), angiopoietins, basic fibroblast growth factor (bFGF), and others [2–4]. However, the interplay between these regulatory factors at different stages of the angiogenic process is not completely understood. The importance of angiogenesis in pathology has led to a

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general interest in clarifying the cellular and molecular mechanisms that are required for the formation of new blood vessels in order to identify novel therapeutic targets. Currently, a number of in vitro and in vivo angiogenesis assays are in general use, each with their own advantages and limitations [5, 6].

Well-established in vivo assays of angiogenesis [7] are the chick embryo chorioallantoic membrane assay (CAM assay) [8, 9], tumor cell injection and implantation [10, 11], the matrix plug assay [12], as well as retina angiogenesis [13, 14] and the ischemic hindlimb [15, 16]. In vivo assays simulate the natural, sequential process of angiogenesis and have the benefit of potentially involving all of the relevant cell types and growth factors. However, despite their relevance, in vivo assays are extremely complex and time consuming and thus not suitable for high throughput analysis, can be difficult to interpret, and results are often confounded by inflammatory responses that have a direct impact on angiogenesis.

While it is clear that in vivo studies cannot be completely avoided, it is important that the most be made of available in vitro studies that closely mimic the in vivo situation. Thus, while complete replacement may not be possible, angiogenesis assays that can assess responses in native endothelial cells can certainly help to reduce and refine in vivo studies.

Several in vitro angiogenesis assays are used widely to investigate and quantify angiogenesis [17, 18]. Perhaps the most frequently used are the endothelial tube (or cord) formation assay, network formation by fibroblast and endothelial co-cultures, or 3D endothelial spheroid sprouting assays [19–22]. However, these cell-based assays are limited by the fact that they require the isolation and culture of the cells of interest and are only able to mimic selective phases of angiogenesis. These models also exclude important contributions from supporting cells such as pericytes as well as the processes involved in sprouting from the parent vessel, invasion and maturation, as well as later remodeling steps.

It follows, therefore, that more biologically relevant information can be obtained by using native endothelial cells, i.e., avoiding the dedifferentiating culture step and organ culture models such as the aortic ring assay. The assay was originally described for the rat aorta by Nicosia and Ottinetti [23] and bridges the gap between in vitro and in vivo methods and is equally applicable to other species [24, 25], including the mouse [26, 27]. This means that it is also useful for the study of a broad spectrum of genetically modified mouse lines [16, 28, 29].

As the name suggests, this model involves the embedding of aortic rings in an extracellular matrix (usually fibrin or collagen gels) and culture in a defined medium. Over a short period of time, i.e., 6–7 days (Fig. 1), hollow capillaries sprout spontaneously from the cut surfaces of the aortic sections, which are thought to be initiated by the wound of the dissection procedure.

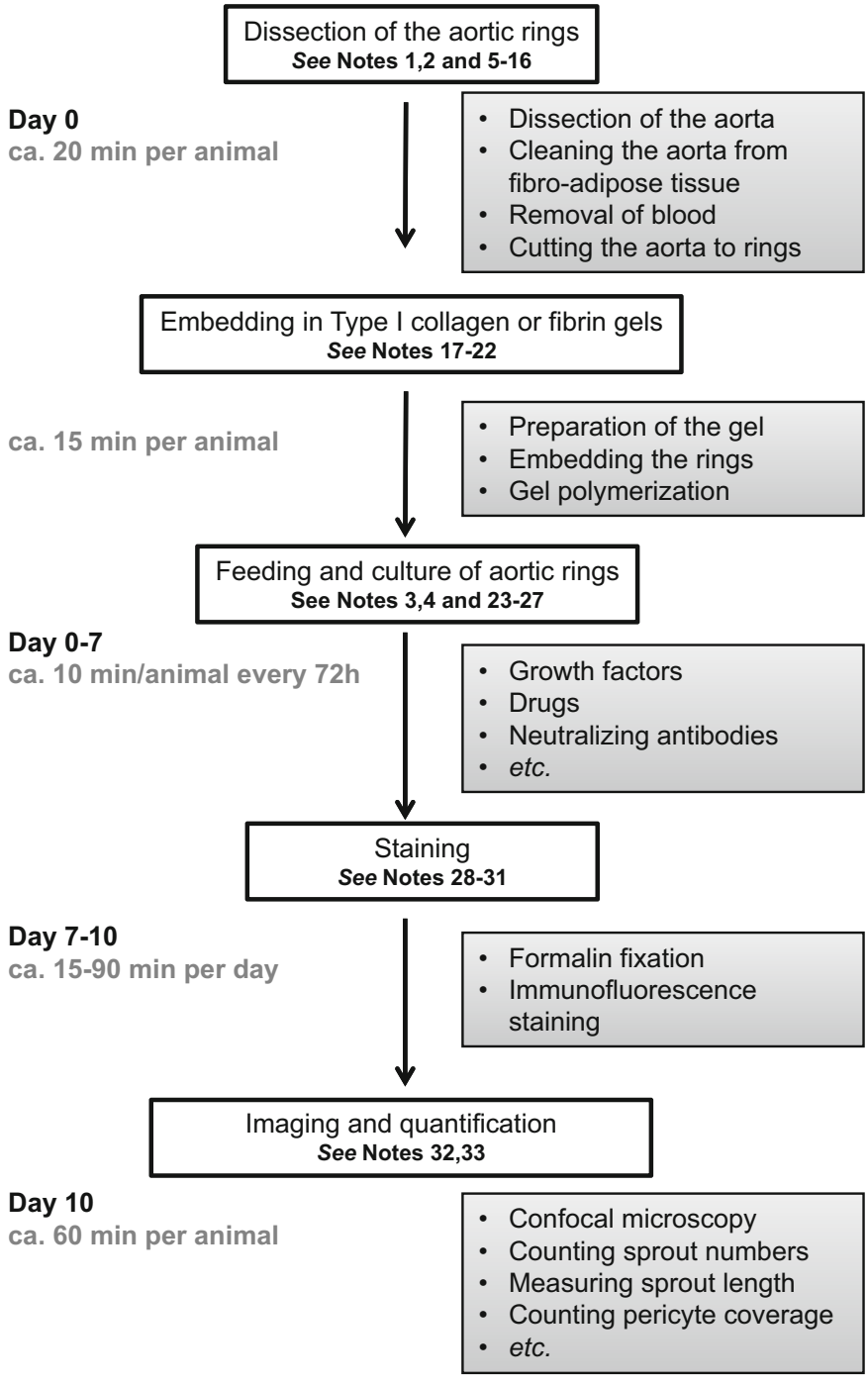


**Fig. 1** Flow diagram showing the different steps of the aortic ring assay. The timing is indicated on the left with the main stages highlighted in solid boxes and single steps in *gray* boxes

The capillary-like tubes formed are made up of endothelial cells as well as supporting cells involved in vessel maturation, such as pericytes. Endothelial sprouts grow out from the aortic sections over a time course comparable to that seen in vivo [30, 31] in an exponential growth phase that can result in a relatively complex network before being followed by a regression phase.

This sensitive assay allows assessment of the cellular and molecular steps in angiogenesis as well as the identification, characterization, or screening of novel angiogenic modulators [32], such as cytokines, peptides [11, 33], anti-angiogenic compounds [34], and neutralizing antibodies. It is important to realize that the vast majority of cells that grow out of the aortic ring are of adventitial origin, i.e., fibroblasts. This means that any conclusions about angiogenesis can only be made after the endothelial cells and/or pericytes have been visualized.

## 2 Materials

### 2.1 Preparation of Aortic Rings

1. Rats or mice of appropriate age, sex, and genetic background (*see* **Note 1**).
2. Microdissection scissors and forceps: bone scissors, a pair of skin forceps, a pair of straight fine forceps (i.e., Dumont #5), and iris scissors (Vannas spring scissors with a blade size of 3 mm for mice, or 4 mm for rat aortae).
3. Dulbecco's modified Eagle's medium/Ham's F12 nutrient mix (DMEM/F12) containing 100 U/mL penicillin/100 μg/mL streptomycin (*see* **Note 2**).
4. Scalpel.
5. Culture dishes (10 cm).
6. Syringe (1 mL).
7. Needles (27-G and 30-G).
8. Dissecting microscope.
9. A culture dish half filled with polymerized silicone (SYLGARD 184 silicone elastomer). Prepare several days in advance to allow full polymerization of the SYLGARD 184.
10. 70 % (v/v) ethanol.

### 2.2 Collagen Gel

1. Rat tail collagen type 1 (Becton Dickenson).
2. Sterile water, 4 °C.
3. Medium 199 (M199) 10× solution.
4. Sterile aqueous 7.5 % sodium bicarbonate ($NaHCO_3$).
5. Sterile aqueous 0.5 N sodium hydroxide (NaOH).

### 2.3 Embedding

1. 48-Well plates for mouse aortae, or 96-well plates for rat aortae.
2. A pair of straight fine forceps (i.e., Dumont #5).
3. Laminar flow tissue culture hood.

### 2.4 Culture Medium

1. Culture medium: Endothelial basal medium (EBM) containing 2.5 % (v/v) autologous (mouse or rat) serum and 100 U/mL penicillin/100 μg/mL streptomycin (*see* **Notes 3** and **4**).
2. Humidified incubator with a 5 % $CO_2$ atmosphere at 37 °C.

### 2.5 Staining, Imaging, and Quantification

1. Dulbecco's phosphate-buffered saline (PBS) solution.
2. Blocking solution: PBS containing 0.5 % (v/v) Triton X-100 and 1 % (w/v) bovine serum albumin (BSA).
3. Anti-CD31 antibodies (e.g., Becton Dickinson, BD 550274).
4. Anti-rat secondary antibodies (e.g., 488 Alexa Fluor).
5. 4 % paraformaldehyde (PFA), pH 7.4.
6. Confocal microscope.

## 3 Methods

The protocol for aortic ring assay involves different steps including the dissection of the aorta, embedding the aortic rings in collagen or fibrin gels, culture of the aortic rings, staining, as well as image capture and the quantification of the capillary sprouts.

### 3.1 Preparation of the Aortic Rings

1. Sacrifice the mouse or rat according to the relevant local ethical guidelines for animal care and experimentation (*see* **Note 5**).
2. Sterilize the surface of the carcass with 70 % ethanol.
3. Lay the carcass on its back and open the ventral skin.
4. Cut through the sternum, and open the rib cage using a bone scissor and skin forceps.
5. Remove the lungs and the esophagus (Fig. 2a).
6. Remove aorta, now visible as a fat-covered vessel tracking down along the spine, rapidly from the sacrificed mouse or rat.
7. Gently detach the thoracic aorta from the spine using closed microdissection forceps starting from the diaphragm toward the heart (Fig. 2b, c).
8. Cut once at the posterior end and once at the anterior end (Fig. 2d–f) (*see* **Notes 6** and **7**).
9. Transfer the dissected aortae immediately to ice-cold serum-free DMEM/F12 with antibiotics. Keep the aortae on ice until dissection.

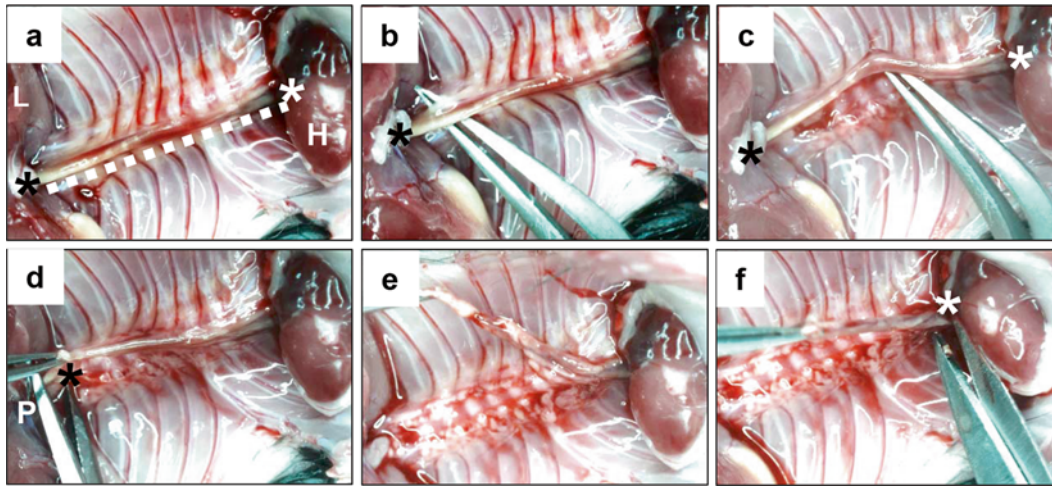


**Fig. 2** Dissection of the thoracic mouse aorta. (**a**) After opening the rib cage and removing the lungs and esophagus, the aorta (*white dotted line*) is visible as it runs along the spine from the heart (*white asterisk*) to the diaphragm (*black asterisk*). The heart (H) and liver (L) are visible. (**b–c**) The aorta is detached from the spine by blunt dissection using closed forceps, beginning at the posterior end moving gently toward the anterior end until the aorta is mostly detached. (**d**) The aorta is cut once at the posterior end (P) and (**e**) lifted gently without applying tension. (**f**) Cut the aorta again at the anterior end (*white asterisk*)

10. For the dissection, add ice-cold DMEM/F12 plus antibiotics to a dish, which is half filled with polymerized silicone.
11. Transfer the aorta to the silicone-filled dissection dish.
12. Pin the ends of the aorta to the silicone with fine 30-G needles without applying tension to the aorta (Fig. 3a) (*see* **Notes 8** and **9**).
13. Carefully remove the surrounding periaortic fibroadipose tissues and branching vessels with fine microdissection forceps and iridectomy scissors under a microscope (Fig. 3b, c), paying special attention not to damage the aortic wall (*see* **Note 10**).
14. Gently flush out blood from the lumen of the aorta with DMEM/F12 using a 1 mL syringe fitted with a 27-G blind needle (Fig. 3d) until the aorta is free of clotted blood (Fig. 3e) (*see* **Notes 11** and **12**).
15. Place a piece of millimeter paper underneath the dish.
16. Using a scalpel blade, cut away the proximal and distal 1 mm segments of the aorta.
17. With help of the scale paper, cut the aorta into equal sections ~1 mm long (Fig. 3f) (*see* **Notes 13** and **14**).
18. Store the aortic rings on ice until the gel has been prepared (*see* **Notes 15** and **16**).

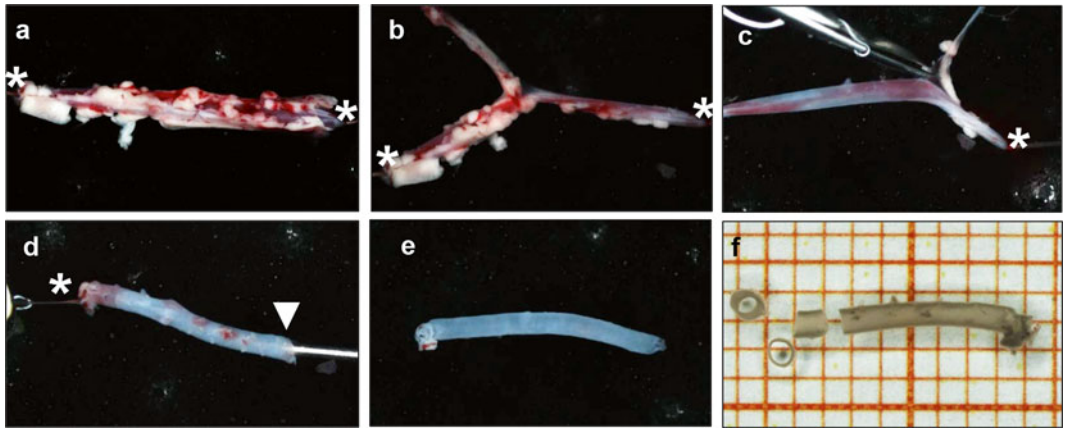


**Fig. 3** Clean and cut the extracted aorta. (**a**) The fat-covered aorta should be pinned at its ends (*white asterisks*) to the silicone bottom of a dissection dish. (**b**–**c**) Clean the aorta of the surrounding fibroadipose tissues by running the scissors parallel to the aorta, thus removing whole strips of fat. (**d**) After removing one pin, the syringe should be partially inserted into the fat-free aorta (*white arrow*) and flushed gently with medium until any remaining blood has been removed (**e**). (**f**) Place a piece of graph paper underneath a culture dish—this helps to cut the aorta into sections of equal length

### *3.2 Preparation of the Collagen Gel*

The use of a collagen matrix is recommended for the aortic ring assay to support the outgrowth of large microvessel sprouts, which are easily distinguishable from fibroblast outgrowth. In addition, a collagen matrix supports the response to growth factors, such as VEGF [27]. However, specific conditions may require the use of a different matrix, as the type of matrix can be important for the angiogenic response to different growth factors [27, 35, 36], for example, sprouting in response to bFGF is stronger in a fibrin matrix [27]. The preparation of a fibrin gel is described as an option in Subheading 4 (*see* **Note 17**).

Prepare the collagen gel on ice to avoid premature polymerization of the matrix. Note that all pipette tips and plates should be stored at 4 °C until use to avoid the premature coagulation of the gel. Work under sterile conditions using a laminar flow.

1. Add rat tail collagen type-1 to a final concentration of 1.5 mg/mL to a 1.5 mL Eppendorf tube.
2. Adjust with sterile water to a volume of 871 µL/mL and invert immediately.
3. Add 100 µL/mL of 10× Medium 199.
4. Add 34 µL/mL of 7.5 % $NaHCO_3$ solution
5. Mix well for 10 s to prevent uneven polymerization. Avoid the formation of air bubbles.

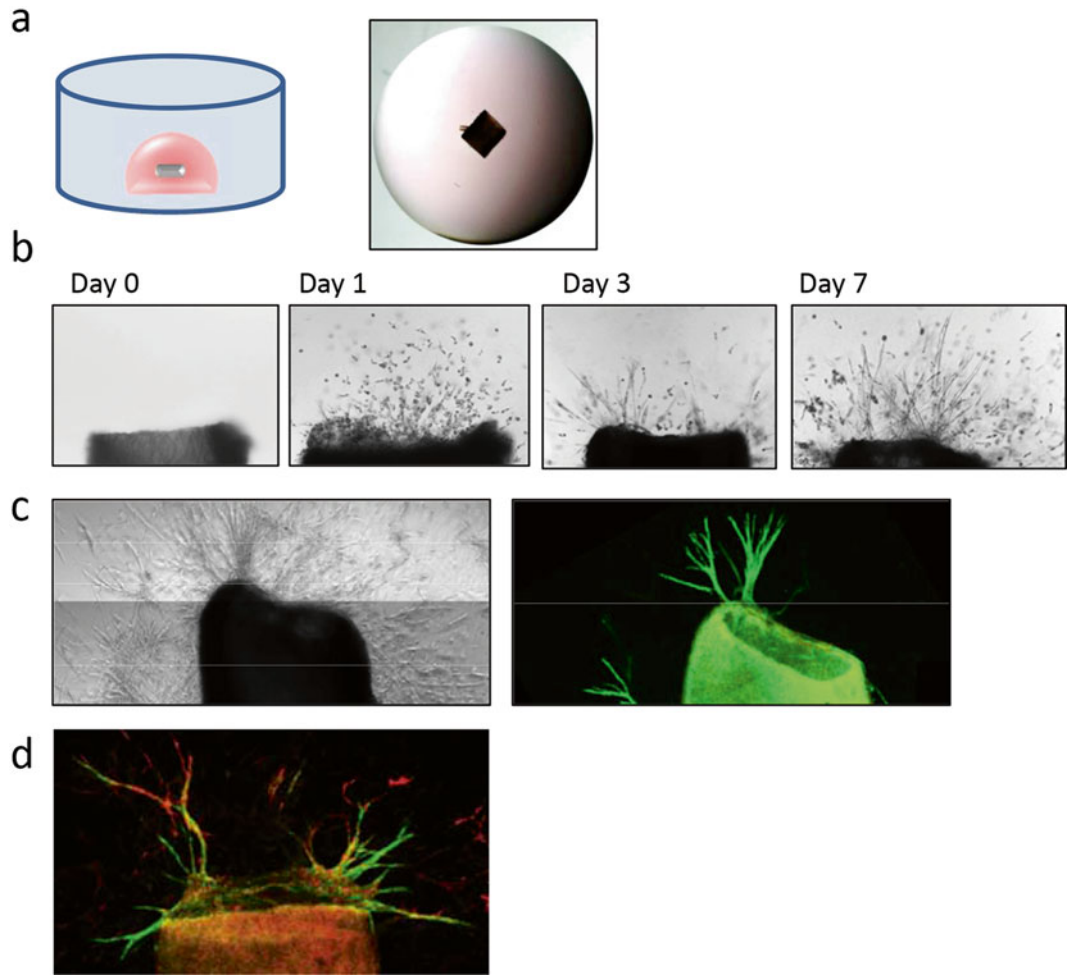


**Fig. 4** Generating and visualizing sprouts. (**a**) Mouse aortic rings are embedded on their sides into a drop of the collagen matrix. (**b**) Phase-contrast images of the aortic rings embedded in collagen at days 0, 1, 3, and 7. (**c**) Comparison of a phase-contrast image and CD31 immunofluorescent staining of the same aortic ring. (**d**) Co-staining with CD31 (*green*) and α-actin (*red*) allows for the simultaneous visualization of endothelial cells and pericytes

6. Adjust the pH with a few drops of 0.5 N NaOH to approximately 7.4. The gel color should turn from orange to cherry red. Put the gel on ice for approximately 1 min until the color no longer changes (*see* **Note 18**).
7. Keep on ice and use within 30 min.

### *3.3 Embedding the Aortic Rings*

#### *3.3.1 Mouse Aortae*

1. Place a 25 μL drop of the gel in the middle of each well of a pre-cooled 48 well plate (*see* **Note 19**).
2. Embed one aortic ring into the middle of each collagen drop using fine forceps (Fig. 4a). The gel should remain its drop-like shape (*see* **Note 20**).

#### 3.3.2 Rat Aortae

1. Add 100 µL of the gel to each well of a pre-cooled 96 well plate (*see* **Note 21**).
2. Embed one aortic ring into the middle of each well using fine forceps (*see* **Note 22**).

### 3.4 Culturing the Aortic Rings

1. Carefully place the plates in an incubator at 37 °C with 5 % $CO_2$ for approximately 45–60 min until the gel has solidified (*see* **Note 23**).
2. Add endothelial basal medium supplemented with 100 U/mL penicillin/100 µg/mL streptomycin, optionally supplemented with 2.5 % autologous serum and/or growth factors (*see* **Notes 24** and **25**).
3. Change the culture medium every 72 h (*see* **Note 26**).
4. Cultivate the aortic sections at 37 °C in a humidified environment for 7 days (*see* **Note 27**).

### 3.5 Fluorescence Staining and Imaging

Although phase-contrast microscopy may provide an overview of microvessel outgrowth (Fig. 4b), it does not allow for discrimination between endothelial cells and other cell types, such as fibroblasts, which grow out from the aortic explants in large numbers. Immunohistochemical staining can be used to identify the interacting cell types, and endothelial sprouts can be quantified following staining with antibodies against CD31 (Fig. 4c). The interaction with supporting pericytes can also be visualized, e.g., by staining against α-actin (Fig. 4d) or αNG2 chondroitin sulfate proteoglycan. Sprout length can be quantified by measuring the number and length of the sprouts (Fig. 5), and coverage by pericytes can be quantified by counting the number of associated pericytes per unit length.

1. Remove the culture medium and wash with PBS (*see* **Note 28**).
2. Fix with 4 % PFA for 60 min at room temperature (*see* **Note 29**).
3. Remove the fixative and wash three times with PBS, each washing step should be at least 5 min.
4. Permeabilize and incubate with blocking solution overnight at 4 °C.
5. Prepare the required primary antibodies, e.g., anti-CD31 for endothelial cells in PBS at an appropriate dilution (*see* **Note 30**).
6. Incubate overnight at 4 °C.
7. Wash five times in PBS for 10 min.
8. Prepare secondary antibodies in PBS 1/500 and incubate overnight at 4 °C or for 2 h at room temperature (*see* **Note 31**).
9. Remove the antibody solution and wash three times for 10 min in PBS.

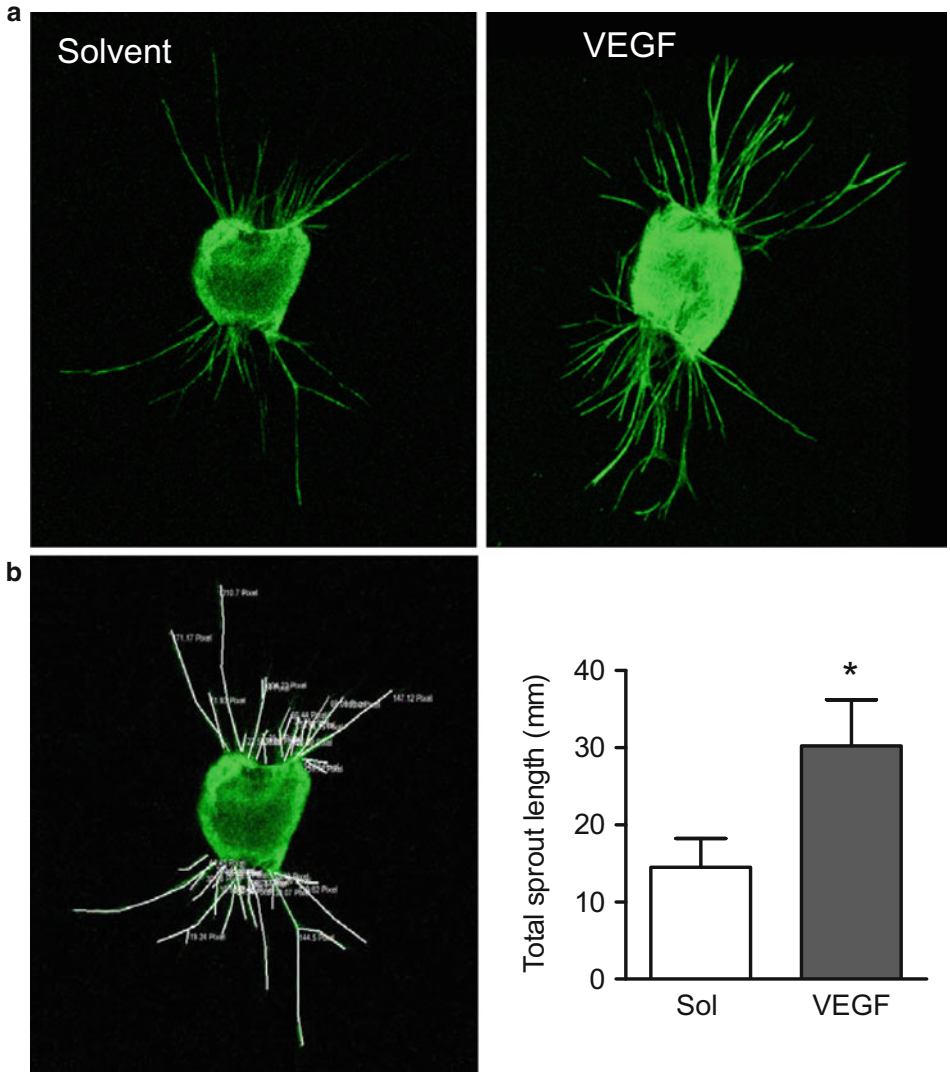


**Fig. 5** Quantification. (**a**) Fluorescence images of endothelial cell sprout outgrowth (CD31 staining, *green*) from solvent- or VEGF-treated rings after 7 days. (**b**) A skeletonized representation of the aortic ring shown in (**a**) generated with the help of quantification software. Total sprout length calculated following the conversion of pixels to mm

10. Postfix for 5 min with 4 % PFA at room temperature.
11. Wash two times for 10 min in PBS.
12. Cover with PBS or mounting medium.
13. Store at 4 °C in the dark.
14. Image aortic sections using a laser scanning microscope with 10 or 20× magnification (*see* **Note 32**).

### 3.6 Quantification

1. Count the number of sprouts that emerge from the main ring as well as the individual branches arising from it as separate vessels [37].
2. Sprout length can be quantified by manually measuring and converting pixels into μm (Fig. 4d) (*see* **Note 33**).

## 4 Notes

1. Outgrowth is optimal in aortic rings from 6- to 12-week-old animals, and with older animals, i.e., 6–10 months of age, sprouting is minimal [35, 38]. If using such animals, the use of collagen gels and medium containing autologous serum is recommended. Fibrin gels, which poorly support sprouting and vascular endothelial growth factor (VEGF) response, or serum-free conditions would not produce meaningful results with these animals. In addition, it has been shown that gender influences outgrowth from aortic explants, and angiogenic response in female rats or mice tends to be impaired compared to males [38]. For these reasons, the age and sex of the animals used within one experiment should be kept as consistent as possible to limit variability. In view of the significant impact that aging and genetic background have on sprouting, aortic rings from littermates should be used.
2. When transfection of the aortic sections is planned, the use of Opti-MEM medium instead of DMEM/F12 is recommended.
3. Do not add endothelial growth medium (EGM) supplements to the basal endothelial medium (EBM).
4. To isolate the autologous serum collect blood (without adding anticoagulants) prior to sacrificing the mouse. This must be performed by trained and licensed individuals according to the relevant ethical guidelines for animal care and experimentation. To obtain the serum, let the blood coagulate at room temperature and centrifuge at 800 × *g* for 10 min. The serum is the supernatant. Transfer the supernatant to a fresh tube and pass through a sterile filter before use. Heat inactivate for 10 min at 54 °C.
5. This must be performed by trained and licensed individuals.
6. Avoid overstretching which could damage the smooth muscle and endothelial cell layers.
7. The abdominal aorta can also be used. However, results do tend to vary between sections from the thoracic and abdominal aorta.
8. Prepare SYLGARD 184-filled dishes according to the manufacturer's protocol in advance, since complete polymerization takes several days.

9. Avoid overstretching which damages the smooth muscle and endothelial cell layers.
10. Handle the aorta only at its ends and avoid stretching the vessel.
11. Make sure all remaining blood is removed since clotted blood impairs sprouting.
12. Insert the syringe only into the tip of the aorta in order to avoid damage to the tissue during syringing. Take care that no air bubbles are introduced into the aorta as this is a great way of removing the endothelial cells.
13. Keep ring width as consistent as possible within the experiment.
14. Approximately 30 rings can be cut from each rat aorta, or ten rings from each mouse aorta. At least three rings per aorta and three different aortae should be used per condition. It is recommended to repeat each experiment several times, since animal experiments contain an inherent variance.
15. If the assay cannot be continued immediately, store the aorta at 37 °C in an incubator with 5 % $CO_2$.
16. If transfection of the aortae is planned, starve the aortic rings in serum-free medium in an incubator at 37 °C with 5 % $CO_2$ overnight prior to transfection, or viral transduction.
17. To prepare a fibrin gel, dissolve lyophilized bovine fibrinogen in endothelial basal medium to a final concentration of 3 mg/mL and add 5.0 mg/mL aprotinin, which prevents the degradation of the fibrin matrix by proteases. Incubate at 37 °C for 1 h to dissolve and pass through a sterile filter (0.45 μm) to remove unpolymerized clumps of fibrinogen. Prepare aliquots of the fibrinogen solution and keep them on ice to prevent premature polymerization when thrombin is added. Add 0.5 U thrombin to each 1 mL aliquot of fibrinogen immediately before use. Prepare only one aliquot at a time. Mix well but avoid the formation of air bubbles. Keep on ice and use within 1 min.
18. A pH of 7.4 is critical for collagen gel polymerization, as well as optimal angiogenic responses in the aortic ring assay [39]; sprouting is markedly delayed at a pH of 6.9 [40].
19. Do not pipette all of the drops at once, but only a few wells at a time to avoid polymerization before there is time to embed the aortic rings.
20. To avoid the dilution of the gel with medium, carefully remove excess medium from the aortic sections by tapping them on an empty dish or sterile filter paper.
21. Rat aortic rings are larger in diameter and thus require a larger amount of matrix, which can no longer be applied in a drop. Although it is also possible to embed mouse aortic sections in

50 μL gel in 96 well plates, this increases the amount of gel needed and the response to growth factors would be impaired.

22. The rings can be embedded in the three-dimensional matrix either with the luminal axis perpendicular or parallel to the bottom of the well. It is preferable to embed the aortic rings on their sides rather than as open rings. This allows for optimal imaging of microvascular sprouting, as the sprouts mostly grow from the cut surfaces along the axis of the lumen. When the rings are placed on their end, the sprouts grow toward the observer and become more difficult to visualize and quantify.
23. The addition of medium before the gel has polymerized can damage the gel. If the gel takes longer than 120 min to set, it will begin to dry out.
24. Add the medium carefully to the side of the well to prevent the fragile gels from lifting or being damaged.
25. Under serum-free conditions, spontaneous angiogenesis in murine aortic rings is usually minimal, whereas rat aortic sections sprout in serum-free conditions. The addition of 2.5 % autologous serum is recommended for murine aortic rings; indeed, the assay rarely functions in its absence. Sprouting can be increased by supplementing small amounts of growth factor (e.g., 10–30 ng/mL VEGF, 10–30 ng/mL bFGF) (Fig. 4d, e); this is particularly useful when anti-angiogenic agents are being tested.
26. If the medium is replaced more often, sprouting is impaired, probably due to the depletion or dilution of endogenous growth factors.
27. Aortic explants can be cultured for up to 14 days before breakdown of the collagen or fibrin matrix occurs [39]. During the first 3 days, fibroblasts grow out of the rings, followed by endothelial cell sprouts. Initially there is an exponential phase of sprouting and growth which peaks approximately at day 7, depending on age and genetic background of the mice and choice of extracellular matrix. In the second week of culture, explants become quiescent and the sprouts begin to regress, probably due to the lack of flow and the depletion of growth factors and other soluble components and the reduction in integrity of the surrounding matrix [30, 41].
28. The PBS buffer should be at room temperature.
29. The 4 % PFA solution should be at room temperature.
30. To co-stain for pericytes, antibodies against α-NG2 chondroitin sulfate proteoglycan (i.e., Millipore, AB5320, 1/200 dilution) are recommended.
31. The addition of 4′,6-diamidino-2-phenylindole (DAPI) or Hoechst 1/5000 to counter stain nuclei can be performed during this step.

32. Imaging can be performed directly in the culture wells if appropriate hardware is available. Alternatively, the gels can be mounted on microscope slides; fill the wells with water and detach the gel from the wells using needles or forceps and a spatula. Carefully arrange the gel on microscope slides and add mounting medium.
33. Quantification of sprout outgrowth from immunostained rings can be performed either by manually counting the number of sprouts and measuring their length as described above or by using computer-assisted image analysis programs, as described elsewhere (*see* refs. [32, 42]). However, if sprouts grow out in large numbers, computer-assisted automatic image analysis is error-prone since it can no longer distinguish between single sprouts, which visually fuse together. In order to obtain meaningful results, manual counting is recommended.

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