Video Article

Chick *ex ovo* Culture and *ex ovo* CAM Assay: How it Really Works

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URL: https://www.jove.com/video/1620
DOI: doi:10.3791/1620

Keywords: Developmental Biology, Issue 33, chick, ex ovo culture, in ovo culture, chorioallantoic membrane, CAM, angiogenesis, tumor, limb buds; grafting

Date Published: 11/30/2009

Citation: Dohle, D.S., Pasa, S.D., Gustmann, S., Laub, M., Wissler, J.H., Jennissen, H.P., Dünker, N. Chick *ex ovo* Culture and *ex ovo* CAM Assay: How it Really Works. *J. Vis. Exp.* (33), e1620, doi:10.3791/1620 (2009).

Abstract

Chicken eggs in the early phase of breeding are between *in vitro* and *in vivo* systems and provide a vascular test environment not only to study angiogenesis but also to study tumorigenesis. After the chick chorioallantoic membrane (CAM) has developed, its blood vessel network can be easily accessed, manipulated and observed and therefore provides an optimal setting for angiogenesis assays. Since the lymphoid system is not fully developed until late stages of incubation, the chick embryo serves as a naturally immunodeficient host capable of sustaining grafted tissues and cells without species-specific restrictions. In addition to nurturing developing allo- and xenografts, the CAM blood vessel network provides a uniquely supportive environment for tumor cell intravasation, dissemination, and vascular arrest and a repository where arrested cells extravasate to form micro metastatic foci.

For experimental purposes, in most of the recent studies the CAM was exposed by cutting a window through the egg shell and experiments were carried out *in ovo*, resulting in significant limitations in the accessibility of the CAM and possibilities for observation and photo documentation of effects. When shell-less cultures of the chick embryo were used[1-4], no experimental details were provided and, if published at all, the survival rates of these cultures were low. We refined the method of *ex ovo* culture of chick embryos significantly by introducing a rationally controlled extrusion of the egg content. These *ex ovo* cultures enhance the accessibility of the CAM and chick embryo, enabling easy *in vivo* documentation of effects and facilitating experimental manipulation of the embryo. This allows the successful application to a large number of scientific questions: (1) As an improved angiogenesis assay[5,6], (2) an experimental set up for facilitated injections in the vitreous of the chick embryo eye[7-9], (3) as a test environment for dissemination and intravasation of dispersed tumor cells from established cell lines inoculated on the CAM[10-12], (4) as an improved sustaining system for successful transplantation and culture of limb buds of chicken and mice[13] as well as (5) for grafting, propagation, and re-grafting of solid primary tumor tissue obtained from biopsies on the surface of the CAM[14].

In this video article we describe the establishment of a refined chick *ex ovo* culture and CAM assay with survival rates over 50%. Besides we provide a step by step demonstration of the successful application of the *ex ovo* culture for a large number of scientific applications.

Daniel S. Dohle, Susanne D. Pasa, and Sebastian Gustmann contributed equally to this study.

Video Link

The video component of this article can be found at https://www.jove.com/video/1620/

Protocol

All equipment and reagents have to be purchased sterile or needs to be heat or steam sterilized or sterilized with 70% ETOH.

The authors state that experiments on animals were performed in accordance with the European Communities Council Directive (86/609/EEC), following the Guidelines of the NIH regarding the care and use of animals for experimental procedures and the regulations set forth by the Institutional Animal Care and Use Committee (IACUC) at the University of Duisburg-Essen (Germany).

Part 1: Incubation of eggs

1. Fertilized eggs can be stored at 13°C for up to one week.
2. Eggs are incubated for 72 hours, lying horizontally in an incubator with a moving tray, rotating the eggs 12 times a day continuously. Humidity is kept at 60 - 62%, incubation temperature at 37.5°C.

Note: Before starting incubation, dirt, feathers and excrement are carefully removed from the egg shells mechanically by dry wiping with common, gray zigzag hand paper towels, which have a rough rather than a soft surface structure. Wiping the eggs with ethanol or disinfectant, however, significantly reduced survival rate of the embryos, irrespective of the liquid used.

Part 2: *Ex ovo* culture

1. After incubation for 72 hours, eggs are removed from the incubator.
   Note: The eggs are incubated for 72 hours because - according to our experiments - for unknown reasons but in agreement with Auerbach et al.1 earlier development stages had significantly lower survival rates. At stages later than 72 hours the yolk sack which is not yet covered by the CAM becomes thinner and tends to adhere to the egg shell leading (i) to small haemorrhages at the area of adherence and (ii) the rupture of the yolk-sack membrane. Therefore the presented method is not applicable to embryos older than 72 hours.
2. The top side of the eggs, where the embryo resides, is marked by pencil since the embryo resists rotation to a certain extent.
3. The egg is held horizontally with the pencil mark on top and cracked on the edge of an 80 mm triangular magnetic stir bar laying oriented perpendicular to the long axis of the egg.
   Note: For a better feeling, no gloves should be used, but hands should be disinfected with 70% EtOH. It is important that the egg shell as well as the egg membrane are opened up. Leaking of egg white is a safe sign that the egg membrane has been perforated.
4. The egg is kept close to the bottom of the Petri dish to avoid further leakage of egg white.
   Note: During the initial opening procedure, it is important that the egg white on the bottom of the Petri dish is still connected to the rest inside the egg as this results in a vacuum inside the egg which holds the yolk with the embryo on top inside the egg. The vacuum can be regulated either by finger pressure which controls the inlet of air into the egg or by lifting the egg which increases the column of connected egg white i.e. the vacuum.
5. By gently applying pressure to the meridian running through the crack and equator of the egg by thumb and middle fingers it is possible to regulate the vacuum. The content of the egg can then be transferred to the Petri dish undamaged, if the egg with the yolk is gently lifted and both halves of the shell are carefully separated. The embryo and the yolk vessels should lie on top of an undamaged yolk.
   Note: If the pencil mark is not kept on top, the embryo might be hurt when cracking the shell. If the eggs are not cracked carefully enough or the embryos are older than 72 hours (see above), the yolk sack sticks to the shell and often ruptures. The same happens when the humidity of the incubator is lower than 60% or eggs are not rotated during incubation.
6. *Ex ovo* cultures are returned to an incubator and kept at 37.5° 38.2°C and 60% humidity. CO_2 or O_2 supply is not necessary if the incubator is not closed hermetically (ventilation grid partially open!) and special Petri dishes with spacers between lid and dish are used.
   Note: Moving of the embryos should be kept to a minimum during the first days of incubation. Too much agitation results in high mortality rates. From incubation day 9 onwards, the embryos are less sensitive to agitation.
   Dead embryos should be removed immediately to avoid infections.
   If cell culture incubators are used, which are hermetically closed, O_2 supply is mandatory for the embryos not to die. Humidity is essential for the CAM not to dry out and if incubation temperature is lower than 37°C, the embryos will develop too slowly.

Part 3: Application of substances for angiogenesis assays

1. Autoclaved folded filter papers (5.5 mm in diameter) are punched by a hole puncher and used as supporting material (carrier) for liquid substances applied to the CAM.
   Note: Nearly each carrier material (see Table below) caused irritation of the CAM, especially when applied before incubation day 10, as it changed the developing CAM vessel architecture. This also included potentially inert substances like hydroxyethyl cellulose foil, Teflon sponges (PTFE-pledgets), collagen sponges, collagen foil, sterile gauze (swabs), round cover slips, and silicon rings, which all caused false positive results. Filter paper seemed to cause the least irritation and was therefore used in our assays.
2. When the CAM is fully developed, up to 6 different samples may be applied and their effects can be compared on a single CAM.
3. To avoid drying filters additional doses (3 µg native angiotropin) or buffer (5 µl PBS) should be re-applied every day.
4. From day 3 after application, blood vessels orientate towards the angiogenic substances on the filters, whereas the pattern of blood vessels surrounding controls is unchanged.
   Note: The CAM should not be mistaken for the yolk-sack. The CAM is a dynamic developing structure while the yolk sack is static with little variances during development. On developing day three the developing CAM can be recognized for the first time at the caudal end of the embryo as a small vesicle. Between day three and day ten the initial vesicle expands, creating a folded two-layered membrane overgrowing the yolk sack at an increasing rate. The application site should be half way between embryo and the fold of the CAM (border) as otherwise the embryo hinders microscopic observation of effects in transmitted light. Alternatively, the filter with the angiogenic substance is internalized when the CAM keeps growing.

Part 4: Inoculation of cells onto the CAM

4.1.Preparation of cells

1. Confluent cells are transferred from culture flask to FALCON tubes by the use of a sterile 25 ml plastic pipette using a pipette aid.
2. To estimate cell number, 15 µl of the cell suspension is transferred to a Neubauer chamber and cells are counted manually under a microscope.
3. Falcon tubes are centrifuged for 3 min at 1000 rpm at room temperature and the supernatant is discarded.
4. For live cell tracking, cells are diluted with PBS to the desired concentration (here: $2x10^7$ cells/ml) and stained with e.g. PKH26 (Sigma), a red fluorescent live cell membrane labelling kit, according to the manufacturers' protocol.
   Cells are re-suspended in cell culture medium (preferentially Dulbecco's modified eagle's medium; DMEM) to the desired concentration for injection (10^7 cell/ml) or inoculation ($3x10^6$ cell/ml). Different cell concentrations are applied for injections vs. inoculation according to concentrations already published in the literature[15-17] and our experience.

4.2 Inoculation of cells onto the CAM

1. Rings (~ 1mm thickness) are cut from a 1ml pipette tip or a peripheral venous catheter (PVL) tube by the use of a sharp scalpel and applied to the CAM of an E6-E14 chick embryo cultured *ex ovo*.
   Note: Try not to "saw" several times but to cut through the pipette tip or catheter tube with one cut using a new, very sharp scalpel to avoid sharp edges on the resulting rings. Check the rings under the microscope for sharp edges resulting from micro-cracks.
2. Depending on the size of the rings 20 µl of $3x10^6$ cell/ml cells are pipetted in total into one ring or as split volumes into multiple rings.
   Note: In some cases traumatisation of the CAM by gentle scraping is a pre-requisite, e.g. to facilitate inoculation of cells[17] and capillarisation of grafts (see below). If, however, traumatisation of the CAM is not intended or contraindicative, e.g. for studies of the invasive potential of tumor cells, rings should not be used for inoculation, but cells should be applied directly as application of rings or rings themselves might induce micro lesion. If rings are used, these should be cut as thin as possible to minimize weight and avoid indentation of the CAM.

Part 5: Intravitreous injection of cells

1. A Hamilton microliter syringe is rinsed several times with 70% EtOH and finally with sterile PBS.
2. The micro syringe is filled with the prepared stained or unstained cell suspension (see Part 4.1).
   Note: The injection site has to be carefully chosen, not to injure blood vessels of the CAM or adjacent structures of the embryo (brain!) by over penetration with the needle. The injection should be done under a dissection binocular.
3. Under microscope control, carefully, but quickly penetrate the CAM and eye layers with the syringe needle and continuously inject 10 to maximal 20 µl cell suspension (preferentially diluted in PBS) into the vitreous of the chick embryo cultured *ex ovo*.
   Note: If the CAM can not be penetrated with the needle, it might be removed at a site with few or no blood vessels by gently tearing it apart with two forceps.
4. The needle should remain 1-2 seconds into the eye to avoid a loss of the injected cell suspension by leakage.

Part 6: Dissection of chicken limb buds

1. Eggs are removed from the incubator after 3 to 4 days of incubation (E3/E4 = Hamburger Hamilton (HH) stage18-23)
2. Eggs are cracked on the edge of a triangular magnetic stir bar and transferred into a Petri dish (details: see Part 2) and the embryo is dissected from the connected yolk vessels by cutting a circle using spring scissors.
3. The embryo is transferred to a fresh Petri dish filled with cold, sterile PBS by use of a small drain spoon and washed by kind agitation.
4. The embryo is transferred to a small Petri dish filled with sterile PBS and freed from surrounding membranes, carefully tearing them apart with fine forceps.
5. Limb buds are detached by microsurgical forceps as close to the body as possible, grasped and transferred to the CAM of an 8-10-day-old host chicken cultured *ex ovo* (see grafting procedure).

Part 7: Preparation of mouse limb buds

1. Time pregnant matings are set up and the morning of the day on which a vaginal plug is detected in females mating is designated gestation day 0.
2. The pregnant female is sacrificed by cervical dislocation when the development of the embryos reached embryonic day (E) 10 to 13 and fixed on a wax board.
3. The abdominal wall is moistened with 70% ETOH, cut along the midline and the skin flaps are fixed laterally by pins.
4. Both horns of the uterus are removed from the abdomen, detached and transferred to a beaker with cold PBS.
5. The embryos are separated, transferred to a Petri dish and uterus wall and embryonic membranes are removed carefully by the use of forceps.
6. Limb buds are cut off by use of spring scissors or detached by fine forceps as close to the body as possible, grasped and transferred to the CAM of 8-10-day-old host chicken cultured *ex ovo* (see grafting procedure).

Part 8: Collection of tumor samples

1. Tumor biopsies are collected in 2 ml Eppendorf tubes filled with tumor cell-specific culture medium (here: Leibovitz's medium) and kept at room temperature.
2. Tumor biopsies are cut into 1-2 mm3 pieces by sterile scalpels.
   Note: If tumor samples were too small, they might not attach to the CAM, if they were too big, the CAM might be injured and the embryo might die.
3. A piece from the core of the biopsy sample is grafted onto the CAM of 8-10-day-old host chicken cultured *ex ovo* (see grafting procedure).
   Note: Taking material from the surface of the biopsy increases the risk of contamination with muscle or connective tissue.

Part 9: Grafting procedure

1. For grafting of limb buds or tumor specimens, the fully developed CAM of 8-10-day-old chick embryos cultured *ex ovo* is used.
2. The grafts are ideally placed on the CAM near a Y bifurcation of a blood vessel.
3. At the desired application site, the CAM is selectively traumatized by gentle scraping off (forceps) the upper peridermal part of the double epithelial layer, leaving the basal layer intact.
   Note: It is essential to avoid bleeding or visible rupture of capillaries.
4. The graft is transferred to the CAM with minimal PBS attached. Depending on the graft, it might be placed once or twice on a site of the CAM, where no final grafting is intended to remove excessive PBS and then finally grafted at the prepared traumatized CAM site.

5. The graft is grasped by fine forceps and layered onto the CAM. Depending on the sensitivity of the graft, light pressure might be applied (not suitable for limb buds) to manoeuvre the graft into a resulting indentation of the CAM (suitable for tumor samples).
   Note: As the chick immune system develops at embryonic day (E) 18, *ex ovo* xenografts cultures should not be prolonged beyond E17 as the host chick embryos frequently die.
   It is important to have available host CAMs, respectively chick embryos cultured *ex ovo,* timed to coincide with a source of donor tissue of your choice.

Part 10: Re-grafting

1. The host chick embryo is killed by decapitation.
2. The CAM with the attached graft is removed by a circular cut with pointed scissors and transferred to a Petri dish filled with PBS.
3. Excessive CAM is cut from the graft by spring scissors except for the former attachment site.
4. In case of bigger tumor samples, the graft is cut in halves or several smaller pieces and re-grafted with the cutting edge facing the new CAM of the second host.

Part 11: Representative Results

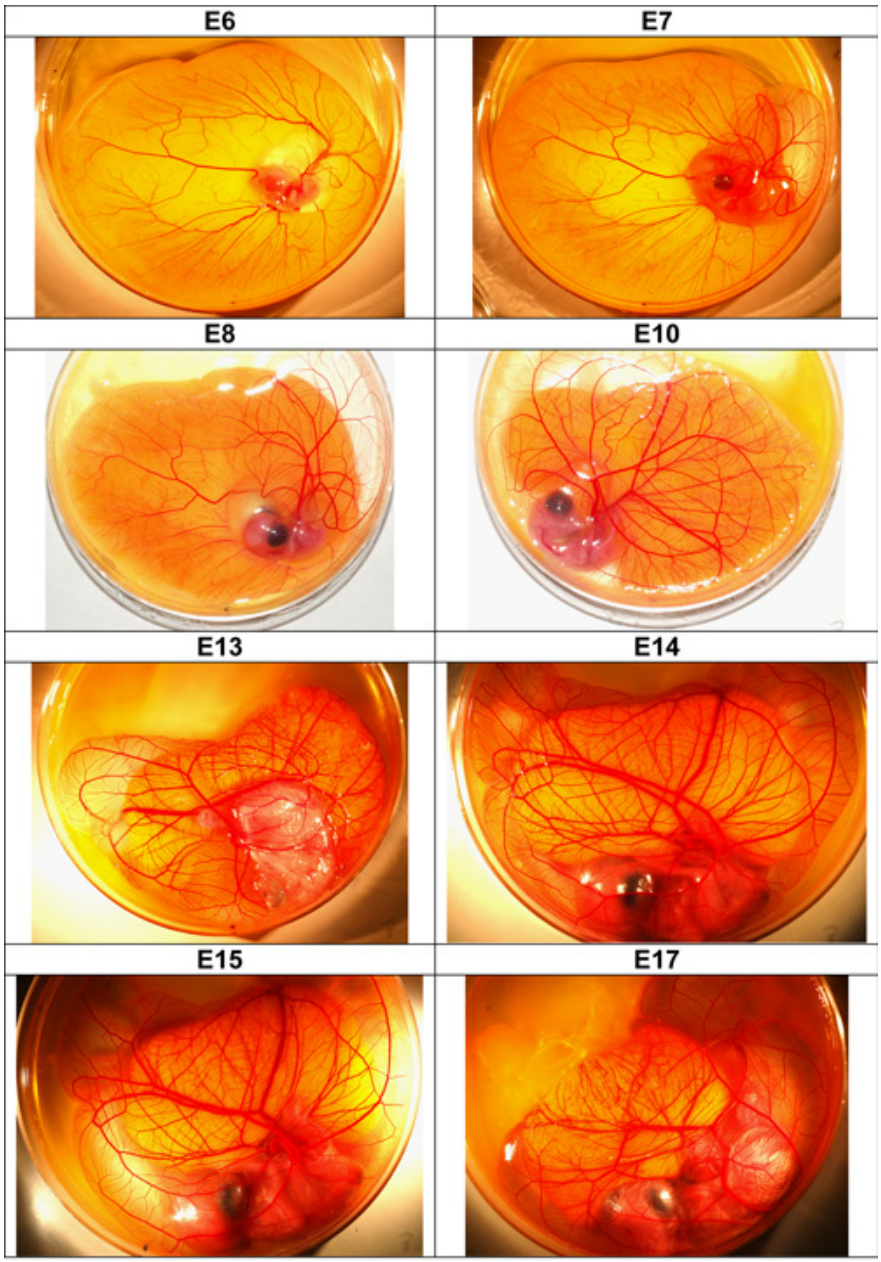

Figure 1. Shell-less chicken culture

Chicken embryos of different development stages cultured *ex ovo* in Petri dishes.

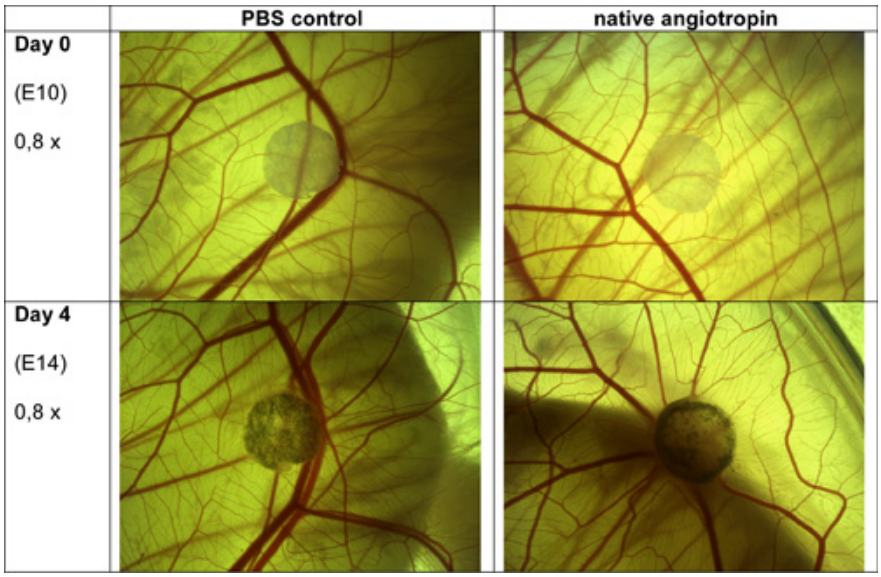

Figure 2. *Ex ovo* CAM angiogenesis assay
Fertilized chicken eggs were incubated horizontally at 37 °C and humidity of 60 - 62% and cracked into Petri dishes at embryonic day 4 (E4). Incubation was continued under the same conditions. At E10 sterile filter papers (5.5 mm in diameter) were layered on top of the CAM and soaked either with 5 µl PBS or 3 µg native angiotropin6. Capillaries showed obvious alignment towards the angiotropin soaked filter paper at E14.

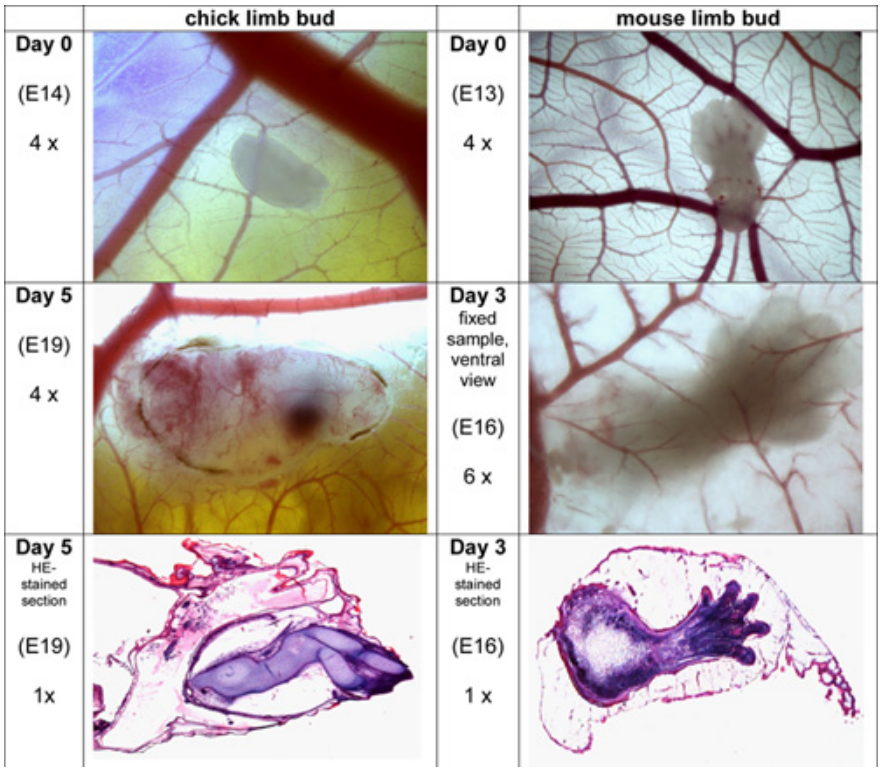

Figure 3. *Ex ovo* grafting of limb buds
Limb buds from chick (E3/E4) and mouse (E13) were grafted onto the CAM of shell-less chicken cultures. Blood vessels from the CAM entered the limb buds after two days. The development of the limb buds continued in these *ex ovo* cultures reaching a two phalange stage in chick and displaying reduction of the interdigital webs in murine limb buds.

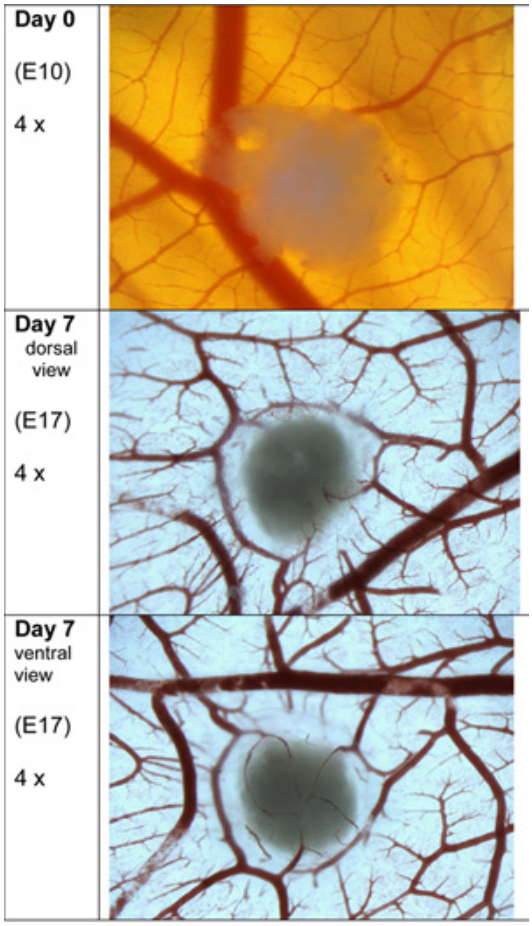

Figure 4. *Ex ovo* inoculation of tumor sample
A biopsy sample of bladder carcinoma was inoculated onto the CAM of a 10-day-old chick embryo cultured *ex ovo*. After two days in culture, the tumor specimen was connected to the CAM vasculature and after 7 days, multiple blood vessels entering the graft were observed.

Discussion

Innovative or just another chick culture protocol?

With former shell-less culture protocols[1-4] the total survival rates of the chick embryos were low, e.g. ca. 30%[1]. The refined *ex ovo* culture protocol described in this video article, by contrast, allows for survival rates over 50%. Compared to classical *in ovo* cultures *ex ovo* culture of chick embryos significantly facilitates the accessibility of the CAM and chick embryo and enables their experimental manipulation and continuous monitoring. This culture method can be used for a wide variety of application ranging from enhanced angiogenesis assays[5,6], to facilitated injections in the vitreous of the chick embryo eye[7-9], intravasation assays[10-12], improved grafting and growth of limb buds[13] and innovative maintenance of tumor samples[14]. Thus, *ex ovo* culture contributes a useful tool in developmental, angionesis and tumor research.

Acknowledgements

The authors would like to thank J. Kueper and J. Wittschier for excellent technical assistance and Prof. Ruebben for generously supplying tumor material.

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