

# Angiogenesis within Stem Cell–Seeded Silk Scaffolds Cultured on the Chorioallantoic Membrane and Visualized by 3D Imaging

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The long-term survival and successful integration of implants for tissue replacement and regeneration highly depends upon the fast ingrowth of blood vessels from the surrounding tissues. Before selecting potential biomaterials for clinical applications, they must be thoroughly tested with proper analytical tools. This unit provides a protocol for studying the potential of cell-seeded scaffolds to attract vessels that will form vascular networks within biomaterials. It includes seeding of stem cells into silk fibroin scaffolds, angiogenesis assay on the chorioallantoic membrane (CAM) of fertilized chicken eggs, a procedure for perfusion with MicroFil, and finally microcomputed tomography ( $\mu$ CT) scanning. This technique can help screen potential biomaterial implants, thereby reducing the amount of animals needed for pre-clinical in vivo studies. © 2017 by John Wiley & Sons, Inc.

Keywords: microcomputed tomography ( $\mu$ CT) • tissue engineering • scaffold biomaterial • stem cells • vasculature

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## INTRODUCTION

Embryonic development as well as tissue homeostasis, pathology, repair, and regeneration are closely dependent on angiogenesis. Vascular networks allow for the circulation of hematopoietic cells, which supply the surrounding tissues with oxygen and nutrients, transport hormones, contribute to waste product and carbon dioxide removal, and protect the body from infections (Carmeliet & Jain, 2011).

As a consequence of population aging, the requirement for tissue and/or organ replacement is continuously growing. Traditional treatment strategies are limited to organ and tissue transplants. However, the availability of grafts is restricted due to donor shortage as well as donor site morbidity. New developments in the field of tissue engineering and regenerative medicine are promising and might offer an alternative treatment of tissue or organ loss caused by injury or disease. One of the main components of tissue engineering is the use of biomaterials, which can optionally carry cells and signaling molecules. There is an increasing need for new biomaterials and reproducible testing protocols for their applicability for tissue regeneration purposes. To guarantee proper integration and long-term survival of implants, it is particularly important to achieve fast vascularization.

**Embryonic and  
Extraembryonic  
Stem Cells**

**1F.19.1**



In this unit, protocols that describe the culture of previously cryopreserved cells (see Basic Protocol 1), seeding of cells on a silk fibroin scaffold (see Basic Protocol 2), performance of a commonly used CAM angiogenesis assay (see Basic Protocol 3), as well as MicroFil perfusion of the vasculature to visualize and analyze the three-dimensional (3D) development of the blood vessel network within the implants cultured on the CAM by  $\mu$ CT (see Basic Protocol 4) are all described.

**BASIC  
PROTOCOL 1**

**CULTURE OF CRYOPRESERVED HUMAN DENTAL PULP STEM CELLS**

This protocol describes how to culture cryopreserved human dental pulp stem cells (hDPSCs), which were previously isolated from non-erupted wisdom teeth (Gronthos, Mankani, Brahim, Gehron Robey, & Shi, 2000; Tirino et al., 2011).

The procedure for anonymized cell collection was approved by the Kantonale Ethikkommission of Zurich and performed with patients' written consent.

**Materials**

Human dental pulp stem cells (cryopreserved hDPSCs)  
Growth medium (see recipe), 4°C

37°C water bath  
15-ml centrifuge tubes (Falcon)  
Centrifuge  
75-cm<sup>2</sup> cell culture flask  
37°C, 5% CO<sub>2</sub> humidified incubator

1. Defrost a vial of cryopreserved hDPSCs containing  $1 \times 10^6$  cells in a 37°C water bath until only a small ice cube is left in the vial (~1 to 2 min).
2. Transfer cell suspension into a 15-ml centrifuge tube and add 5 ml cold growth medium dropwise while slightly shaking tube.
3. Centrifuge cell suspension 5 min at  $400 \times g$  (1500 rpm), room temperature.
4. Prepare a 75-cm<sup>2</sup> cell culture flask by adding 12 ml cold growth medium.
5. Remove supernatant from centrifuge tube and resuspend cell pellet in 1 ml cold growth medium and then transfer into cell culture flask prepared in step 4.
6. Culture cells in a 37°C, 5% CO<sub>2</sub> humidified incubator. Change medium every 3 to 4 days. Optionally, passage cells at 80% to 90% confluency.

**BASIC  
PROTOCOL 2**

**SEEDING HUMAN DENTAL PULP STEM CELLS ONTO SILK FIBROIN SCAFFOLDS**

This protocol describes seeding hDPSCs onto silk fibroin scaffolds. Before starting the procedure, ensure that the biomaterial and instruments are sterile (autoclave silk fibroin scaffolds and instruments).

**Materials**

Growth medium (see recipe)  
hDPSCs (see Basic Protocol 1)  
Trypsin  
Phosphate buffered saline (PBS)

37°C water bath  
70- $\mu$ m strainer  
Centrifuge

Neubauer chamber (or other cell counting device)  
Microscope  
Sterile silk fibroin scaffold (5-mm diameter, 3-mm height, pore-size of 200 to 300  $\mu\text{m}$ , 90% porosity)  
Sterile filter paper  
24- and 96-well plates  
Sterile tweezers  
37°C, 5% CO<sub>2</sub> humidified incubator  
50-ml centrifuge tubes (Falcon)

#### ***Prepare cells and scaffolds***

1. Warm up growth medium to 37°C, wash with PBS, and trypsinize cells when cells are 80% to 90% confluent. Use a 70- $\mu\text{m}$  strainer and a 50-ml centrifuge tube to obtain a single cell suspension and count cells using a Neubauer chamber and a microscope.
2. Centrifuge cell suspension 5 min at 400  $\times$  g room temperature.
3. Resuspend cell pellet in growth medium at a concentration of  $1 \times 10^6$  cells/50  $\mu\text{l}$ .
4. Place sterile scaffolds onto a piece of sterile filter paper to remove most of the residual liquid and then move them into wells of a 96-well plate (one scaffold/well) using sterile tweezers.
5. Pipet 50  $\mu\text{l}$  of cell suspension on top of each scaffold. Pipet cell suspension up and dispense it over the scaffold several times. Repeat for all samples.

#### ***Attach cells***

6. For cell attachment, place the 96-well plate 30 min in a 37°C, 5% CO<sub>2</sub> humidified incubator.
7. Remove plate from incubator and open it under a sterile fume hood. Carefully turn the scaffolds upside down and pipet the remaining cell suspension on top of the scaffolds. Then place the plate back into the incubator and incubate for an additional 30 min.
8. Prepare a 24-well plate by adding 1 ml of growth medium into each well. Use one well to add 2 ml of growth medium.
9. Before placing the scaffolds into the wells of the 24-well plate, wash away non-adherent cells by dipping the scaffold a few times into the well filled with 2 ml of growth medium.
10. Culture for a desired period of time and change medium every 3 to 4 days.

### **PREPARATION OF FERTILIZED CHICKEN EGGS FOR CAM ASSAY AND PLACING CELL-SEEDED SAMPLES ONTO THE CAM**

This protocol describes how to prepare fertilized chicken eggs for the chorioallantoic membrane (CAM) assay and how to place the cell-seeded samples onto the surface of the CAM. Before starting the procedure, ensure that all instruments are sterile. Note that steps 10 through 12 are performed 4 days after steps 1 through 9.

According to Swiss animal care guidelines, experiments performed in chicken embryos until ED 14 do not need ethical approval (TSchV, Art. 112).

#### ***Materials***

Fertilized Lohman white Lohman Selected Leghorn (LSL) chicken eggs  
70% ethanol  
Cell-seeded scaffolds (see Basic Protocol 2)

**BASIC  
PROTOCOL 3**

**Embryonic and  
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**1F.19.3**

38°C egg incubator (e.g., Bruja 3000, Brutmaschinen-Janeschitz)  
60-mm petri dishes  
Autoclave tape  
5-ml syringes  
21-G × 1 ½-in. (40 mm × 0.8-mm) needles  
Sterile pointy scissors  
Clear tape (e.g., Scotch tape)  
500-ml glass beaker  
Pencil  
37°C, 0% CO<sub>2</sub> humidified incubator  
Silicone rings (taken from sterile cryovials; e.g., Sigma-Aldrich, cat. no. CLS430488-500EA)

#### ***Pre-incubate and prepare eggs***

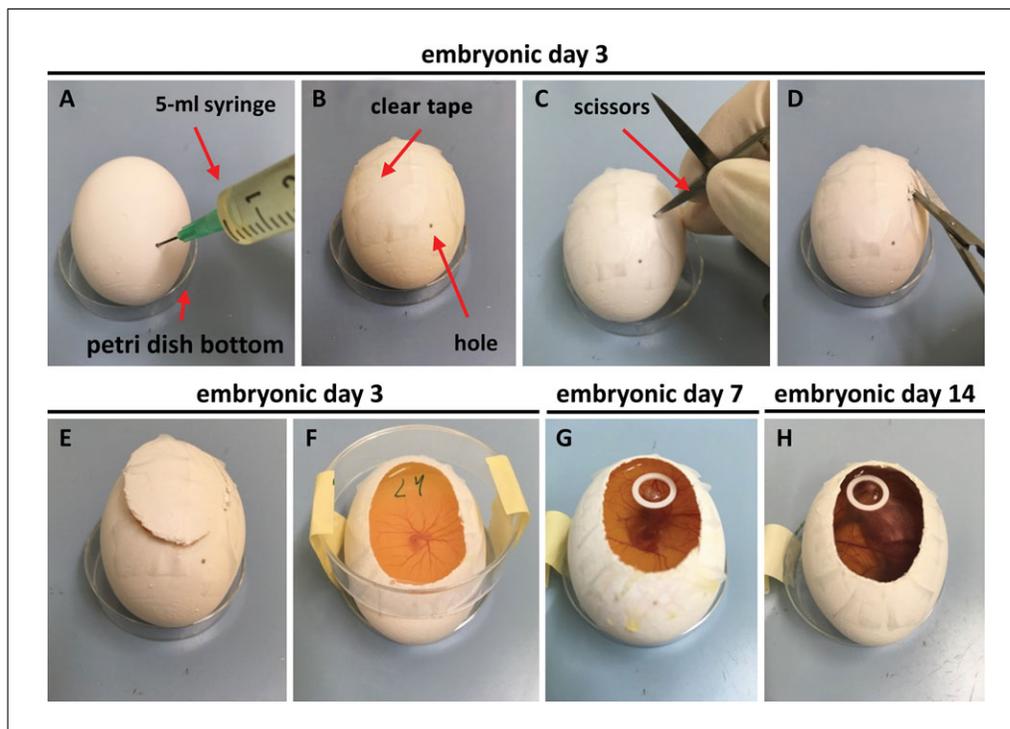
1. For pre-incubation, place fertilized Lohman white LSL chicken eggs horizontally in an egg incubator for 3 days at 38°C at a rotation speed of 360°/4 hr.
2. Stop the rotation in the morning of embryonic day 3 (ED 3) and keep eggs in this position for 3 hr to ensure that the embryo is located on the top of the egg.
3. Using a sterile clean bench, prepare 60-mm petri dishes (two dishes per egg; label one dish bottom per egg on its top side and one dish bottom per egg on its rim), 5-cm long pieces of autoclave tape (three pieces per egg; spray a longer piece of autoclave tape with 70% ethanol before cutting into 5-cm pieces), 5-ml syringes (one syringe per ten eggs), 21-G × 1 ½-in. needles (one needle per egg), sterile scissors, clear tape, and a 500-ml glass beaker (for egg white disposal). Make a loop out of the 5-cm tape (sticky side out) and attach it inside in the middle of the bottom dish, which is marked at its rim (one per egg).
4. Mark the top of the egg with a pencil and carefully wipe the egg-shell with 70% ethanol without turning it.

#### ***Expose embryo***

5. Place the egg horizontally (with the pencil mark up) into the bottom dish onto the tape to stabilize it. Carefully make a small hole in the shell at the bigger end of the egg with the tip of sterile pointy scissors and remove ~4 ml of albumen using a syringe and a needle to lower the developing embryo (Fig. 1F.19.1A).
6. Place two pieces of clear tape on the area where the window is desired (Fig. 1F.19.1B).
7. Make another small hole through the tape and the shell, carefully insert the scissors, and start cutting an oval hole while turning the egg with the other hand (Fig. 1F.19.1C-E).
8. Remove the shell and use the 60-mm petri dish bottom labeled on the top side to cover the opening. Fix the cover to the dish under the egg with two pieces of autoclave tape, and incubate in a 37°C, 0% CO<sub>2</sub> humidified incubator (Fig. 1F.19.1F).
9. Check eggs once per day and discard dead eggs.

#### ***Place cell-seeded scaffolds on CAM***

10. On ED 7, place the cell-seeded scaffolds on the vascularized CAM. Prepare 60-mm petri dishes (one dish per egg; label the dish bottom on its top side), 5-cm long pieces of autoclave tape (two pieces per egg; spray a longer piece of autoclave tape with 70% ethanol before cutting it), and silicone rings (one ring per egg) by removing them from the cryovial using sterile tweezers inside the clean bench and collecting them in a sterile petri dish.



**Figure 1F.19.1** Chorioallantoic membrane (CAM) assay procedure. **(A)** Removal of the egg white to lower the developing embryo. **(B)** Egg covered with clear tape, where the shell will be opened. Ensure the tape seals the first hole. **(C)** Make another hole for the starting point of shell cutting. **(D)** Keep scissors flat while cutting to ensure no harm to the embryo. **(E)** Cut an oval window into the shell. **(F)** Cover the open egg with the second petri dish bottom and fix the two dishes with autoclave tape. **(G)** Positioning of the silicone ring and sample on the CAM on ED 7. **(H)** Egg with sample on ED 14.

11. After placing an egg inside the clean bench, remove the Petri dish covering the egg, place a silicone ring on the CAM to ensure a flat surface, and position the cell-seeded sample in the middle of this ring (Fig. 1F.19.1G). Re-cover the egg with a new petri dish and reattach it to the lower one with two pieces of autoclave tape.
12. Incubate for a maximum of 7 days until ED 14 (Fig. 1F.19.1H) in a 37°C, 0% CO<sub>2</sub> humidified incubator.

## PERFUSION OF THE DEVELOPING VASCULATURE

This protocol describes how the vasculature within the biomaterial can be perfused with a radiopaque contrast agent to visualize the vascular network using  $\mu$ CT scanning.

### Materials

- Chicken embryos (see Basic Protocol 3)
- MicroFil set (MV-diluent, MV-curing agent, yellow silicone rubber injection compound; Flow Tech)
- Superglue
- PBS
- 4% paraformaldehyde
- Small containers (e.g., Sterilin 7-mL Polystyrene Bijou Containers, Thermo Fisher Scientific)
- 5-ml syringes
- Three-way valves (Discofix C; B. Braun Melsungen AG)
- 10-cm tube

**BASIC  
PROTOCOL 4**

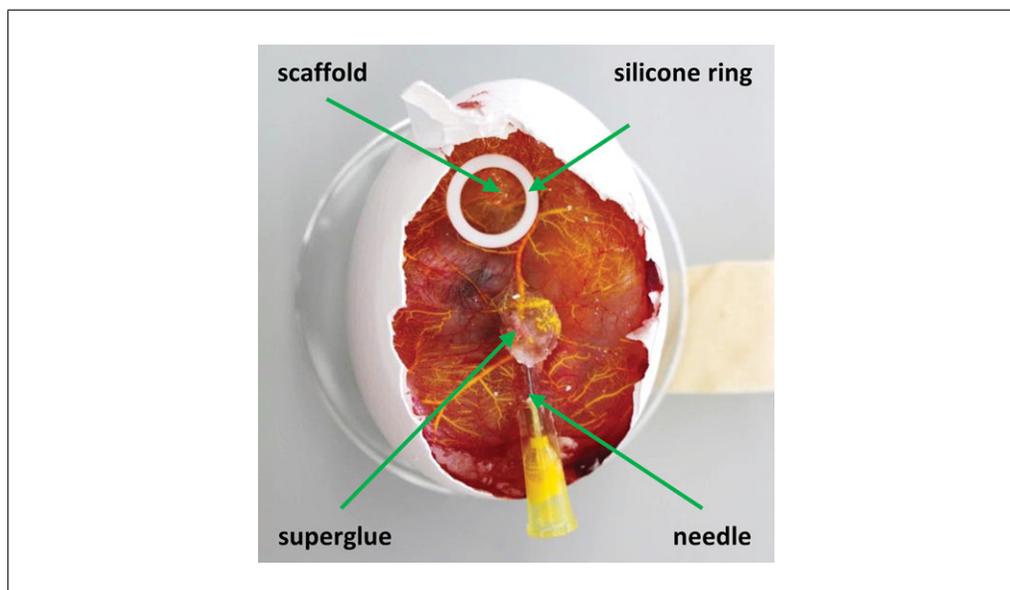
**Embryonic and  
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**1F.19.5**

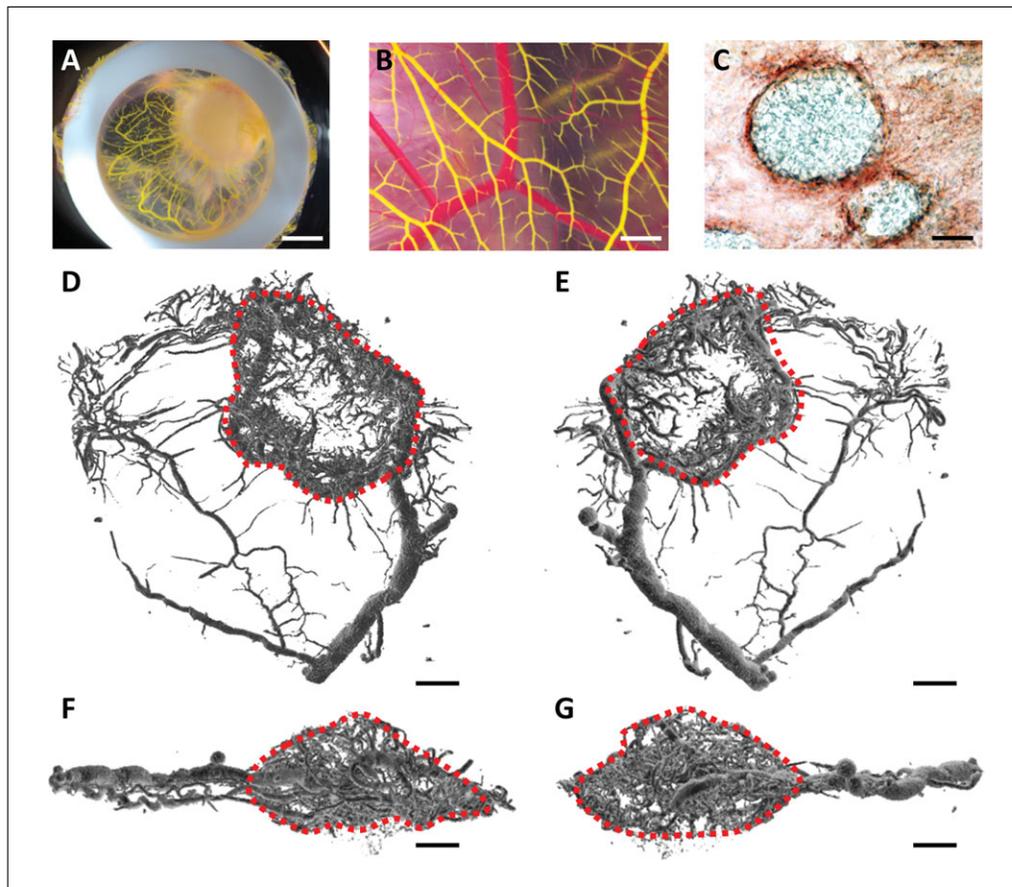
30-G × 1-in. (25 × 0.3–mm) needles  
24-well plates  
Stereo microscope  
Sterile, blunt-end tweezers  
Microcomputed tomography scanner

***Perfuse chicken embryos with mix of MicroFil components (ED 14)***

1. In a small container, dilute the silicone rubber injection compound (yellow) ten-fold in MV-diluent and add 10% MV-curing agent right before use and mix well—working time is a minimum of 20 min and starts with the addition of curing agent. Prepare 5 ml per egg.
2. Draw up the mixture with a 5-ml syringe and attach a three-way valve with a 10-cm tube for more flexibility between the 30-G needle and syringe.
3. Using a stereo microscope, hold a branch of the vitelline vasculature matching the diameter of the needle with sterile blunt-end tweezers and carefully insert the needle into the vessel. To secure the needle within the vessel, apply a small drop of superglue where the needle enters the vasculature (Fig. 1F.19.2).
4. Carefully inject the MicroFil mix into the vasculature until the pressure increases.  
*If the filling of the vasculature is not sufficient, repeat the injection at a non-perfused site. First detach the tube from the needle, which will remain in the CAM, and attach a fresh needle to the tube. Repeat step 3.*
5. Store the perfused chicken embryo overnight at 4°C for complete curing of the MicroFil.
6. On the next morning, cut the CAM around the perfused scaffold samples, wash the samples in 1 ml of PBS, fix in 1 ml of 4% paraformaldehyde, wash in PBS, and store up to 1 week in 70% ethanol at 4°C until  $\mu$ CT imaging is performed. Use wells of a 24-well-plate for these steps.
7. Perform  $\mu$ CT scanning at an isotropic resolution of 20  $\mu$ m, an energy level of 70 kVp, an intensity of 114  $\mu$ A, and 300 msec integration time (Fig. 1F.19.3D-G).



**Figure 1F.19.2** MicroFil perfusion setup. For proper perfusion, a 30-G needle is fixed with a drop of superglue before MicroFil (yellow) is injected.



**Figure 1F.19.3** Imaging of perfused samples. (A) Sample after MicroFil perfusion. Scale bar = 2 mm. (B) CAM after MicroFil perfusion. Scale bar = 1 mm. (C) Histological section of a perfused sample showing vessels (brown, anti-vWF) containing MicroFil. Scale bar = 20  $\mu$ m. (D–G) Top, bottom, left and right view of the  $\mu$ CT image of a MicroFil-perfused sample. The red dotted line marks the area of the silk scaffold. Scale bar = 2 mm.

## REAGENTS AND SOLUTIONS

*For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see SUPPLIERS APPENDIX.*

### Growth medium

Dulbecco's Modified Eagle's Medium/F12 (500 ml) containing:  
 10% fetal bovine serum  
 1% penicillin/streptomycin  
 0.5  $\mu$ g/ml fungizone  
 Prepare fresh

## COMMENTARY

### Background Information

Complete 3D visualization of the microvasculature within in vivo models is often complicated due to the dimensions of the samples/organisms. Non-optical techniques like X-ray, magnetic resonance imaging, and positron emission tomography allow for full body imaging depth with a rather low spatial resolution, whereas optical

methods like single- and two-photon fluorescence microscopy or orthogonal polarization spectral imaging provide better resolution at the expense of penetration depth (Upputuri, Sivasubramanian, Mark, & Pramanik, 2015). The development of new visualization methods using  $\mu$ CT has improved the advancement on tissue anatomy, physiology, and pathology. Combining  $\mu$ CT scanning with perfusion

techniques of tissues and organs allowed for the 3D visualization of the vascular network, its analyses at the histological level, and additionally generated big sets of volumetric and quantitative data (Garcia-Sanz, Rodriguez-Barbero, Bentley, Ritman, & Romero, 1998). These can be used to determine the number, density, length, branching, and diameter of vessels and study the pro- or anti-angiogenic effects of treatments with the goal to either improve or limit vascularization. Therefore, this method has been applied in various research fields, including embryonic vascular development (Anderson-Berry et al., 2005), cancer research (Xuan et al., 2007), and tissue engineering (Stoppato et al., 2013).

However, all previous studies were performed on rodents, which necessitate extensive animal care-taking, animal licenses, as well as surgical skills and equipment. Furthermore, the analysis of the implant vasculature in these studies required a minimum of a 10-day incubation time. Compared to assays performed in rodents, the CAM assay is simple to perform, cost-effective, and highly reproducible. Additionally, ethical approval when performing experiments in chicken embryos until embryonic day 14 (Swiss Animal Care Guidelines, TSchV, Art. 112) is not required. The CAM assay together with MicroFil perfusion followed by  $\mu$ CT imaging allows for studying the early phases of angiogenesis on the microvascular level within implanted scaffolds. This technique could be used for evaluating the angiogenic potential of cells seeded in biomaterial scaffolds before being tested in pre-clinical studies. In the context of tissue engineering, biomaterial properties like pore size and number, stiffness, as well as the seeding density of various cell populations could be optimized using the described protocol. At the same time, this method could be used to accelerate vascularization following implantation and could also help to reduce animal use in the field of tissue engineering following the 3Rs principle (replacement, refinement, and reduction) (Woloszyk, Liccardo, & Mitsiadis 2016).

### Critical Parameters and Troubleshooting

When performing the CAM assay, it is critical to work under an as-sterile-as-possible environment to prevent any infection of the chicken embryo, which eventually will cause the embryo's death before the end of the experiment. Therefore, the egg incubator should also be wiped before each experiment and the

water should be changed. Furthermore, it is important to open the door of the incubator at least once per day to provide the chicken embryos with oxygen. It is enough to keep the door open while checking for potential dead embryos and removing them. Keeping the door open too long will increase the risk for infection.

If removal of the egg white (see Basic Protocol 3, step 5) is not complete the first time, change the needle before trying a second time. Make sure not to harm the embryo.

During the CAM assay, the eggs should be kept at 37°C as long as possible. Therefore, the work at room temperature should be performed as quickly as possible with only one egg at a time.

### Anticipated Results

The visualization method described in this unit results in the acquisition of 3D images of vascular networks growing into a potential biomaterial implant (Fig. 1F.19.3A). It allows for the testing of different conditions and/or properties of a biomaterial, e.g., cell-seeded versus empty scaffolds or the comparison of the same biomaterial with different pore sizes. Therefore, this method is suitable as a first screening before choosing the biomaterials for further *in vivo* testing. Additionally, the perfused samples can be used for histology and immunohistochemistry (Fig. 1F.19.3C) after the  $\mu$ CT scan (Fig. 1F.19.3D-G) has been performed, thereby reducing the amount of samples and gaining more information from each individual sample.

### Time Considerations

This assay requires at least 14 days for completion of the CAM assay. Depending on the growth behavior of the selected cells, additional days may be needed before D0 of the CAM assay to grow the required number of cells. The CAM assay alone takes 14 days with the following time points to be considered. Day 0 (D0): The placing of 40 eggs in the egg incubator takes ~15 min. Day 3 (D3): The opening of 40 eggs requires ~2.5 to 4 hr. Day 6 (D6): Seeding cells on the scaffolds takes ~2 hr. Day 7 (D7): Placing samples on the CAM of the chicken embryos requires 1 to 2 hr, depending on the number of surviving eggs. Day 14 (D14): Per egg, plan ~10 to 20 min for the perfusion.

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### Key Reference

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*The presented method was first published in the referenced paper. However, in Current Protocols the procedure is described in more detail and with more figures giving step-by-step instructions.*