Modifications to the classical rat aortic ring model to allow vascular degeneration studies

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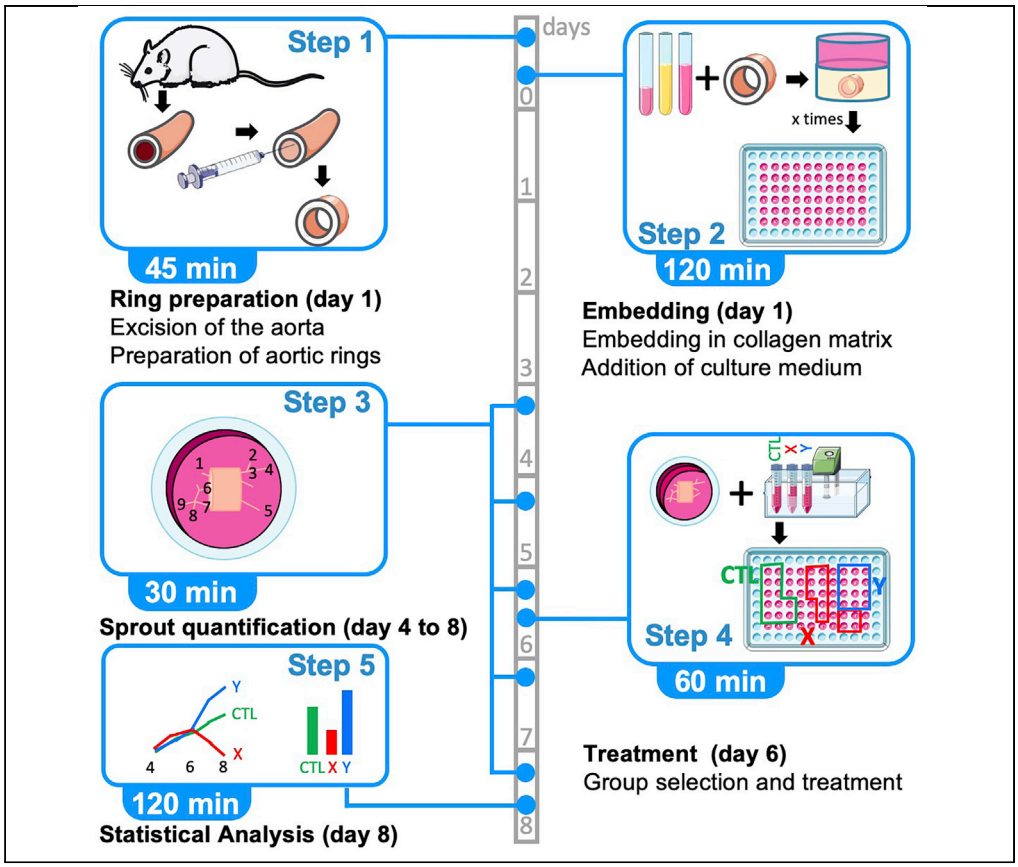

The classical aortic ring model is well suited for deciphering pro-angiogenic processes. Here, we propose simple modifications of the standard protocol to study various anti-angiogenic processes from growth arrest to capillary degeneration. Aortic rings are cultured under basal conditions for 6 days to allow physiological vessel sprouting and then split into treatment groups to follow capillary growth or degeneration for an additional 2 days.

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HIGHLIGHTS

Aortic rings sprout under basal conditions until day 5 and are treated on day 6 only

Growth rate analysis allows evaluation of vascular degeneration

The protocol can distinguish angiostatic from vascular degenerative processes

Paired statistical analysis confers additional power to the analysis

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Modifications to the classical rat aortic ring model to allow vascular degeneration studies

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SUMMARY

The classical aortic ring model is well suited for deciphering pro-angiogenic processes. Here, we propose simple modifications of the standard protocol to study various anti-angiogenic processes from growth arrest to capillary degeneration. Aortic rings are cultured under basal conditions for 6 days to allow physiological vessel sprouting and then split into treatment groups to follow capillary growth or degeneration for an additional 2 days.

BEFORE YOU BEGIN

Preparation of collagen stock solution

Timing: 10 min

⚠ CRITICAL: All described procedures are performed under a laminar flow cabinet to maintain sterility.

⚠ CRITICAL: It is important to maintain the collagen solution at 4°C to avoid polymerization.

1. Prepare a 0.2% acetic acid stock solution by dissolving pure acetic acid in distilled water. Store at 4°C.
2. Prepare a collagen stock solution (2 mg/mL) by dissolving 5 mg collagen powder in the cold 0.2% acetic acid.
3. Place the solution at 4°C for several hours with gentle agitation (alternatively, on a shaking platform for 12–18 h) to allow complete solubilization of the collagen.

Note: When tightly closed, the collagen stock solution can be stored for up to 6 months at 4°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
ISOLECTIN B4 (10 µg/mL)	Merck	Cat# L2895
Rabbit polyclonal IBA1 (1:200, 2.5 µg/mL)	Wako	Cat#019-19741; RRID:AB_839504

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Donkey anti-rabbit Alexa Fluor 546 (1:400, 5 μg/mL)	Thermo Fisher Scientific	Cat# A10040, RRID:AB_2534016
Hoechst 33342 (1:1,000)	Thermo Fisher Scientific	Cat# H3570
Chemicals, peptides, and recombinant proteins		
Acetic acid (0.2%)	VWR	Cat# 20103.330
Lyophilized collagen from rat tail tendon	Roche	Cat# 11179179001
Sodium hydroxide (NaOH)	Merck	Cat# 1.09913
DMEM, no glucose	GIBCO	Cat# 11966-025
EBM medium	Lonza	Cat# CC-3121
Primocin	Invitrogen	Cat# Ant-pm
PBS	Thermo Fisher Scientific	Cat# 10010056
Formaldehyde solution (4%, buffered, pH 6.9)	Merck	Cat# 1.00496
Triton X-100	Merck	Cat# T8787
Experimental models: organisms/strains		
5 week-old Sprague-Dawley rat	Janvier Labs	N/A
Other		
Pursept A Xpress	Brand	Cat# 44830
Standard forceps	VWR	Cat# 232-0092
Standard scissors	VWR	Cat# 233-1421
Thin forceps	VWR	Cat# OUTI0-AXAL
Micro scissors	VWR	Cat# HAMMHSB520-12
Collector tube	VWR	Cat# 216-1805
Petri dishes	Merck	Cat# CLS430588
Sharp blade	VWR	Cat# 233-0115
Micropipets, 10 μL and 200 μL	Bio-Rad	Cat# 166-0505; 166-0508
Filtered micropipet tips, 10 μL and 200 μL	Dutscher	Cat# 015020CL; 014220CL
Hemolysis tube	VWR	Cat# 212-1823
96-well plate	Thermo Fisher Scientific	Cat# 10687551
CO_2 incubator set at 37°C	Thermo Fisher Scientific	Cat# 4110
Stereo microscope	Omano	Cat# OM9959
Inverted bright-field microscope	Olympus	Cat# CKX53
Inverted confocal microscope	Olympus	Cat# FV100; RRID:SCR_016840

⚠ CRITICAL: Formaldehyde is toxic and must be used while wearing gloves and under a fume hood. Hoechst is a DNA intercalator and must be used while wearing gloves. Formaldehyde and Hoechst waste must be collected for specific elimination.

⚠ CRITICAL: High-quality collagen is required to obtain proper sprouting. We recommend using lyophilized collagen from rat tail tendon (Roche, Cat# 11179179001).

Alternatives: References for basic chemicals, laboratory materials, dissection tools, and microscopes in the Key resources table can be replaced by similar products from various providers.

STEP-BY-STEP METHOD DETAILS

Rat aorta excision

Timing: 25 min per rat

Here, we explain how to remove the aorta from the rat.

1. Sacrifice five-week-old Sprague-Dawley male rats by CO_2 asphyxiation and cervical dislocation.
2. Disinfect the abdominal skin with Pursept A Xpress.

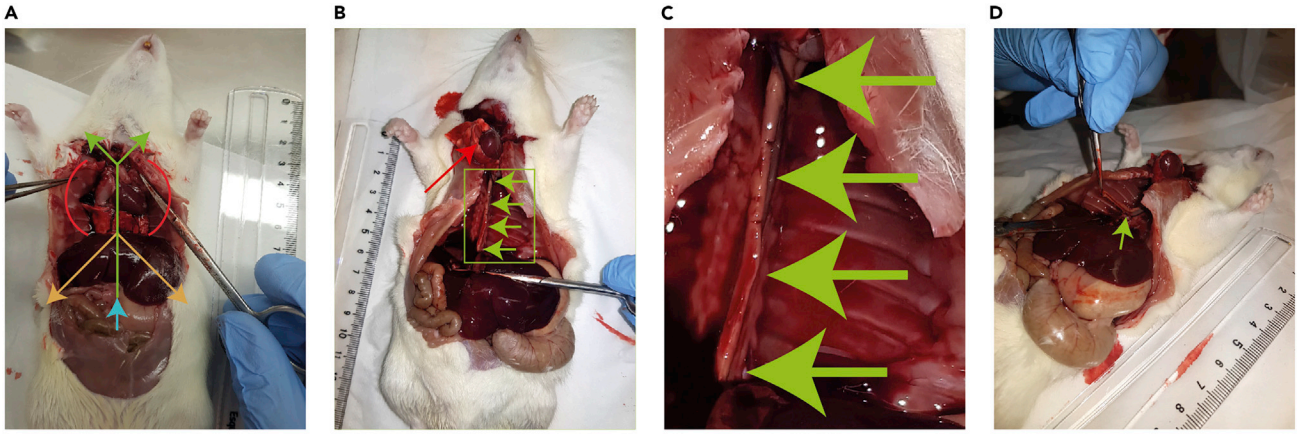

Figure 1. Rat aorta excision

(A) The site for the initial incision (step 3) is highlighted by the blue arrow. The Y-shaped incision (step 4) is highlighted by the green arrows. Incisions under the thoracic cage (step 5) are highlighted by the yellow arrows. The ribs are pushed to the side to provide access to the heart and lungs (step 6), as highlighted by the red curves.

(B) The heart and lungs are displaced (step 8) (yellow arrow) to provide access to the aorta (green arrows in the green region of interest).

(C) Higher magnification of the region of interest.

(D) The distal thoracic aorta is cut with fine scissors while being held with forceps and excised from the rat by progressive progression of the scissors alongside the vertebral column, as highlighted by the green arrow.

The ruler is graduated in centimeters.

3. Lift the skin at the bottom of the abdomen with forceps and make an incision in the skin and underlying muscles using scissors.
4. Make a Y-shaped incision through the rat's thoracic cage, starting at the previous incision, running up to the manubrium of the sternum and then on each side to the shoulders (Figure 1A).
5. Make an incision on each side, just under the thoracic cage (Figure 1A).
6. Push the ribs to the side to have access to the intrathoracic organs (heart and lungs) (Figure 1A).
7. Cut the diaphragm, the vena cava, and the esophagus.
8. Displace the heart and lungs to clear the thoracic cavity (Figure 1B).
9. Displace the stomach, liver, and intestines to clear the abdominal cavity (Figure 1B).
10. Absorb blood using an absorbent tissue.
11. The aorta is surrounded by fibroadipose tissue and appears as a whitish vessel along the vertebral column (Figure 1B).

Note: Be careful not to mistake the aorta for the esophagus. The aorta is the vessel that is tightly bound to the vertebral column, whereas the esophagus is only maintained by smooth connective tissue.

12. Cut the distal end of the aorta using fine scissors.
13. Hold the distal end of the aorta using fine forceps and cut the subaortic tissue with scissors (Figure 1C; Methods Video S1: Aorta excision, steps 13 and 14).

14. Cut the aorta at the proximal end (Methods Video S1: Aorta excision, steps 13 and 14).
15. Place the aorta in a Falcon tube containing EBM.

Note: Steps 7–15 are also illustrated in Methods Video S2: preparing aorta steps 7–15.

Preparation of aortic rings

Timing: 20 min per aorta

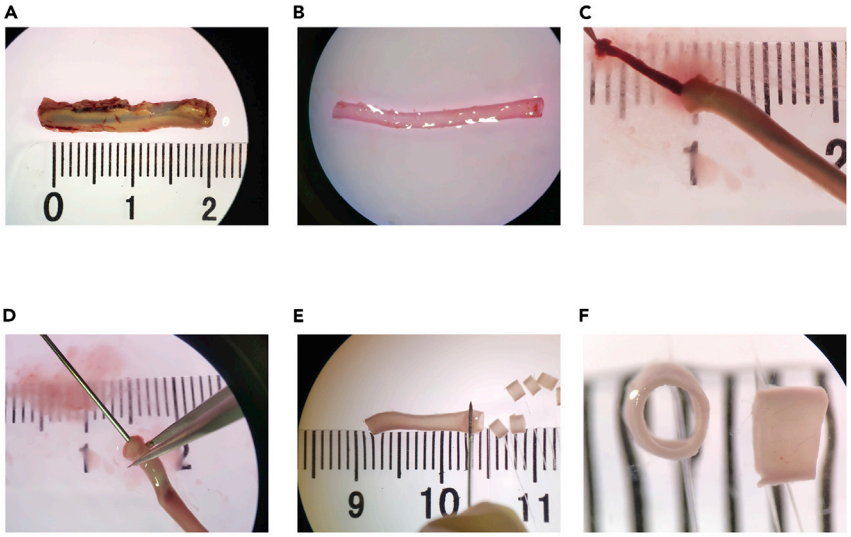

Figure 2. Preparation of aortic rings

(A) Freshly excised aorta in a Petri dish filled with EBM.
(B) Aorta after fibroadipose tissue removal.
(C) Intraluminal blood clot removal.
(D) Intraluminal flushing.
(E) Cutting of 1-mm wide aortic rings.
(F) Higher magnification of aortic rings.
The ruler is graduated in centimeters

These steps describe how to prepare and cut the aorta to obtain aortic rings.

16. Place the aorta in a Petri dish containing EBM to keep it wet (Figure 2A).

    Note: The isolated five-week-old Sprague-Dawley rat aorta is approximately 2–3 cm long.

    Note: Dissection is easier to practice in a moderately filled Petri dish to avoid floating movements of the aorta during dissection. Troubleshooting 1

17. Under a stereo microscope, remove the fibroadipose tissue (Figure 2B; Methods Video S3: fibroadipose tissue removal, step 17).

    Note: Due to the large size of the rat aorta, dissection is easier to practice at low (6–10×) magnification.

18. Remove any intraluminal blood clots with forceps (Figure 2C; Methods Video S4: intraluminal clot removal, step 18).

19. Hold the extremity of the aorta using forceps and insert a 2.5-mL syringe with a 23G needle containing EBM into the lumen. Flush EBM into the aorta to remove intraluminal blood (Figure 2D; Methods Video S5: aorta flushing, step 19).

    Note: When finished, the aorta should be pale pink and translucent.

20. Cut the aorta into 1-mm wide rings with a sharp blade using a single straight motion (Figures 2E and 2F; Methods Videos S6 and S7: cutting aorta in rings, step 20).

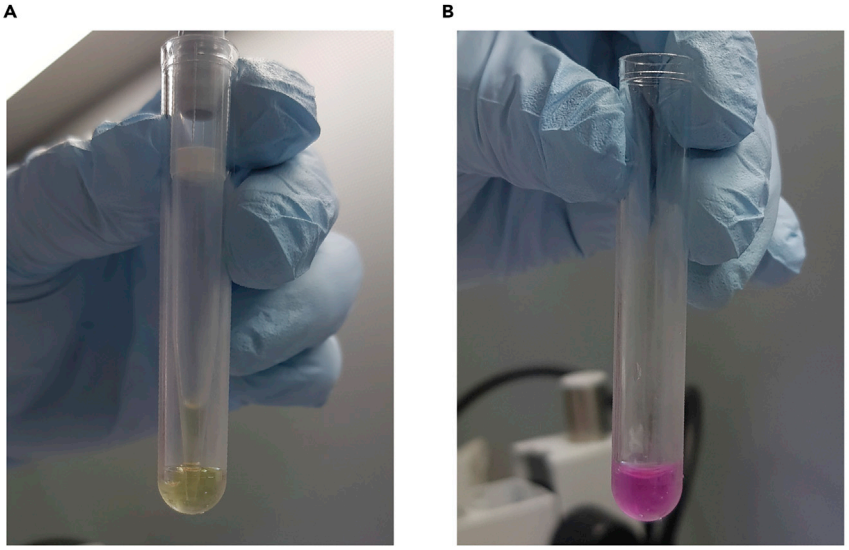

Figure 3. Preparation of working solution of collagen

(A) After mixing 200 μL collagen stock solution with 200 μL "DMEM, no glucose" medium, the solution turns yellow.

(B) After the addition of a sufficient volume of 5 N NaOH (typically 1–2 μL), the solution turns back to a persistent pink.

21. Discard the most proximal and distal aortic rings and keep only the sharp-cut rings.

Note: Up to 20 to 30 rings can be obtained, depending on the length of the aorta.

Preparation of working solutions of collagen

Timing: 30 min per aorta

Aortic rings are embedded in 1 mg/mL collagen gel.

22. Collagen polymerizes rapidly at 19°C–21°C. Prepare a small volume and use it immediately: mix 200 μL of the collagen stock solution with 200 μL DMEM, without glucose, in a hemolysis tube (the solution turns yellow [Figure 3A; Methods Video S8: Collagen solution preparation, step 22]) and equilibrate the pH with a few microliters of 5 N NaOH (Figure 3B; Methods Video S9: pH equilibration, step 22). This working volume of 1 mg/mL collagen solution is sufficient for the embedding of six rings.

Note: After the addition of collagen, the DMEM solution will turn yellow due to acidification. 5 N NaOH is used to equilibrate it to a pH of 7 to 7.4. The amount of NaOH to use is the minimal volume required that allows the red phenol of the DMEM to remain persistently pink. This volume should be empirically determined for each stock solution of collagen.

⚠ CRITICAL: Physiological pH is required to culture aortic tissue. If NaOH is gradually added (i.e., 0.5 μL by 0.5 μL), the color of the red phenol is a sufficient indicator for equilibrating the pH (the presence of sodium bicarbonate in the DMEM will help to maintain a physiological pH when incubated in a 5% CO_2 incubator). We do not recommend using a pH meter or pH strips to avoid unnecessary time-consuming procedures during critical steps.

⚠ CRITICAL: Gently mix and homogenize the solution to avoid the formation of bubbles, which impairs proper sprouting. (Troubleshooting 2 and 3)

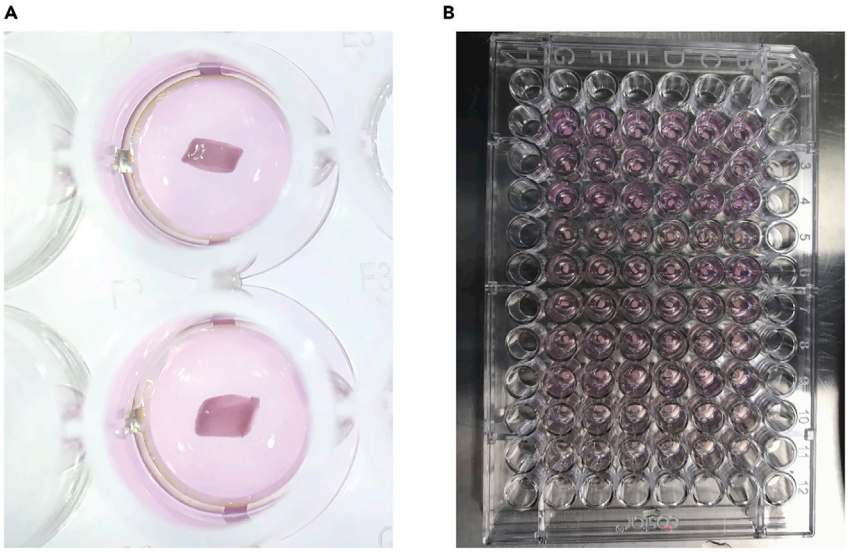

Figure 4. Aortic rings embedded in a collagen matrix
(A) Global view of a culture plate with 60 aortic rings embedded in a collagen matrix.
(B) Higher magnification of two aortic rings embedded in a collagen matrix in the center of their wells.

⚠ CRITICAL: Always keep the collagen stock solution, "DMEM, no glucose" medium, and the prepared working solution of collagen on ice to prevent polymerization. (Troubleshooting 3)

Embedding of the aortic rings in the collagen matrix

Timing: 45 min per aorta (plus 30 min of incubation).

Aortic rings are embedded using a 1 mg/mL working collagen solution.

23. Off the ice, fill the wells of a 96-well culture plate with 60 µL of the working collagen solution.

⚠ CRITICAL: Off the ice, collagen will rapidly polymerize inside the well. We recommend filling only three wells at a time.

⚠ CRITICAL: The tube of the working solution should be kept on ice between each pipetting step. (Troubleshooting 3)

⚠ CRITICAL: We do not recommend using the external wells of the plate because of excess evaporation from the peripheral wells.

24. Immediately, gently pick up an aortic ring with the forceps and dry the ring and the tips of the forceps with an absorbent tissue to remove excess medium.

⚠ CRITICAL: Removal of all culture medium from the ring and the forceps is essential, as the addition of culture medium near the aortic ring significantly decreases the local collagen concentration and dramatically impairs vascular sprouting. (Troubleshooting 3)

25. Embed the aortic ring into a collagen-filled well before it polymerizes. The ring should be placed in the center of the well and the walls of the aortic vessel should face the bottom (Figures 4A and 4B).

⚠ CRITICAL: We recommend embedding the ring within 5 min following the addition of collagen to prevent polymerization. Do not attempt to keep the plate on ice during embedding to prevent matrix polymerization. At 4°C, the gel viscosity is reduced and rings do not stay still. (Troubleshooting 3 and 4)

⚠ CRITICAL: Do not damage the collagen matrix by stirring with forceps. If the ring does not stay in the required position, hold it for 30 s with the forceps until the gel starts to polymerize. Then, gently remove the forceps from the gel while checking that the ring stays still. If the ring starts moving, hold it again for an additional 30 s.

Note: The use of a stereo microscope may help in placing the ring in the center of the well.

26. Discard the hemolysis tube when it is emptied.

⚠ CRITICAL: Do not re-use tubes, as collagen may have polymerized inside the tube.

27. Once all the rings are embedded, add 200 μL PBS to all the empty surrounding wells to prevent evaporation in wells of interest.
28. Incubate the plate at 37°C in 5% CO_2 for 30 min to allow complete collagen polymerization.

Note: If more than one culture plate is needed for the experiment, we recommend starting incubation of the first plate as soon as all the wells have been filled with aortic rings.

Addition of culture medium

Timing: 15 min per aorta (plus 4 days of incubation)

29. Prepare EBM Medium by adding a 1:1,000 volume of Primocin.
30. Warm the medium in a water bath at 37°C.
31. Add 150 μL warmed medium to each well.
32. Allow vessel sprouting by incubating the plate for four days at 37°C and 5% CO_2.

Note: The EBM will provide all nutrients necessary for paracrine secretion of factors needed for aortic ring sprouting.

⚠ CRITICAL: The rings produce paracrine pro-angiogenic molecules that are necessary for the initiation of sprouting. Therefore, changing the medium before day 4 is not recommended.

Quantification of sprouting and changing of the medium

Timing: 30 min per aorta from day 4 to day 8 (20–30 rings)

Here, we describe how to count sprouts and change the growth medium.

33. Starting on day 4, sprouts are counted every day under a bright field or phase-contrast inverted microscope with a 40× or 100× magnification.
    a. Sprouts grow from the cut sides of the ring.
    b. Microvessels are composed of endothelial cells associated with pericytes. A small number of fibroblast and macrophages migrate into the gel around neovessels.

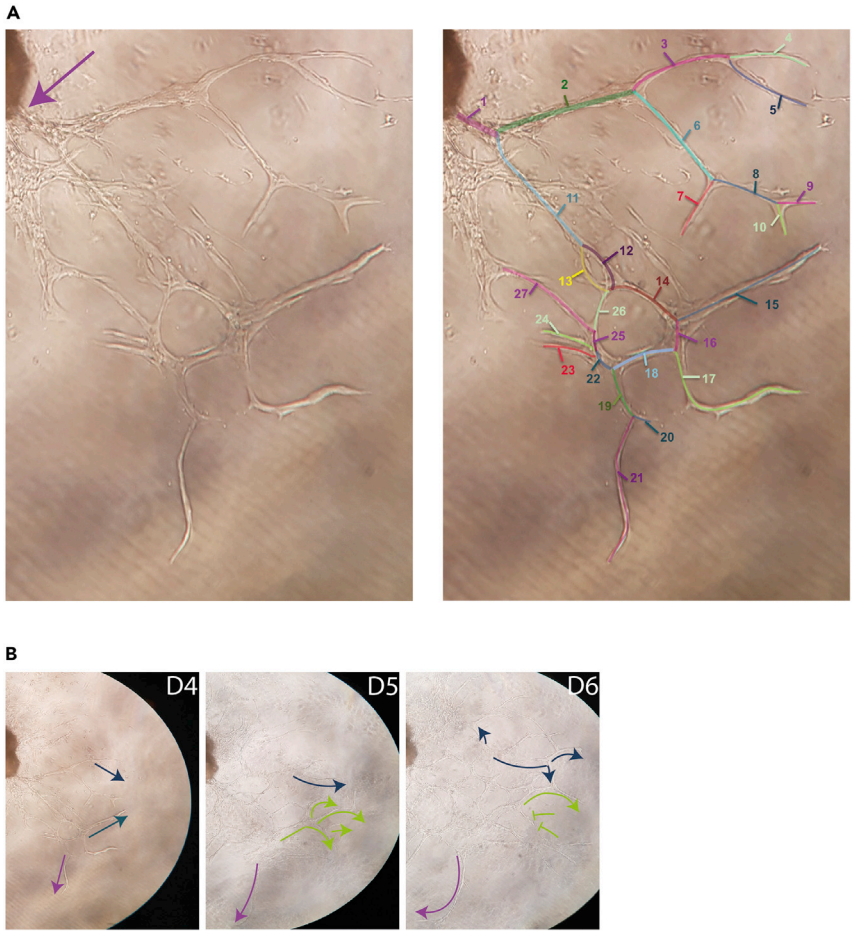

Figure 5. Quantification of sprouts

(A) Quantification of sprouts on day 4. A single sprout originating from the aortic ring (purple arrow in the left panel) has grown into 27 individual countable sprouts.

(B) Evolution of sprouting of the same area from day 4 to day 6. Arrows highlight the growth and regression of three independent vessels.

⚠ CRITICAL: Neovessels and fibroblasts can be hard to tell apart. Frequently readjusting the focus should help to definitively identify neovessels, which are thicker and longer than isolated macrophages and fibroblasts.

Note: The serum-free culture condition strongly favors the sprouting of endothelial cells over the proliferation of fibroblasts.

c. Counting is performed as follows (as previously described in Aplin et al., 2008) (Figure 5A):
   i. Every branching generates two new vessels. Thus, each branch of a vessel is counted as a separate vessel.
   ii. Sprouts can merge and form loops. Therefore count each loop as two vessels.
d. Each ring is counted daily until day 8 (Figure 5B).

Note: To determine the sprouting growth rate, it is recommended that the same experimenter count the vessels every day at the same time of the day.

Note: If you have more than one plate to count, take only one plate out at a time and leave the others in the incubator.

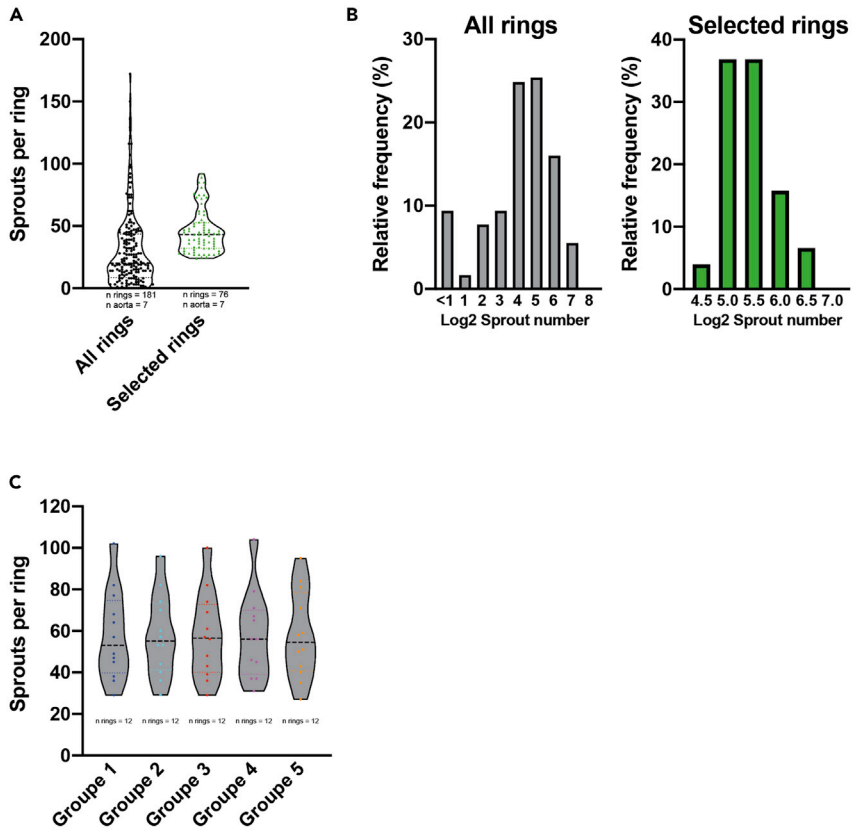

Figure 6. Selection of aortic rings for testing

Quantification of sprouts per ring on day 6 for group constitution.

(A) Violin plot representation of 181 rings (from 7 aorta) and stringent selection of 76 rings (42%) that will be used for the test.

(B) Frequency log2 distribution of sprouts per ring for all rings (left panel) or selected rings (right panel).

(C) An example of the distribution of aortic rings across homogeneous groups.

In (A) and (C), dots represent individual aortic rings and the dashed lines represent the median and quartiles.

34. The medium has to be renewed at day 4 (and then every 2 days thereafter if necessary).
    a. Prepare EBM Medium with 1:1,000 Primocin and heat it in a water bath at 37°C.
    b. Remove 130 µL of the old medium and add 150 µL of the fresh medium into each well.

Note: EBM Medium with 1:1,000 Primocin can also be prepared in a larger quantity on D0. However, the exact total volume of medium used for the entire experiment will not be known, as certain aortic rings will not be selected after D6 (see step 36).

Selection of suitable aortic rings for testing compounds

Timing: 1 h

Here we describe how to select the rings that will be used to test compounds.

35. At day 6, select the sprouting rings that will be used for the rest of the experimentation. We advise selecting only rings that produce a minimum of 20 sprouts (Figure 6A).

Note: Approximately 10% of rings fail to sprout. For the remaining rings, the number of sprouts typically follows a log-normal distribution (Figure 6B). The percentage of rings that

grow and the number of sprouts per ring depends on the skill of the experimenter. Given the log-normal distribution, some rings sprout very heavily relative to the others, making it difficult to equally distribute them among the different groups. We recommend discarding them (in our example, we did not select rings with >95 sprouts on day 6; Figure 6A). (Troubleshooting 5, 6, and 7)

36. Create groups in which the rings are assigned evenly according to their number of sprouts (Figure 6C).

Note: We recommend using a sufficient number of rings per group to allow proper parametric statistical analysis at the end of the experiment.

37. After group assignment, change the medium and stimulate them with the compounds to be tested.

Fluorescence imaging (optional)

Timing: 1 week

At day 8 after the last live-counting, aortic rings can be fixed for further fluorescence imaging.

38. Remove the culture medium from all the wells.
39. Wash each culture well with 200 µL PBS three times (with 15-min intervals between each wash).
40. Fix the culture with 100 µL 4% formaldehyde for 30 min.

⚠ CRITICAL: Formaldehyde is toxic and must be used while wearing gloves under a fume hood.

41. Wash each culture well with 200 µL PBS three times (with 15-min intervals between each wash).

Note: Formaldehyde waste must be collected for further specific elimination.

42. Dilute primary IBA1 antibody at 1:200 (2.5 µg/mL) and ISOLECTIN B4 (10 µg/mL) in PBS 0.1% Triton X-100 .
43. Incubate for 3 to 7 days at 4°C.
44. Wash three times (15 min) with 200 µL PBS.
45. Add secondary antibody at 1:400 (5 µg/mL) and renew the ISOLECTIN B4 treatment (10 µg/mL).

Note: Imaging through the gel is challenging and may result in a poor signal for macrophages (IBA1 positive cells), depending on the microscopy equipment. We do not recommend using lower concentrations of secondary antibodies without testing the signal using your set-up.

46. Incubate for a minimum of 12 h at 4°C.
47. Add Hoechst 33342 at 1:1,000 and incubate for a further 30 min.

⚠ CRITICAL: Hoechst is a DNA intercalator and must be used while wearing gloves.

48. Wash three times (15 min) with 200 µL PBS.

⚠ CRITICAL: Hoechst waste must be collected for further specific elimination.

49. Vessels and macrophages can be observed directly in the well using an inverted fluorescence microscope.

Note: Collagen gel does not generate as much fluorescent noise as a Matrigel-based matrix and high-quality pictures can be obtained with a confocal microscope. However, aortic rings are autofluorescent and images from an epifluorescent microscope are still very noisy.

Note: If an inverted confocal microscope is not available, an upright confocal microscope can be used after removing the gel from the well. We recommend using needle to cautiously detach the gel from the well. Disruption of the gel may result in alteration of the vessel structure.

EXPECTED OUTCOMES

The above protocol allows detection of the growth arrest of capillaries, as well as dynamic vascular degenerative processes.

The growth rate of aortic rings is determined by daily counting of the number of sprout under an inverted bright-field microscope. In contrast to the classical aortic ring protocol, it allows the identification of compounds with various angiogenic properties. In our example, group 1 and group 3 compounds do not change the basal growth rate, group 5 compounds show anti-angiogenic and angiostatic properties, and group 2 and 4 compounds show vascular degenerative properties (Figure 7A).

Note: The proposed protocol makes it possible to differentiate groups 2 and 4 from group 5, which would not be possible with day 0 treatment in the classical aortic ring protocol. In the original model, these groups would all result in the absence of sprouting.

Confocal fluorescence imaging allows visualization of vascular degeneration at the cellular level (Figure 7B).

Note: Macrophages are important in the sprouting process of the aortic ring model (Gelati et al., 2008; Zorzi et al., 2010). The macrophages are positive for ISOLECTIN B4 and can be further distinguished from vessels due to their also being positive for IBA1.

QUANTIFICATION AND STATISTICAL ANALYSIS

In contrast to the original protocol in which the rings are treated on day 0, this protocol allows stringent selection of solely actively sprouting aortic rings before their inclusion in a test group. Approximately 60% of the rings do not develop a satisfying sprouting response (> 20 sprouts per ring). Such selection thus highly improves the statistical power of analysis and decreases the required quantity of the tested compounds.

The number of sprouts of each aortic ring is compared between day 6 (just before the treatment) and day 8 (two days after). This allows the use of paired statistical analysis, which improves the statistical power of the analysis. The number of sprouts per aortic ring follows a normal log distribution (Figure 6B). We thus recommend analyzing the growth rate as the fold change for each individual ring using the following formula:

$$\text{Log2}(\text{sprouts counted on day 8}/\text{sprouts counted on day 6})$$

Growing aortic rings will have values >0 and degenerative aortic rings will have values < 0.

If a sufficient number of replicates is used in the experiment (i.e., n = 12 per group), we recommend using parametric tests (t-test for two group comparisons or one-way ANOVA for comparisons of more than two groups) (Figure 8A). If parametric test assumptions are not met, nonparametric tests

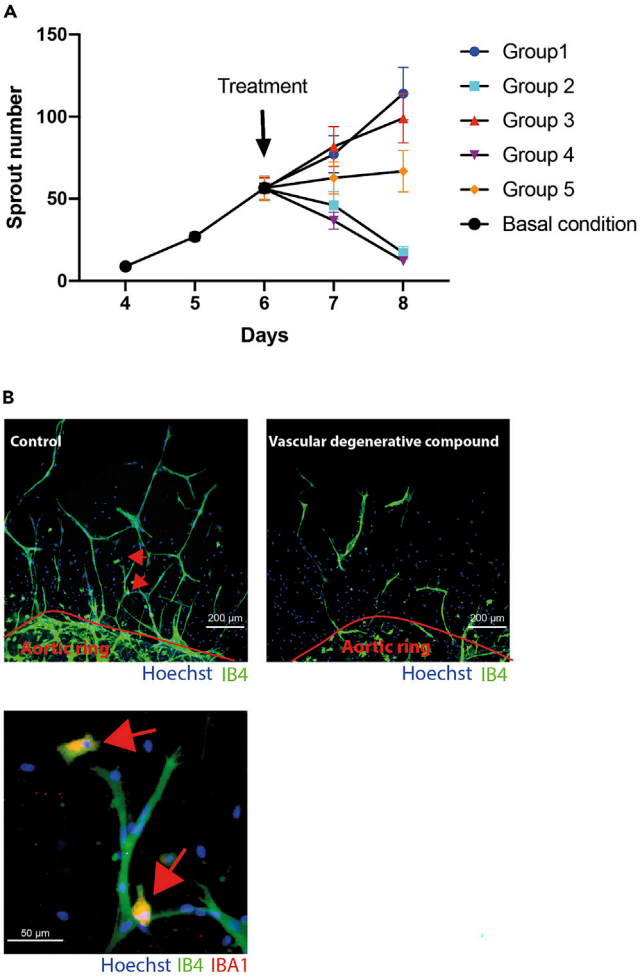

Figure 7. Expected outcomes

(A) Time course representation of the mean number of sprouts per ring for selected aortic rings for testing compounds. Between D4 and D6 all aortic rings were cultured under basal conditions and then five different treatments were tested.

(B) Confocal microscopy images of a group 1 (left panels) and group 4 aortic ring (right panel). Endothelial cells are labeled with ISOLECTIN B4 (green) and nuclei with Hoechst (blue). The bottom left panel shows an enlargement of the upper left panel, highlighting the presence of IBA1 positive cells (red, macrophages).

can be used for comparisons (Mann-Whitney for two groups or Kruskal-Wallis for more than two groups).

LIMITATIONS

Sprouts mainly originate from the sharp-cut side of aorta, thus the aorta have to be cut into rings to generate sprouts. However, manually cutting the aorta may result in irregularly sized aortic rings, which appears to correlate with sprouting capacity. We tested the use of a 1 and 2 mm puncher to produce aortic discs of regular shape and size to overcome this difficulty. Moreover, these 2D discs would be easier to observe and a single aorta could provide approximately 2 to 3 times more discs than those cut as rings. However, aortic punches failed to grow in the collagen matrix. Only half generated vessels and none generated enough vessels to be analyzed.

Thus, the ring shape spears to be necessary for sprouting for unknown reasons. Irregular size of the rings, which leads to wide log-normal distribution, is still one of the main limitations of this

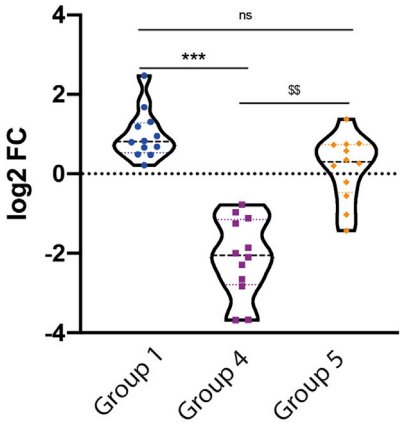

Figure 8. Quantification

Violin plot representation of log2 fold change in the number of sprouts per ring between D6 (before stimulation) and D8 (2 days after stimulation). Dots represent individual aortic rings and dashed lines represent the median and quartiles. p values were determined using two-tailed Kruskal-Wallis tests ($p < 0.0001$), followed by Dunn's multiple comparison test. Group 1 versus group 5: ****p-adjusted < 0.0001; group 1 versus group 4: $p = 0.2436$; and group 4 versus group 5: $^{\$\$}p = 0.0051$.

technique, with approximately only 40%–50% of embedded rings suitable for treatment and subsequent statistical analysis.

TROUBLESHOOTING

Problem 1

Floating aorta are difficult to clear from fibroadipose tissue or to cut into regular rings.

Potential solution

Keep the aorta in small volumes of culture medium during dissection and cutting to prevent it from drying and floating. It is also easier to cut the aorta on the lid of a Petri dish.

Problem 2

Presence of bubbles in the collagen matrix

Potential solution

The release of trapped air within the pipet has to be avoided, as the solution rapidly polymerizes around the bubbles which thereafter remain captive in the matrix.

Problem 3

Collagen polymerizes unevenly, resulting in impaired sprouting.

Potential solution

This situation may be due to inefficient homogenization of the collagen, "DMEM no glucose" medium and NaOH. Always carefully homogenize the collagen working solution.

This situation may be the consequence of the collagen having already polymerized before embedding of the aortic ring. Always maintain the collagen solution on ice to prevent polymerization, prepare small volumes of collagen, do not fill more than three wells at a time with collagen, and use new tubes for each 400 µL of working collagen solution. If the collagen has already started to polymerize in the hemolysis tube, do not use it and prepare a new solution. If the collagen has started to polymerize in the culture well before the addition of the ring, do not use the well.

This situation may be due to presence of culture medium on the aortic ring or forceps when the aortic ring is embedded in the collagen solution. This will result in an inadequate concentration of collagen in the immediate proximity of the aortic ring for proper vessel structure formation. Always dry the forceps and aortic ring with absorbent tissue before embedding aortic ring in the collagen solution.

Problem 4
The ring does not stay still in the collagen solution.

Potential solution
Keep the ring in position with the forceps until the collagen starts to polymerize (30 s or 1 min should be sufficient) and then carefully remove the forceps. Do not keep the culture plate on ice, as this will prevent polymerization.

Problem 5
Rings do not sprout as much as anticipated

Potential solution
A substantial fraction (50%–60%) of the rings will not grow sufficiently for experimentation and will have to be excluded before treatment. Always consider embedding more rings than the number required for the final analysis.

Problem 6
Rings do not sprout at all.

Potential solution
Make sure not to have mistaken the aorta for the esophagus. Methods Video S2 illustrates the steps to isolate the aorta (if a hemorrhage occurs while dissecting, use absorbent tissue to remove the blood).

Make sure that the aorta has not been damaged or has dried during the process. Do not wait more than 45 min before adding the culture medium to the embedded aortic rings (Step 28).

Make sure that the aortic rings are well embedded in the collagen matrix.

Problem 7
Bacterial/fungal contamination

Potential solution
The entire procedure should be carried out in a sterile cabinet. Avoid any contact of the aorta with instruments that have touched the fur and do not make incision of the intestine. We recommend using Primocin at a 1:1,000 dilution during dissection and in the EBM culture medium added after gel polymerization (unless contraindicated for the experiment).

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xavier Guillonneau (xavier.guillonneau@inserm.fr).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate any unique datasets or code.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2020.100281.

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AUTHOR CONTRIBUTIONS

Conceptualization, G.B. and X.G.; methodology, G.B. and X.G.; validation, G.B. and T.-M.S.; formal analysis, G.B. and X.G.; investigation, G.B., T.-M.S., and X.G.; writing – original draft, G.B., T.-M.S., and X.G.; writing – review & editing, G.B., T.-M.S., X.G., and F.S.; visualization, G.B., T.-M.S., and X.G.; supervision, G.B., F.S., and X.G.; project administration, G.B. and X.G.; funding acquisition, F.S. and X.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Aplin, A.C., Fogel, E., Zorzi, P., and Nicosia, R.F. (2008). The aortic ring model of angiogenesis. Methods Enzymol. *443*, 119–136.

Gelati, M., Aplin, A.C., Fogel, E., Smith, K.D., and Nicosia, R.F. (2008). The angiogenic response of the aorta to injury and inflammatory cytokines requires macrophages. J. Immunol. *181*, 5711–5719.

Zorzi, P., Aplin, A.C., Smith, K.D., and Nicosia, R.F. (2010). Technical Advance: The rat aorta contains resident mononuclear phagocytes with proliferative capacity and proangiogenic properties. J. Leukoc. Biol. *88*, 1051–1059.