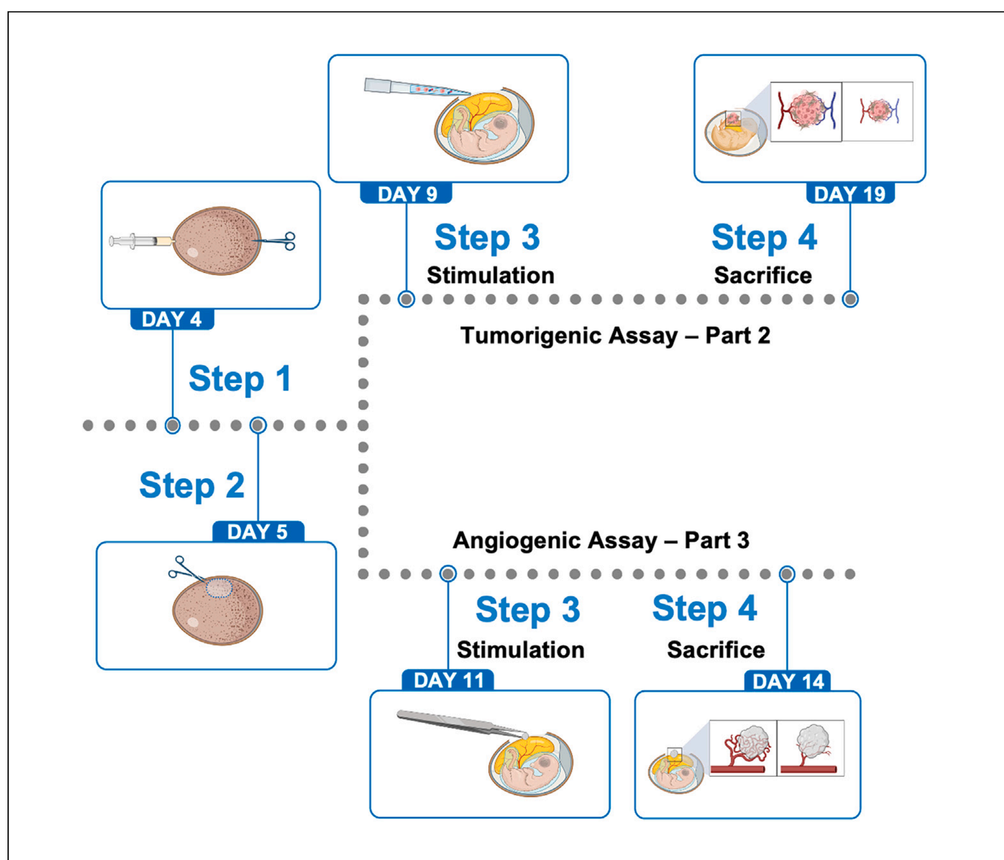
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for performing angiogenic and tumorigenic assays using the *in ovo* chick embryo chorioallantoic membrane model



Tommy Chastel, Serena Filiberti, Stefania Mitola, Roberto Ronca, Andrei Turtoi, Michela Corsini

michela.corsini@unibs.it

Highlights

Steps for egg preparation for angiogenic assays

Instruction for tumor cell engraftment on the CAM

Steps for analyzing angiogenic and tumorigenic outcomes

The chick embryo chorioallantoic membrane (CAM) is a viable alternative *in vivo* model to explore cancer biology due to its simplicity, accessibility, and cost-effectiveness. Here, we present a protocol for performing angiogenic and tumorigenic assays using the *in ovo* chick embryo CAM model. We describe steps for initial general preparation and for the angiogenic and tumorigenic assays in two distinct sections.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Chastel et al., STAR Protocols 6, 103663
March 21, 2025 © 2025 The Authors. Published by Elsevier Inc.
https://doi.org/10.1016/j.xpro.2025.103663

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for performing angiogenic and tumorigenic assays using the *in ovo* chick embryo chorioallantoic membrane model

Tommy Chastel,[1,3] Serena Filiberti,[2,3] Stefania Mitola,[2] Roberto Ronca,[2] Andrei Turtoi,[1] and Michela Corsini[2,4,5,*]

[1]Tumor Microenvironment and Resistance to Treatment Lab, INSERM U1194, 34090 Montpellier, France

[2]Department of Molecular and Translational Medicine, University of Brescia, 25123 Brescia, Italy

[3]These authors contributed equally

[4]Technical contact

[5]Lead contact

*Correspondence: michela.corsini@unibs.it
https://doi.org/10.1016/j.xpro.2025.103663

SUMMARY

The chick embryo chorioallantoic membrane (CAM) is a viable alternative *in vivo* model to explore cancer biology due to its simplicity, accessibility, and cost-effectiveness. Here, we present a protocol for performing angiogenic and tumorigenic assays using the *in ovo* chick embryo CAM model. We describe steps for initial general preparation and for the angiogenic and tumorigenic assays in two distinct sections.

BEFORE YOU BEGIN

The chick embryo chorioallantoic membrane (CAM) is a highly vascularized extraembryonic membrane that mediates gaseous exchange and calcium transport within the embryo and is endowed with high potential to explore tumor biology. The development of the CAM begins between days 3.5 and 4 post fertilization (dpf) through the fusion of the allantois and the chorion and continues developing until around day 12.[1,2]

Due to its naturally extended and highly organized vascular network, the CAM model offers a unique and versatile platform for studying angiogenesis and tumor biology. This vascular structure provides an excellent system to follow blood vessel formation, remodeling, and interactions. For this reason, the CAM supports rapid and dynamic vascular development, making it highly sensitive to angiogenic stimuli. Additionally, the CAM is readily accessible for experimental manipulation, allowing for precise interventions and real-time observation. Its transparency facilitates direct visualization and high-resolution imaging of vascular growth, angiogenic responses, and tumor-vascular interactions without invasive procedures. Moreover, the CAM model is cost-effective, does not require dedicated animal facilities, and is endowed with limited ethical issues, thus making it an attractive alternative for preclinical studies and easily transferable to most laboratories. Its versatility extends to applications such as drug screening, testing pro- and anti-angiogenic compounds, and investigating tumor metastasis, offering a powerful tool for basic and translational research.

This protocol describes an *in ovo* CAM assay, which represents a valuable *in vivo* model for advancing cancer research. It provides a platform for investigating angiogenic and tumorigenic mechanisms and exploring treatment efficacy. The CAM assay is distinguished by its simplicity,

 © 2025 The Authors. Published by Elsevier Inc.
This is an open access article under the CC BY-NC license (http://creativecommons.org/licenses/by-nc/4.0/).

accessibility, rapid developmental growth, and cost-effectiveness compared to traditional *in vivo* animal models.

The following protocol outlines a high-throughput assay designed to assess tumor cells' angiogenic and tumorigenic characteristics. This protocol has been successfully applied to several human cell lines, including the breast cancer cell line MCF7, the melanoma cell lines Sk-Mel-31 and A2058, the hepatocellular carcinoma cell line HLE, the endometrial adenocarcinoma cell line HEC1B, and the pancreatic carcinoma cell line PANC01. Additionally, murine cancer models, such as the melanoma B16BL6 cell line, have been successfully used with this protocol.

The described method consists of three parts:

Part 1) The initial egg preparation phase.

Part 2) The tumorigenesis assay section, including cell culture preparation.

Part 3) The angiogenesis assay section, including stimuli preparation.

⚠ CRITICAL: To ensure repeatability and robust statistical output, a minimum number of eggs must reach the experimental endpoint, represented by at least eight/ten conditioned eggs. The inability to purchase eggs from certified centers, such as the Veterinary Research Center (e.g., The Animal and Plant Health Agency [APHA] in the United Kingdom), may lead to a 5–10% loss of the eggs because they may be unfertilized, non-viable, or dead due to trauma or excessive nutrient loss.

⚠ CRITICAL: Egg manipulation can easily be done in non-confined bench areas. However, egg contamination by bacteria or fungi is a potential risk. Contamination should be accounted for when calculating the experimental sample size.

Institutional permissions

All experiments in this protocol were performed per local and institutional regulations that were compliant with EU directive 2010/63. Users of this protocol should consult their institution for guidance on their local and institutional regulations.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Alginic acid	Sigma-Aldrich	180947
Calcium chloride	Merck	223506
Ethanol	Carlo Erba	414635
Penicillin-streptomycin (5,000 U/mL)	Thermo Scientific	15070063
0.25% Trypsin EDTA	Thermo Scientific	15050065
Fetal bovine serum (FBS)	Thermo Scientific	A3160501
DPBS	Gibco	14190-094
DMEM/F-12 + Glutamax media	Gibco	31331-028
Millipore express plus 0.22 μm PES 500 mL	Sigma-Aldrich	S2GPU05RE
10 mL syringe without needle	Terumo	SS+10ES1
0.22 μm Filter Millex-GP filter unit	Sigma-Aldrich	SLGP033RS
Experimental models: Cell lines		
DAOY	ATCC	HTB-186

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Fertilized chicken egg	Veterinary Research Center	Earl Les Bruyers, 28190 Dangers, France
Software and algorithms		
Fiji	ImageJ	https://imagej.net/software/fiji/
Other		
Egg incubator	Fiem	MG316/rep evo ua
Humidifier	Fiem	MG200/300
Microsurgery scissors	Fine Science Tools	No. 14084-08
Straight forceps	Fine Science Tools	No. 11252-00
Curved forceps	Fine Science Tools	No. 11274-20
Paper punch	Bruneau	13241
Coverslip	Sarstedt	No.83.1840.002
Centrifuge	Eppendorf	54030 R
Cryopure tube	Sarstedt	72.380.006
1.5 mL tubes	Sarstedt	72.706.400
12 wells plate	Sarstedt	83.3921
Duct tape	Scotch	313
Milli-Q water production system	Millipore	ZIQ7000T0C
15 mL polypropylene conical tube	Falcon	352096
50 mL polypropylene conical tube	Falcon	352070

STEP-BY-STEP METHOD DETAILS

Part 1: Procedure for egg preparation and opening

Timing: 4 days

Timing: 1 h (for step 1)

Timing: 4 h for 150 eggs (for step 2)

Timing: 3 h for 150 eggs (for step 3)

CRITICAL: Use sterile tweezers before making all the procedures.

CRITICAL: To maintain the embryo in the correct position and minimize the risk of trauma, leave the eggs in a prone position.

Note: See Figure 1 for egg anatomy and orientation.

1. Day 1: Eggs Reception.
   a. Wash the incubator and the trays with water and soap. Then, thoroughly wipe them down with 70% ethanol before starting. Clean the eggs with lukewarm water to remove all contaminants (feathers, feces, dust, etc.). See troubleshooting – problem 1.
   b. Let eggs dry in a prone position on the bench in an egg carton.
   c. Spray the eggs with 70% ethanol. See troubleshooting – problem 1.
   d. Allow the ethanol to evaporate.
   e. Turn the eggs on the other side, spray them with 70% ethanol, and allow the ethanol to evaporate. See troubleshooting – problem 1.
   f. Place the egg cartons in the incubator with the eggs in a prone position.
   g. Ensure the humidity is around 40–60% and the temperature is 37°C. See troubleshooting – problem 1.

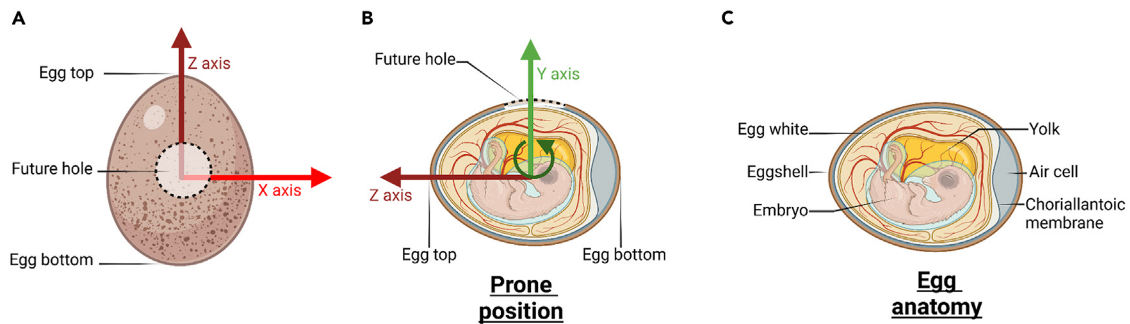


Figure 1. Representation of egg orientation and anatomical structures

(A) Schematic depiction of the egg orientation along the X (light red) and Z (dark red) axes, highlighting the location of the future opening for accessing the CAM.

(B) The prone position of the egg (hole on top) shows the Y axis and the alignment of the internal structures relative to the external egg orientation.

(C) Cross-sectional view of the egg anatomy, illustrating key structures including the eggshell, air cell, albumen, yolk, embryo, and chorioallantoic membrane.

2. Day 4: Albumen aspiration.
   a. Identify and mark the upper part of the eggs, as shown in Figure 2A.
   b. Using surgical tweezers, pierce the egg's air chamber at the bottom to facilitate the embryo's detachment from the eggshell's internal membrane. Insert approximately 1 cm of the tweezer into the egg (Figure 2B). See troubleshooting – problem 1.
   c. Seal the hole with waterproof adhesive tape (Figures 2C and 2D).
   d. Using surgical tweezers, pierce and remove a small piece of the eggshell (Figure 2E′) to create an opening approximately the diameter of a 10 mL syringe tip (Figure 2E″). See troubleshooting – problem 1.

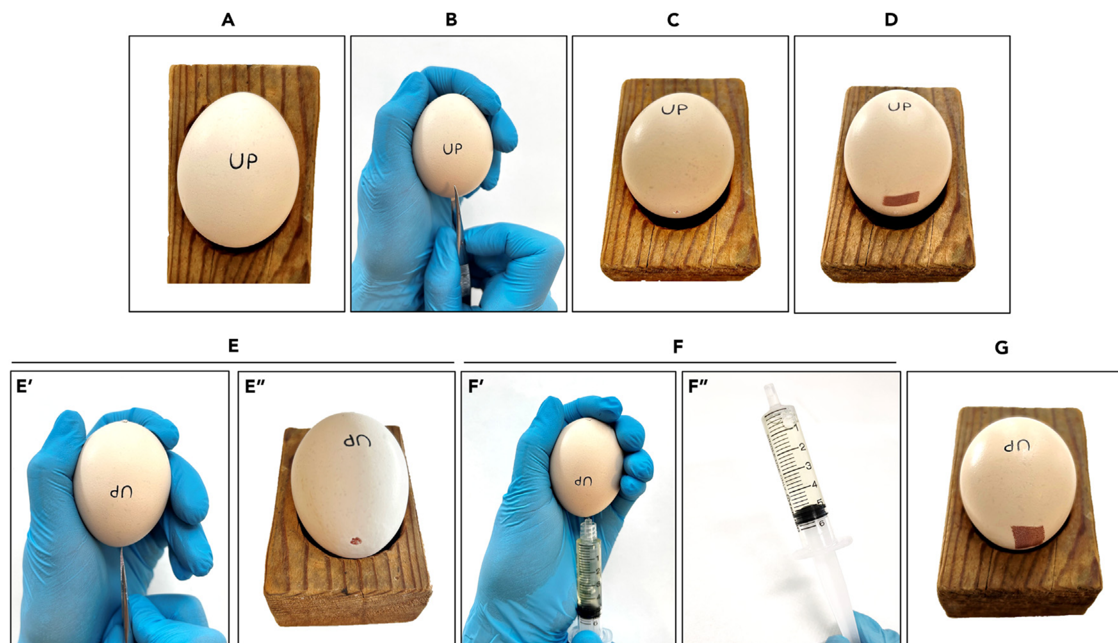


Figure 2. Eggs preparation

(A) Steps of egg preparation. Marked egg (UP) to indicate the correct orientation.

(B and C) Creation of a small incision in the eggshell using scissors at the "bottom" of the egg.

(D) Closing of the incision in the eggshell with waterproof adhesive tape.

(E and F) Albumen removal using a syringe.

(G) Closing of the incision in the eggshell with waterproof adhesive tape.

e. Slightly tilt the egg to allow the albumen to start flowing out (Figure 2F′).
f. Using a 10 mL syringe, extract 5 mL of albumen from the opening, ensuring it does not penetrate the egg (Figure 2F).
g. Seal this small hole with transparent adhesive tape (Figure 2G).
h. Avoid removing any yolk, as this could be fatal to the embryo. See troubleshooting – problem 1.

⚠ CRITICAL: Ensure that the humidity and water level in the incubator are always maintained correctly to prevent dehydration.

⚠ CRITICAL: Be careful to avoid cutting too deeply with the scissors during fenestration.

3. Day 5: Fenestration.
   a. Apply a small piece of duct tape, approximately 3 × 4 centimeters, to the portion of the eggshell identified as the upper part (as in Figure 3A). Although some folds are inevitable, minimize them as much as possible. This duct tape helps to prevent excessive shell fragments from falling inside the egg in the next steps.
   b. Using sterilized scissors, create a circular window in the eggshell with a diameter of at least 1.5–2 cm. Using straight forceps, remove the cut eggshell (Figure 3B). See troubleshooting – problem 1.
   c. If the air cell was not correctly punctured during the previous day's procedure (indicated by a still-high volume), use straight forceps to pierce it, keeping the forceps as high as possible and directing them towards the bottom of the egg (Figure 3C). See troubleshooting – problem 1.
   d. Apply additional duct tape over the window to seal the egg as tightly as possible. Ensure the hole is completely airtight, as prolonged exposure to air can be fatal to the embryo (Figure 3D). Avoid having any folds in contact with the hole.

Note: After this step of the protocol, the eggs are incubated for the subsequent days. During this period, the eggs can be left in the incubator under appropriate humidity and temperature conditions. The operator may monitor the embryonic development and remove any non-viable eggs daily. This monitoring helps to prevent potential contaminations (typically fungal) that can arise in one embryo and rapidly spread to the others.

Part 2: Procedure for tumorigenic assay

⏲ Timing: 10 days

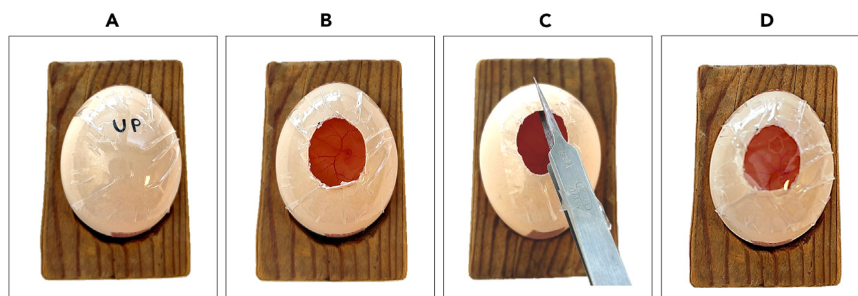


Figure 3. Fenestration procedure
Steps for creating a window in the eggshell to access the CAM.
(A) Application of the transparent tape to the eggshell.
(B) Removal of the eggshell to expose the CAM.
(C) Breaking of the air window with forceps.
(D) Closure of the window with transparent tape.

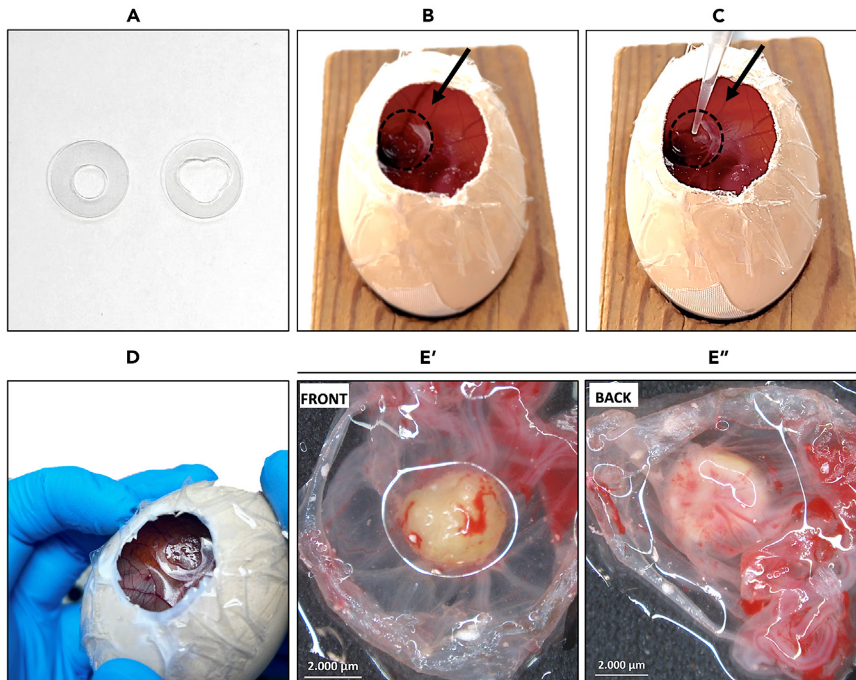


Figure 4. Tumor cell implantation in the CAM for tumorigenic assay
Steps for the tumorigenic assay.
(A) Representative image of the rings used to stabilize the cancer cells implant on the CAM.
(B) Placement of the ring on the CAM (arrow indicates the ring's position).
(C) Implant of cancer cells into the CAM (arrow indicates the ring's position).
(D) Control of the implant.
(E′) Front view of the tumor inside the ring after placement on the CAM at day 19.
(E″) Back view of the tumor inside the ring after placement on the CAM at day 19.
Scale bar 2000 µm.

Timing: 4 h for 150 eggs (for step 4)

Timing: 4–5 h for 150 eggs (for step 5)

4. Day 9: Cell harvesting and engraftment - Tumorigenesis assay.

Note: Estimate carefully the total number of cells required for the experiment (it is recommended to use 2 to 5 $\times$ 10^6 cancer cells per egg) and prepare an excess sufficient for at least four additional samples.

Note: Plastic coverslips are used to confine the implantation of tumor cells on the CAM. To prevent contamination, the coverslips must be prepared, thoroughly washed, and stored in a sterile environment, ready for use as outlined below.

a. Use an ear punch to create a hole in the plastic coverslip (13 mm diameter). The hole can be single or triple, as shown in Figure 4A.
b. Place the coverslip in 70% ethanol overnight using a standard cell culture plate.
c. Sterilize the plate containing the coverslip by exposure to ethanol and UV light.
d. Transfer the sterilized coverslip into sterile Dulbecco's Phosphate-Buffered Saline (dPBS) and store it at 4°C.
e. Open the eggs' windows by removing the duct tape.

f. Gently place one sterilized coverslip onto the CAM through the window, avoiding large preformed blood vessels (Figure 4B).
g. Using a 200 μL pipette tip, gently wound the CAM with a single scratch within the hole of the coverslip to facilitate cell attachment.
h. Harvest the cells according to the established protocol.
i. Centrifuge the cells at 500*g for 5 min.
j. Discard the supernatant and wash the cell pellet with sterile Dulbecco's Phosphate-Buffered Saline (dPBS).
k. Repeat the centrifugation and washing steps.
l. Count the cells.
m. Centrifuge the cells and resuspend them in dPBS to achieve the desired number of cells per egg in a final volume between 30 and 50 μL. Ensure to account for the volume of the cell pellet, aiming for the total volume (cell pellet volume + dPBS + treatment volume) to be within the 30–50 μL range.
n. Add the prepared cell suspension into the hole of the coverslip (Figure 4C).
o. Allow the eggs to rest undisturbed on the bench for at least 30 min. This step enables the cells to adhere to the membrane, minimizing the risk of the suspension spilling over the edges of the coverslip.
p. Apply a new piece of duct tape over the previously opened window to reseal the egg as tightly as possible.
q. Place the egg cartons in the incubator with the eggs in a prone position until day 19.
r. Maintain humidity at 40–60% and temperature at 37°C.

Note: If treatment is to be applied during the tumorigenic experiment, it can either be added at the time of engraftment by directly mixing it with the cell suspension or administered dropwise onto the tumor during the experiment.

Note: Clearly label each egg with its condition name and number using a marker.

Note: Allow the eggs to rest undisturbed on the bench for at least 30 min. This step enables the cells to adhere to the membrane, minimizing the risk of the suspension spilling over the edges of the coverslip.

Note: During the incubation period of the experiment, it is possible to monitor if the cells are still inside the disk positioned on the membrane and if they are forming the tumor mass (Figure 4D).

Note: Due to the timing of opening, stimulating, and closing the eggs, we recommend performing a maximum of one or two conditions at a time.

Note: Determine the number of cells per egg based on their proliferative capacity *in vivo*. For cells with limited growth potential *in vivo*, a maximum of 5 million cells per egg is recommended. Conversely, a minimum of 2 million cells per egg is sufficient for cells with robust proliferative capacity.

5. Day 19: Sacrifice of embryos and recovery of organs and tumors.
   a. Prepare a 6 cm dish filled with dPBS for rinsing the blood from the tumors (replace the dPBS when it becomes too bloody to ensure proper cleaning) and a desired multiwell plate to store the samples.
   b. Open the eggs' windows by cutting away the duct tape.
   c. Using microsurgical scissors, carefully cut around the coverslip, detaching the chorioallantoic membrane. See troubleshooting – problem 1.

d. Using straight and/or curved forceps, remove the entire tumor tissue together with the surrounding CAM. See troubleshooting – problem 1.
e. Rinse the collected tissue in the pre-prepared 6 cm dish containing dPBS.
f. Gently remove the coverslip by carefully pulling it away with the forceps.
g. Once thoroughly rinsed, transfer the tumor tissue and the surrounding CAM into the multiwell plate.
h. Capture images of both sides of the tissue using a stereomicroscope (as shown in Figures 4E′–4E″).
i. Depending on the desired data, the tissue can be processed as follows.

6. For Embedding.
   a. Place the samples in ten volumes (approximately 5–10 mL) of 4% PFA in dPBS and let them sit at 4°C for 24–48 h.
   b. Use your own protocol or send the samples to a Pathology Lab for tissue embedding.
7. For Snap-Freezing.
   a. Carefully remove as much of the chorioallantoic membrane as possible from the excised tissue.
   b. Snap-freeze tissue in liquid nitrogen.
   c. Once fully frozen, transfer the tumor into a cryotube.
   d. Store samples at −80°C.
8. Tumor size measurement.

   Note: When a caliper measurement of the tumor masses is not possible, the analysis of the tumor volumes can be quantified by image analysis using ImageJ/FIJI.

   a. Open each image in ImageJ/FIJI.
   b. Use the "Straight" tool to measure the tumor size. Record both the length and width of the tumor in pixels.
   c. Enter the measured values (in pixels) into an Excel sheet.
   d. To convert the pixel measurements into millimeters (mm), measure the scale present on each image and apply a cross-multiplication for the conversion.
   e. Calculate the tumor volume using the following formula, where "width" is the smaller value of the two measurements:

$$V = \frac{\text{length} \times \text{width}^2 \times \pi}{6}$$

   Note: Apply the formula consistently and input the converted values into your calculations for accurate tumor volume quantification.

Part 3: Procedure for angiogenic assay

Timing: 3 days (for step 9)

Timing: 4–5 h for 150 eggs (for step 10)

Timing: 3–5 min per egg (for step 11)

9. Day 10: Alginate & calcium chloride preparation.

   Note: Alginate and calcium chloride are used to embed stimuli during angiogenesis assays. To prevent contamination, both solutions must be prepared under sterile conditions the day before egg treatment, as outlined below.

   a. To maintain sterility, prepare the 6% alginic acid solution by dissolving acid alginic in LPS-free water in a 50 mL tube under a biological hood. See troubleshooting – problem 1.

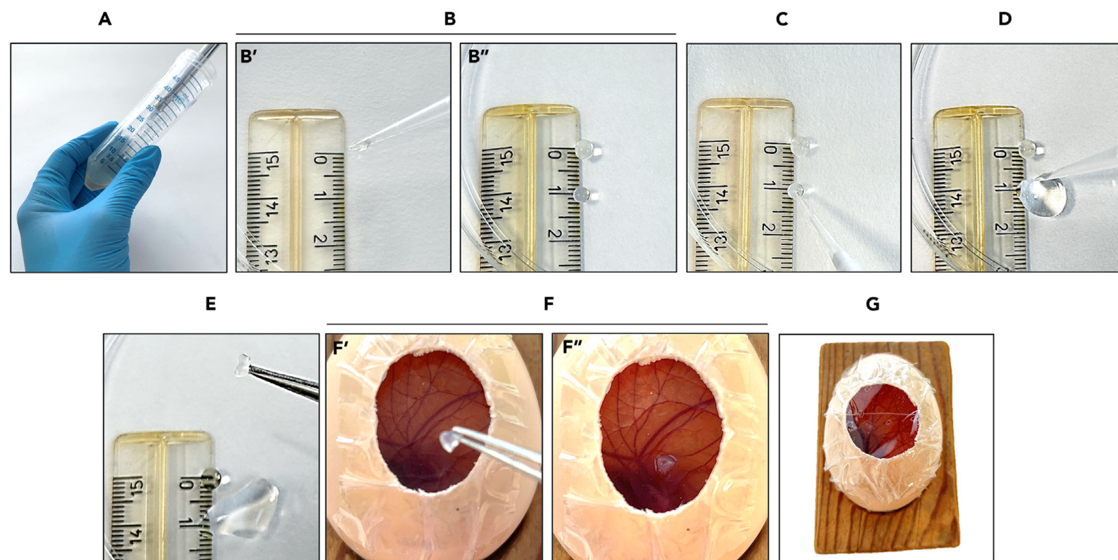


Figure 5. Stimulation of the CAM for the Angiogenesis assay
Steps for the angiogenesis assay.
(A and B) Preparation of the alginic acid spheres.
(C) Cell suspension addition inside the alginic acid sphere.
(D) Coverage with $CaCl_2$ to allow the alginate polymerization.
(E) Removal of the excess of $CaCl_2$.
(F′ and F″) Placement of the alginic sphere on the CAM.
(G) Closure of the window with transparent tape.

b. Vortex the solution thoroughly.
c. Place the 50 mL tubes on a Rotating Mixer Shaker for test tubes at 4°C, and allow them to rotate overnight at 4°C.
d. Prepare a 0.1 M calcium chloride ($CaCl_2$) solution in LPS-free water.
e. Sterilize the solution by filtration with a 0.22 μm syringe filter.
f. Store the alginic acid and calcium chloride solutions at 4°C until use.

⚠ CRITICAL: For optimal results, a fresh solution should be prepared each time. However, the alginate solution should be used for two consecutive experiments.

10. Day 11: Alginic acid sphere preparation, cell treatment, and engraftment.
    a. Harvest the cells as previously described (Day 9: Tumorigenesis - Cell harvesting).
    b. Resuspend the harvested cells in dPBS. A final volume of 3–5 μL containing between 10,000 and 20,000 cells per egg is generally sufficient to achieve optimal angiogenesis stimulation.
    c. Use the inside of the cell culture plate lid to prepare the alginic acid spheres.
    d. Soak a 200 μL pipette tip in the alginic acid solution (Figure 5A).
    e. Deposit the alginic acid onto the plate lid to form spheres approximately one to two millimeters in diameter, as shown in Figures 5B′–5B″.
    f. Prepare an excess of droplets to ensure enough well-formed alginic acid spheres. See troubleshooting – problem 3.
    g. Add 3–5μL of the cell suspension directly to one droplet of alginic acid. This volume should contain the desired number of cells at any treatment (see Figure 5C).
    h. Cover each alginate droplet dropwise with $CaCl_2$ to facilitate the polymerization of alginic acid, converting it into sodium alginate. This process will form hardened and collectible alginate spheres (Figure 5D).
    i. Carefully use forceps to remove the well-shaped (uniform and round) alginate spheres from the $CaCl_2$ solution (see Figure 5E). See troubleshooting – problem 1.

j. Remove as much excess $CaCl_2$ as possible to prevent the formation of calcium chloride crystals. Carefully take the sphere out of the $CaCl_2$ solution and gently blot it on dry areas of a Petri dish to dry it. See troubleshooting – problem 2.
k. Open the eggs' windows by cutting the duct tape.
l. Place one alginate sphere onto the CAM of each egg (Figure 5F'), ensuring it is not positioned near large-caliber vessels (see Figure 5F'').
m. Seal the eggs as tightly as possible using duct tape over the window (Figure 5G).
n. Place the eggs back in the incubator and do not manipulate them for 72 h (see step 7).

Note: The bottom of the 10 cm dish is negatively charged to facilitate cell attachment. If alginic acid droplets are placed directly on the dish bottom, they will flatten and fail to maintain their spherical shape.

⚠ CRITICAL: Always include the appropriate controls in the experimental plan. It is crucial to remember that even small concentrations of contaminants, such as LPS, can induce strong angiogenic responses, potentially skewing the results. Also, dPBS solution, culture medium, or the medium where the stimuli are dissolved may provoke a response. These factors must be considered to ensure accurate results.

⚠ CRITICAL: Ensure that all spheres are the same size for consistency in the experiment.

⚠ CRITICAL: Do not place the alginate sphere near large, pre-formed blood vessels, as this can interfere with measuring angiogenesis.

11. Day 14: Vessel counting.
    a. 72 h after the application of the stimuli, proceed to count the number of new vessels that contact the alginate sphere using a stereomicroscope.
    b. Open the eggs from one condition.
    c. Carefully count the neo-vessels that reach the alginate sphere (Figure 6). See troubleshooting – problem 2 and problem 3.

Note: Several image-based angiogenesis quantification software programs are available.[3–5] However, in this protocol, we recommend manual counting to allow focus correction during the counting process, which ensures maximal accuracy. It is essential to count only the vessels reaching the alginic acid sphere and differentiate them from any pre-existing vessels. If two vessels merge into one before reaching the sphere, it should be counted as one vessel. Conversely, if one vessel bifurcates into two upon reaching the sphere, it should be counted as two distinct vessels.

Note: We suggest having at least two independent experiments to confirm the trend in your experiment.

EXPECTED OUTCOMES

Immunohistochemistry.[6–8]

Tumor size.[9,10]

Omics (i.e., transcriptomics, metabolomics, lipidomic).[11]

RT-qPCR.[7,8]

The outcome of the angiogenesis assay will be the number of neo-vessels formed that reach the alginic acid spheres. These data determine the angiogenic effect and potential of the implanted stimuli/tumor cells compared to the control represented by dPBS.

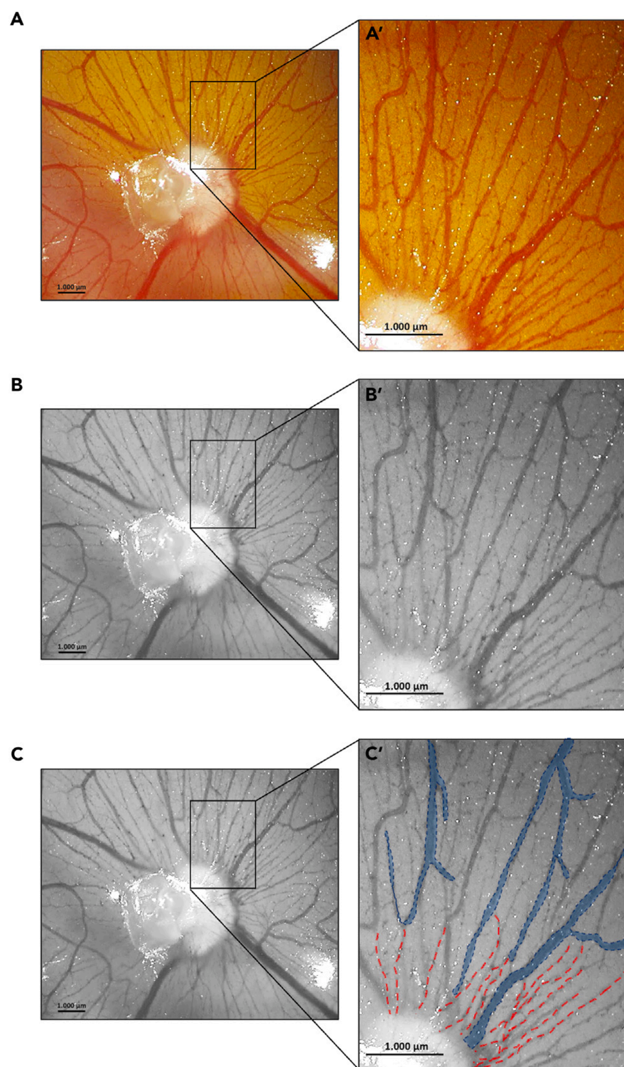


Figure 6. Overview of new neo-vessels formation on the CAM at day 14

Representative images of newly formed vessels converging toward the alginate sphere containing stimuli on the CAM at day 14 (A: 7X bright-field image; A′: 28X bright-field image; B–B′: grayscale images;). Newly formed neo-vessels are highlighted in red, while pre-existing vessels, which are larger and do not penetrate the region containing the stimuli, are highlighted in blue (C–C′). Scale bar 1000 µm.

LIMITATIONS

The CAM assay protocol we have presented ensures accurate results for both angiogenesis and tumorigenesis studies. However, it can have some limitations.

For the angiogenesis assay, the main limitation is represented by the manual counting of newly formed vessels under the microscope. A semi-quantitative approach can be adopted to improve accuracy. Different platforms[12] or in-house scripts can help determine the angiogenic responses or inhibitions. However, this approach may miss smaller vessels, which can be of significant interest. Therefore, ongoing improvements in imaging techniques and evaluation methods are needed. One potential strategy involves incorporating sequential photography to document new vessels' progressive formation meticulously. The difficulty distinguishing true neovascularization from increased vascular density caused by vessel rearrangement highlights the importance of timing in assessing angiogenic responses. Our protocol addresses this issue by concluding the angiogenesis

experiment on dpf 13, thus avoiding prolonged incubation and the risk of extended inflammatory reactions.

The tumorigenesis protocol is designed to assess both normal and cancer cells that exhibit rapid growth *in vivo*. Hence, slow-proliferative cell lines might have problems growing properly during the limited experimental window. For cell lines with a slow *in vivo* growth rate, a larger quantity of cells can be used, and a mid-point analysis can be performed to confirm cell proliferation using RT-qPCR, for instance.

Another limitation mentioned in the literature is the nonspecific inflammatory reaction observed in CAM, especially when experiments extend beyond a 15-day incubation period.[13] Due to the host's immature immune system, this inflammatory response is significantly reduced when grafting occurs during the early stages of CAM development.

Lastly, it is important to recognize that a single *in vivo* model for studying angiogenesis or tumorigenesis is not fully exhaustive. The chick embryo CAM assay should be combined with additional *in vitro* and/or *in vivo* models to account for species differences (e.g., zebrafish vs. mouse), diverse anatomical locations, and specific microenvironments.

TROUBLESHOOTING

Problem 1

High number of dead embryos following the egg opening procedure (>10%) and grafting procedure (>10%) due to contaminants (related to Part 1: The initial phase; Part 2: Tumorigenic essay; Part 3: Angiogenic essay).

Potential solution

- Work in aseptic conditions as much as possible to minimize contamination.
- Clean the incubator thoroughly before starting the incubation process (part 1, day 1, step a).
- Use sterilized water to maintain humidity in the incubator (part 1, day 1, step g).
- Use 70% ethanol to sterilize or clean all tools that will come into contact with the eggs (e.g., tweezers, scissors, and coverslips) (several steps – see protocol).
- To prevent contamination during the stimulation process, prepare alginate and calcium chloride under sterile conditions (preferably under a biological hood) (part 3, day 10, step a).

Problem 2

Difficulty in counting vessels due to calcium chloride crystal formation (related to Part 3: Angiogenic essay).

Potential solution

- Pay attention to wash the alginic sphere correctly (part 3, day 11, step j).
- When counting vessels, add a few μL of dPBS to the surface of the CAM. This can help dissolve any calcium chloride crystals and improve visibility, enabling more accurate vessel counting (part 3, day 14, step c).

Problem 3

Inconsistent shape and size of alginate spheres (related to Part 3: Angiogenic essay).

Potential solution

- The alginic acid solution is prepared in large excess. Practice making as many spheres as possible to standardize the shape and size of the alginate spheres (part 3, day 11, step f).

- Discard embryos where alginate spheres deviate significantly in size from those in other conditions to maintain experimental consistency (part 3, day 14, step c).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Michela Corsini (michela.corsini@unibs.it).

Technical contact

For any detailed technical inquiries, please contact Michela Corsini at michela.corsini@unibs.it.

Materials availability

All materials are available as mentioned in the materials section.

Data and code availability

Upon request, the lead contact will share all original pictures reported in this protocol. This protocol does not report original data or code.

ACKNOWLEDGMENTS

R.R. was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC IG 2019 – ID. 23151) and Consorzio Interuniversitario per le Biotecnologie (CIB). M.C. is funded by the European Union – Next Generation EU, Mission 4, Component 2, CUP B93D21010860004. We are grateful to Dr. Marianna Cerasuolo (University of Sussex) for her editing assistance.

AUTHOR CONTRIBUTIONS

Conceptualization, M.C., A.T., S.M., and R.R.; methodology, M.C., S.F., and T.C.; writing – original draft, S.F. and T.C.; writing – review and editing, all authors; funding acquisition, M.C., A.T., and R.R.; resources, A.T. and R.R.; visualization, S.F. and T.C.; funding acquisition, R.R.; supervision, M.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Deryugina, E.I., and Quigley, J.P. (2008). Chick embryo chorioallantoic membrane model systems to study and visualize human tumor cell metastasis. Histochem. Cell Biol. *130*, 1119–1130. https://doi.org/10.1007/s00418-008-0536-2.
2. Gabrielli, M.G., and Accili, D. (2010). The chick chorioallantoic membrane: a model of molecular, structural, and functional adaptation to transepithelial ion transport and barrier function during embryonic development. J. Biomed. Biotechnol. *2010*, 940741. https://doi.org/10.1155/2010/940741.
3. McDonald, D.M., and Choyke, P.L. (2003). Imaging of angiogenesis: from microscope to clinic. Nat. Med. *9*, 713–725. https://doi.org/10.1038/nm0603-713.
4. Guerra, A., Belinha, J., Mangir, N., MacNeil, S., and Natal Jorge, R. (2021). Simulation of the process of angiogenesis: Quantification and assessment of vascular patterning in the chicken chorioallantoic membrane. Comput. Biol. Med. *136*, 104647. https://doi.org/10.1016/j.compbiomed.2021.104647.
5. Annese, T., Tamma, R., and Ribatti, D. (2023). IKOSA(®) CAM Assay Application to Quantify Blood Vessels on Chick Chorioallantoic Membrane (CAM). Methods Mol. Biol. *2572*, 129–139. https://doi.org/10.1007/978-1-0716-2703-7_10.
6. Rezzola, S., Loda, A., Corsini, M., Semeraro, F., Annese, T., Presta, M., and Ribatti, D. (2020). Angiogenesis-Inflammation Cross Talk in Diabetic Retinopathy: Novel Insights From the Chick Embryo Chorioallantoic Membrane/Human Vitreous Platform. Front. Immunol. *11*, 581288. https://doi.org/10.3389/fimmu.2020.581288.
7. Corsini, M., Ravelli, C., Grillo, E., Dell'Era, P., Presta, M., and Mitola, S. (2021). Simultaneously characterization of tumoral angiogenesis and vasculogenesis in stem cell-derived teratomas. Exp. Cell Res. *400*, 112490. https://doi.org/10.1016/j.yexcr.2021.112490.
8. Ribatti, D. (2014). The chick embryo chorioallantoic membrane as a model for tumor biology. Exp. Cell Res. *328*, 314–324. https://doi.org/10.1016/j.yexcr.2014.06.010.
9. Pinto, M.T., Ribeiro, A.S., Conde, I., Carvalho, R., and Paredes, J. (2020). The Chick Chorioallantoic Membrane Model: A New In Vivo Tool to Evaluate Breast Cancer Stem Cell Activity. Int. J. Mol. Sci. *22*, 334. https://doi.org/10.3390/ijms22010334.
10. Ademi, H., Shinde, D.A., Gassmann, M., Gerst, D., Chaachouay, H., Vogel, J., and Gorr, T.A. (2021). Targeting neovascularization and respiration of tumor grafts grown on chick embryo chorioallantoic membranes. PLoS One *16*, e0251765. https://doi.org/10.1371/journal.pone.0251765.
11. Al-Mutawa, Y.K., Herrmann, A., Corbishley, C., Losty, P.D., Phelan, M., and Sée, V. (2018). Effects of hypoxic preconditioning on neuroblastoma tumour oxygenation and metabolic signature in a chick embryo model. Biosci. Rep. *38*, BSR20180185. https://doi.org/10.1042/bsr20180185.
12. Pion, E., Asam, C., Feder, A.L., Felthaus, O., Heidekrueger, P.I., Prantl, L., Haerteis, S., and Aung, T. (2021). Laser speckle contrast analysis (LASCA) technology for the semiquantitative measurement of angiogenesis in in-ovo-tumor-model. Microvasc. Res. *133*, 104072. https://doi.org/10.1016/j.mvr.2020.104072.
13. Ribatti, D. (2017). The chick embryo chorioallantoic membrane (CAM) assay. Reprod. Toxicol. *70*, 97–101. https://doi.org/10.1016/j.reprotox.2016.11.004.