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## The chicken ex ovo embryo and CAM vessels as in vivo model for contrast-enhanced ultrasound imaging and microbubble-mediated drug delivery studies --Manuscript Draft--

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## TITLE

The preparation of chicken ex ovo embryos and chorioallantoic membrane vessels as in vivo model for contrast-enhanced ultrasound imaging and microbubble-mediated drug delivery studies

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## KEYWORDS

Ultrasound Imaging, Contrast-enhanced Ultrasound Imaging, Microbubbles, Drug Delivery, ex ovo chicken embryo, chorioallantoic membrane (CAM) vessels

## SUMMARY

This protocol describes three methods on how to obtain and use 5 to 8-day old chicken embryos and their chorioallantoic membrane (CAM) as an in vivo model to study contrast-enhanced ultrasound imaging and microbubble-mediated drug delivery.

## ABSTRACT

The chicken embryo and the blood-vessel rich chorioallantoic membrane (CAM) is a valuable in vivo model to investigate biomedical processes, new ultrasound pulsing schemes, or novel transducers for contrast-enhanced ultrasound imaging and microbubble-mediated drug delivery. The reasons for this are the accessibility of the embryo and vessel network of the CAM as well as the low costs of the model. An important step to get access to the embryo and CAM vessels is to take the egg content out of the eggshell. In this protocol, three methods for taking the content out of the eggshell between day 5 to 8 of incubation are described thus allowing the embryos to develop inside the eggshell up to these days. The described methods only require simple tools and equipment and yield a higher survival success rate of 90% for 5-day, 75% for 6-day, 50% for 7-day, and 60% for 8-day old incubated eggs in comparison to ex ovo cultured embryos (~50%).

The protocol also describes how to inject cavitation nuclei, such as microbubbles, into the CAM vascular system, how to separate the membrane containing the embryo and CAM from the rest of the egg content for optically transparent studies, and how to use the chicken embryo and CAM in a variety of short-term ultrasound experiments. The in vivo chicken embryo and CAM model is extremely relevant to investigate novel imaging protocols, ultrasound contrast agents, and ultrasound pulsing schemes for contrast-enhanced ultrasound imaging, and to unravel the mechanisms of ultrasound-mediated drug delivery.

## INTRODUCTION

Ex ovo chicken embryos and the blood-vessel rich chorioallantoic membrane (CAM) have proven to be a suitable model to investigate various biological and biomedical processes like embryogenesis, oncology, and drug delivery<sup>1-4</sup>. Ultrasound has been used for imaging of the embryonic heart development<sup>4,5</sup> and for activating cavitation nuclei upon injection, such as microbubbles, for vascular drug delivery<sup>6,7</sup>. Chicken embryos are inexpensive, require less infrastructure and equipment, and have less strict legislation compared to other animal models<sup>8</sup>. The chicken embryo and CAM vessels are easily accessible after opening the egg whereas this proves to be much more difficult with mammalian embryos and vessels. Besides this, the chicken embryo and CAM vessels provide a heartbeat and a pulsating blood flow. The CAM shows similarities in vessel anatomy with mammals and can be used for drug screening<sup>8-10</sup>. Because of these characteristics, the CAM vessels have also proven to be a suitable model to investigate contrast enhanced ultrasound imaging (CEUS)<sup>11-16</sup>. In addition, the model can be used to optically investigate the behavior of ultrasound contrast agents in an ultrasound field using an ultra-high-speed camera and the effect of acoustic radiation force on propelling, binding and extravasation of drugs<sup>7,17-19</sup>. Although the chicken embryo and CAM are less suitable for long term experiments, they can be beneficial for short term in vivo experiments.

To increase visibility and controllability over the chicken embryo and CAM during experiments, it is important to take the egg content containing the embryo and CAM out of the eggshell<sup>18</sup>. Previous chicken embryo studies involving ultrasound contrast agents used 5 to 6-day old embryos<sup>7, 11, 12, 17, 19</sup> and 14 to 18-day old embryos<sup>13-16</sup>. Multiple approaches have been described in detail to take the egg content out of the shell<sup>18, 20, 21</sup>. However, to the best of our knowledge, the previously published approaches focus on taking the egg content out of the eggshell after 3 days of incubation (i.e., Hamburger & Hamilton (HH) stage 19-20<sup>22</sup>), and continue the culture ex ovo. This ex ovo culture approach has multiple disadvantages including increased risk of fatalities during culture (~50%)<sup>1,18</sup>, the use of antibiotics<sup>18, 20</sup>, and decreased total vessel length in comparison to in ovo growth<sup>23</sup>. Since culturing the embryo within the eggshell is providing the most natural environment, it is easiest to incubate the embryo within the eggshell until the day of the experiment. For this reason, an approach in which the egg content is taken out of the eggshell at 5 to 8 days of incubation would be beneficial especially for experiments on 5 to 8-day old embryos.

In this protocol, we describe three methods to take the egg content out of the eggshell when the embryo is at day 5 to 8 of development (HH 26-35<sup>22</sup>) allowing the embryo to develop within the eggshell until the day of the experiment. The CAM vessel size ranges from 10-15  $\mu\text{m}$  in diameter,

in the smaller capillaries of an 8-day old embryo<sup>24</sup> to 115-136  $\mu\text{m}$  in diameter in the larger vessel of 6 and 8-day old embryos<sup>24, 25</sup>. The three described methods only require basic lab tools and reduce the risk of complications before the experiment has begun, thereby reducing unnecessary costs and labor. We also detail a method to separate the membrane containing the embryo and CAM from the yolk sack making the CAM optically transparent for microscopy studies. Because the membrane containing the embryo and CAM can be pinned down on for example a holder with an acoustic membrane, the setup can also be made acoustically transparent<sup>26</sup>, allowing the combination of microscopy and ultrasound studies when the light path will be affected by the yolk. Finally, we describe several other ultrasound setups that can be used for ultrasound or CEUS imaging.

## PROTOCOL

All animal experiments were conducted in accordance with the Netherlands Experiments on Animals Act and in accordance with the European Council (2010/63/EU) on the protection of animal use for scientific purposes.

### 1. Embryo preparation protocol

#### 1.1. Incubation of the fertilized chicken eggs

1.1.1. Store freshly fertilized chicken eggs at 15 °C for up to one week.

1.1.2. To incubate the fertilized eggs, place them vertically with the pointy side down into a 37 °C, humidified incubator. Turning the eggs during incubation is not necessary.

NOTE: Write the starting date of incubation of top of the egg using a permanent marker.

#### 1.2. Preparing up to 5-day (120 h) old embryo's (HH stage 26-28)<sup>22</sup>

##### 1.2.1. Preparation of working area

1.2.1.1. Warm up a heating plate to 37 °C.

1.2.1.2. Place a metal egg holder (**Figure 1A,B**), a metal weighing boat holder (**Figure 1C,D**), and a 10 mL Erlenmeyer filled with PBS on the heating plate.

1.2.1.3. Fill a weighing boat (85 mm  $\times$  85 mm  $\times$  25 mm) with a 10 mm layer of ultrasound gel and place the filled weighing boat in the prewarmed metal weighing boat holder.

NOTE: Filling the weighing boat with ultrasound gel will raise the embryo and CAM. This can be beneficial for the injection or imaging of the embryo and CAM but is not necessary for taking the embryo and CAM out of the eggshell.

1.2.1.4. Prepare a few pieces of tape (around 3 cm length) with part of one end folded back on itself so that does not stick anymore.

## 1.2.2. Taking the egg content out of the eggshell

1.2.2.1. Take a 5-day old incubated fertilized egg and transfer it to the prewarmed metal egg holder (**Figure 1A,B**). Make sure to keep the egg in the same orientation (i.e., date on top).

NOTE: It is important to keep the egg in the same orientation to keep the air sac and the embryo and CAM at the same position in the top of the egg.

1.2.2.2. Use the pointy back of a tweezer (or similar; **Figure 1E**) to make a small indent on the very top of the egg (where the date is written) (**Figure 2A**).

1.2.2.3. Use the pointy back of the tweezers to make a second indent on the side of the egg around 2/3 down the egg (**Figure 2B**).

NOTE: Be careful not to make the indent too big and create a hole. If by accident a hole is created, seal the hole with tape and do not make another indent.

1.2.2.4. Using the larger tweezers (**Figure 1E**), take out a small piece of eggshell from the indented area on top of the egg (with written date). Make sure the air sac in the top of the eggshell makes contact with the air outside the egg, but do not penetrate the shell too deep.

NOTE: If the shell is penetrated too deep when making the top indent, the embryo and CAM could be damaged and the embryo will not survive the removal from the shell. It is important that the small hole on the top creates air contact between the inside and outside of the egg. If this is not done, a vacuum will be created in the next steps of the procedure which will result in large air bubbles getting trapped under the CAM making the embryo and CAM useless. To check the position of the air sac inside the egg, a light source can be used since its position is not always exactly at the top and can also be more to the side.

1.2.2.5. Use a 5 mL syringe and 19 G needle to penetrate the shell through the second indent on the side 2/3 down the egg and withdraw ~2 mL of egg white (**Figure 2C**).

NOTE: Make sure the needle is pointing down towards the bottom of the egg to prevent the chance of damaging the embryo and CAM. This step creates a larger air pocket in the top of the egg needed for removal of the egg content. If a hole is accidentally created instead of an indent in step 1.2.2.3, puncture the tape with the needle for the withdrawal of the egg white. Reseal the puncture with another piece of tape.

1.2.2.6. Take out the needle and use tape to seal the gap on the side (**Figure 2D**).

NOTE: To prevent egg white from leaking out of the egg, the top hole can be closed using a finger before taking the needle out. If egg white keeps leaking out with the tape already in place, first remove the egg white with a piece of tissue to make sure the tape sticks properly.

1.2.2.7. Empty the syringe by adding the egg white to the weighing boat.

1.2.2.8. Use the large tweezers (**Figure 1E**) to enlarge the small opening on the top of the egg (**Figure 2E**). When looking inside the egg through the opening on the top, the embryo and CAM are visible. Keep locating the embryo and CAM while taking away as much of the eggshell as possible (**Figure 2F**).

NOTE: Keep on moving the egg to maintain maximum visibility on the position of the embryo and CAM inside the shell. Make sure the edge of the opening in the shell does not go lower than the CAM. Besides this, do not penetrate the inner membrane and prevent sharp edges.

1.2.2.9. After creating the opening, turn the egg 180° and place the egg back in the egg holder in such a way that the created opening at the top of the egg is now facing towards the bottom. The embryo will float up and will become invisible from the bottom (**Figure 2G**) which takes 1-2 min. Make sure the whole embryo and CAM (including all the vessels) have disappeared and only yolk is visible before proceeding to the next step (**Figure 2H**).

NOTE: If the embryo is still visible from the bottom after 2 min, turn the egg clockwise for 1-2 min. This will help the embryo and CAM to float up.

1.2.2.10. Remove the tape from the side opening. Look whether the inside of the egg now bulges out of the bottom opening. If this is the case, proceed to the next step. If not, use the needle on the syringe to puncture the opening on the side once more to release the vacuum in the egg. Make sure to point upwards with the needle to prevent the chance of puncturing the yolk sack. Continue until the egg bulges out of the bottom opening.

1.2.2.11. While holding the bottom of the egg close to the weighing boat in the metal weighing boat holder (**Figure 1C,D**), gently but quickly make a horizontal scratch in the membrane over the entire width of the opening using one of the sharp points of the small tweezer (**Figure 1F**) and gently drop the egg content into the weighing boat (**Figure 2I**).

NOTE: If the egg content does not come out, use the needle on the syringe to puncture the side opening again with the needle pointing upwards.

1.2.2.12. If the embryo is in the weighing boat sideways, it will usually go up by itself. If this does not happen, use a piece of tissue paper to reposition the embryo. Put one side of the tissue paper on the embryo, drag the tissue paper to the other end, and release the tissue paper with a few drops of ~30 µL of PBS (37 °C) using a plastic Pasteur pipet.

1.2.2.13. Visually check whether the embryo is alive by ensuring the heartbeat is still present, the CAM vessels are intact and there is no bleeding, and there is no leakage of yolk. If one of these things is not correct, discard the embryo and CAM because it will not be viable.

1.2.2.14. Make sure the embryo and CAM are kept at 37 °C and do not dry out because this will make the CAM vessels deteriorate and eventually the embryo will die. To prevent this, regularly put small drops of ~30 µL of 37 °C PBS on the embryo and CAM.

### 1.3. Preparing 6 to 7-day (144-168 h) old embryo's (HH stage 28-32)<sup>22</sup>

#### 1.3.1. Preparation of working area

1.3.1.1. Prepare the stage as described in section 1.2.1.

#### 1.3.2. Taking the egg content out of the eggshell

1.3.2.1. Two hours in advance of the experiment, take a 6 to 7-day old incubated egg and rotate the egg 180° inside the incubator so that the top of the egg is facing towards the bottom. After 1 h, rotate the egg back to its original position and leave for another 1 h.

NOTE: Rotating the egg 2 h in advance of the experiment will make it easier to take the egg content out of the shell.

1.3.2.2. After rotating, take the egg from the incubator.

1.3.2.3. Perform step 1.2.2.2 until step 1.2.2.4.

1.3.2.4. Use a 5 mL syringe and 19 G needle to penetrate the shell through the second indent on the side 2/3 down the egg and withdraw between 5-6 mL of egg white. Make sure the needle is pointing down towards the bottom of the egg.

NOTE: With the 5 mL syringe we used, it is possible to withdraw up to 6 mL so only one penetration is needed.

1.3.2.5. Take out the needle and use a piece of tape to seal the gap on the side (**Figure 2D**).

1.3.2.6. Empty the syringe by adding the egg white to the ultrasound gel in the weighing boat.

1.3.2.7. Use the large tweezers (**Figure 1E**) to enlarge the small opening on the top of the egg (**Figure 2E**). Try to make the opening as big as possible but make sure the edge of the opening in the shell does not go lower than the CAM. Besides this, do not penetrate the inner membrane and try to prevent sharp edges.

263  
264 1.3.2.8. Fill a syringe with ~1 mL more of 37 °C PBS than the withdrawn volume during step  
265 1.3.2.4.

266  
267 1.3.2.9. Take off the tape from the side gap, penetrate the gap with the filled syringe, and  
268 empty it into the shell. Make sure the needle is pointing down towards the bottom of the egg.

269  
270 NOTE: Since egg white has a higher viscosity (~160 cP)<sup>27</sup> than PBS (~1 cP), substituting the egg  
271 white with PBS reduces both tension and stress on the embryo and CAM while taking the egg  
272 content out of the shell.

273  
274 1.3.2.10. Take out the needle and quickly reseal the gap with a piece of tape (**Figure 2D**).

275  
276 1.3.2.11. Turn the egg 180° and place the egg back in the egg holder in such a way that the  
277 created opening at the top of the egg is now facing towards the bottom. Turn the egg clockwise  
278 until the whole embryo and CAM (including all the vessels) have disappeared and only yolk is  
279 visible.

280  
281 1.3.2.12. Perform step 1.2.2.10 until step 1.2.2.14.

282  
283 1.4. Preparing 8-day (192 h) old embryo's (HH stage 32-35)<sup>22</sup>

284  
285 1.4.1. Preparation of working area

286  
287 1.4.1.1. Warm up a heating plate to 37 °C.

288  
289 1.4.1.2. Place a metal weighing boat holder (**Figure 1C,D**) and a 10 mL Erlenmeyer filled  
290 with PBS on the heating plate.

291  
292 1.4.1.3. Take a shallow container of 170 x 110 x 70 mm, or similar, and fill the container  
293 with 1 L of 37 °C PBS.

294  
295 1.4.1.4. Place a weighing boat (85 × 85 × 25 mm) in a 90 mm diameter Petri dish. Place the  
296 Petri dish and the weighing boat at the bottom of the container and make sure they are  
297 completely submerged.

298  
299 1.4.2. Taking the egg content out of the eggshell

300  
301 1.4.2.1. Two hours in advance of the experiment, take an 8-day old incubated egg and  
302 rotate the egg 180° inside the incubator so that the top of the egg is facing towards the bottom.  
303 After 1 h, rotate the egg back to its original position and leave for another 1 h.

304  
305 NOTE: Rotating the egg 2 h in advance of the experiment will make it easier to take the egg  
306 content out of the shell.

307  
308 1.4.2.2. Take an 8-day old incubated egg from the incubator.

309  
310 1.4.2.3. Hold the egg horizontally and use the pointy back of the large tweezer (**Figure 1E**)  
311 to make a small indent 1/2 down the egg. Continue making small indents in a ring pattern 360°  
312 round the eggshell. Use a spacing of ~10 mm between the indents.

313  
314 NOTE: During this procedure, small cracks can start to form between the indents.

315  
316 1.4.2.4. After creating the small indents all around the shell, make one bigger hole by  
317 cracking the shell between two small indents using the pointy back of the large tweezer.

318  
319 1.4.2.5. Completely submerge the egg in the 37 °C PBS and keep it submerged for 5 min.  
320 After 5 min, keep the egg close to the weighing boat inside the container. Put the top of both  
321 thumbs in the large hole and gently open the egg. The egg will crack along the small indents.

322  
323 1.4.2.6. When the crack is formed all the way around the eggshell, gently try to pull the  
324 two eggshell pieces apart and keep on gently moving the two pieces back and forth until the egg  
325 content is separated from the shell. Then, gently drop the egg content into the weighing boat.

326  
327 NOTE: By moving the two pieces of eggshell back and forth, more PBS will flow into the eggshell,  
328 which will help in separating the egg content from the shell. Sometimes, a bit of egg white will  
329 stick to the inside of the eggshell. When this happens, use the tweezers to separate the egg white  
330 from to shell.

331  
332 1.4.2.7. Slowly raise the Petri dish containing the weighing boat and egg content from the  
333 PBS. When out of the PBS, slightly tilt the weighing boat to remove the excess PBS.

334  
335 1.4.2.8. Place the weighing boat containing the egg content into the metal weighing boat  
336 holder and move to the desired experimental setup.

## 337 338 2. Selected Applications

339  
340 2.1. Injecting microbubbles and/or other solutions into the CAM vessels

341  
342 2.1.1. Preparing the injection setup

343  
344 2.1.1.1. Pull glass needles from glass capillary tubes using a micro forge (**Figure 3A**) or buy  
345 pulled glass capillary needles.

346  
347 2.1.1.2. In case the tip of the glass capillary needle is not beveled, break off a small part of  
348 the tip of the needle. Fill the glass needle with mineral oil and place it in a micro-injection system.  
349 Make sure there are no air bubbles in the mineral oil in the glass needle.

350



NOTE: The mineral oil is added as per instruction of the manufacturer of the injection system we used.

2.1.1.3. Empty the pulled-glass capillary needle as far as the micro-injection system allows and partly refill the glass needle with air.

NOTE: The small bit of air will prevent the mixture of the mineral oil and the to-be-injected solution.

2.1.1.4. Put 10  $\mu\text{L}$  of the desired solution, in this protocol microbubbles, on a piece of waxy film (**Figure 3B**). If more than one solution is needed, solutions can be mixed before pipetting<sup>7</sup>.

NOTE: Before filling the needle with microbubbles, leave the microbubble drop on the waxy film for  $\sim 1$  min so that the microbubbles will float to the top of the drop and get concentrated. For the F-type custom-made ultrasound contrast agent<sup>28</sup>, this concentration step will increase the to-be-injected microbubble concentration with  $\sim 30\%$ . The post-injection concentration in the chicken embryo blood will be between  $32 \times 10^3$  microbubbles/ $\mu\text{L}$  for 5-day old embryos and  $19 \times 10^3$  microbubbles/ $\mu\text{L}$  for 6-day old embryos.

2.1.1.5. Fill the glass needle with the microbubble and/or other solution by positioning the glass needle tip into the drop on the waxy film. When aspirating microbubbles, make sure to position the needle tip in the top of the liquid drop to aspirate the microbubble enriched solution.

NOTE: Before injecting microbubbles, raise the tip of the glass needle to its highest point and wait for  $\sim 2$  min. This will ensure the microbubbles will concentrate into the tip of the glass needle.

## 2.2. Injection into the CAM vessels

2.2.1. Before injection, look at the CAM under a stereo microscope and select the best vessel to inject. Always inject into one of the **veins** of the embryo. These are the vessels in which the blood flow moves towards the embryo. Veins are lighter in color than the arteries due to the oxygenated blood<sup>29</sup>. In addition, veins are always on top of the artery with two exceptions namely the anterior and posterior vitelline veins (i.e., the less branched veins, indicated with asterisks in **Figure 6A,B**) which do not have an artery in their surroundings.

NOTE: Injecting into one of the branches will limit the obstruction of the blood flow during injection. Good injections sites have been indicated with arrowheads in **Figure 6A,B**. It is crucial to inject into the vein, since this will force the injected substance to flow towards the embryo. Besides this, injecting into the artery will result in a massive bleeding when removing the glass needle which will kill the embryo.

2.2.2. Position the glass needle and the embryo in such a way that the glass needle tip and the selected vein are in the same focal plane and in the same line of direction. Try to position the

needle as horizontal as possible parallel to the selected vein. The needle tip should touch the vessel wall.

NOTE: By positioning the glass needle as horizontal as possible, the chance of piercing through the entire vessel is lower.

2.2.3. After positioning, slowly advance and penetrate the vessel wall with the glass needle. During penetration, the CAM will first be pushed away by the movement of the glass needle. Keep advancing the glass needle until the vessel wall is penetrated.

NOTE: If by accident the vessel is pierced through and through, slowly retract the needle to get back into the lumen. When back inside the lumen, slightly lift the needle up and move forward along the vessel to reposition the needle.

2.2.4. After penetration, slightly retract the glass needle to better position the tip inside the vessel lumen and move the glass needle sideways to check it is not attached to the vessel wall. Slowly inject a small amount of the solution to confirm that the tip is positioned inside the vessel lumen (**Figure 3C**).

2.2.5. Make sure the injected solution follows the blood flow. If it does not, slightly move the glass needle and keep injecting small amounts until the glass needle is positioned correctly<sup>17</sup>.

2.2.6. When the desired amount is injected, leave the glass needle in the vessel for ~15 s to prevent a massive bleeding. Then, move the glass needle a little bit sideways, up and down, and back and forth a few times to allow a gently retraction of the glass needle.

NOTE: Some bleeding is normal. For every injection use a new glass needle because the glass needle gets easily clogged with and blunt from egg white.

### 2.3. Microscopy imaging of the embryo and/or CAM vessels

#### 2.3.1. Preparing holder with acoustic membrane

2.3.1.1. Take a cell culture chamber consisting of a square plastic holder with two parallel 50  $\mu\text{m}$  thick acoustically transparent polycarbonate membranes<sup>26</sup>, further referred to as holder with acoustic membrane. Close both ports with a lid.

2.3.1.2. Use a scalpel to remove one of the two membranes from the holder with acoustic membrane.

NOTE: To remove the membrane, cut the membrane just next to the glue line on the plastic. Be careful not to slip off from the edge to prevent damage to the other membrane.

2.3.1.3. Prepare ~15 mL of 2% agarose in demi water solution by heating to between 80-95 °C in a small glass beaker. Cool the glass beaker with the dissolved agarose solution under a running cold-water tap.

NOTE: If the agarose is too hot it will melt the acoustic membrane which will create an uneven surface.

2.3.1.4. When the solution is cooled down to around 37 °C, slowly pour the solution in the holder with acoustic membrane until it fills up the entire holder. Slightly tilt the holder with acoustic membrane so the agarose layer will distribute evenly inside the plastic frame (**Figure 4A**). Make sure the agarose layer is flat and let the agarose set at room temperature.

2.3.2. Removing embryo and CAM from yolk sack and placing on holder with acoustic membrane

2.3.2.1. Take out the egg content out of the egg as described in section 1.2, 1.3, or 1.4.

2.3.2.2. If needed, inject the CAM with microbubbles and/or other solution(s) as described in section 2.1.2.

2.3.2.3. Fill a 1 L Petri dish with ~500 mL of 37 °C PBS and place the holder with acoustic membrane with agarose on the bottom of the dish. Make sure the agarose layer is facing up.

2.3.2.4. Use small scissors to **quickly** cut into the membrane of the yolk sack, also called Vitellus membrane, around the entire CAM while the egg content is in the weighing boat (**Figure 4B**). Keep the scissors in the same position and rotate the weighing boat while cutting for better precision and more speed.

NOTE: From the moment the first cut is made, the yolk will start to leak. This reduces the visibility of the embryo and CAM. Try to cut all the way around the CAM within 6-7 cuts. This should not take much longer than 20 s. The small tweezers (**Figure 1F**) can be used to hold the edge of vitelline membrane and prevent cutting into the CAM.

2.3.2.5. Use a tablespoon to scoop up the cut-out membrane containing the embryo and CAM from the weighing boat. Slowly raise the spoon from the weighing boat and visually inspect whether the cut-out membrane containing the embryo and CAM are still attached to the remainder yolk sack membrane.

2.3.2.5.1. When this is the case, use the scissors to make an extra cut. While scooping, slightly tilt the spoon to get rid of as much yolk as possible but do not let it dry out. Transfer the cut-out membrane containing the embryo and CAM to the 1 L Petri dish, submerge in the 37 °C PBS, and remove the spoon.

2.3.2.6. When the membrane containing the embryo and CAM is submerged in the 37 °C PBS, use the small tweezers (**Figure 1F**) to grab one edge of the membrane and gently swirl around the membrane to get rid of the yolk that is still attached.

2.3.2.7. When all the yolk is removed, use the small tweezers to move the membrane containing the embryo and CAM and position it above the holder with acoustic membrane.

2.3.2.8. Use one insect specimen pin to pin down the membrane containing the embryo and CAM in one corner. Avoid piercing the vessels in the CAM and only pin down the membrane.

2.3.2.9. Use a second insect specimen pin to pin down the membrane containing the embryo and CAM on the diagonally opposite corner.

2.3.2.10. Slowly lift the holder with the acoustic membrane containing the embryo and CAM from the 37 °C PBS. Slightly tilt the holder to get rid of most of the PBS.

2.3.2.11. Use the small tweezers (**Figure 1F**) to stretch and evenly distribute the membrane containing the embryo and CAM over the holder with acoustic membrane and pin down the rest of the membrane. Make sure the membrane containing the embryo and CAM is slightly stretched to assure it is flat (**Figure 4C**).

2.3.2.12. Place the holder with acoustic membrane with the pinned down membrane containing the embryo and CAM into a microscopy setup which is kept at 37 °C.

2.3.2.13. Place a coverslip or an acoustically and optically transparent membrane (depending on the desired objective and use of ultrasound or not) on top of the region of interest on the embryo or CAM (**Figure 4D**) to allow optical visualization.

## 2.4. Ultrasound imaging of the chicken embryo and/or CAM vessels

### 2.4.1. Ultrasound imaging from the side of the chicken embryo and CAM vessels

2.4.1.1. Take out the egg content as described in section 1.2, 1.3, or 1.4. However, do not use a standard weighing boat. Instead, use a custom-made weighing boat with one acoustically transparent wall.

NOTE: The standard weighing boat was adjusted by cutting off one side of the weighing boat and replacing it with a window of polyester foil which was glued together using epoxy glue.

2.4.1.2. Submerge the preferred ultrasound transducer in a 37 °C water bath and position in the desired spot with the required standoff distance.

2.4.1.3. Place the weighing boat in the water bath in such a way that the transparent wall is facing the transducer. Make sure the weighing boat is deep enough to be level with the transducer but avoid water getting into the weighing boat (**Figure 5A**).

2.4.1.4. If desired, add another setup to the top of the embryo or CAM vessels, like a microscope or a laser (**Figure 5A**).

2.4.2. Ultrasound imaging from the top of the embryo and CAM vessels without acoustic interference

2.4.2.1. Fill a 2 L beaker glass with 37 °C PBS. Place a 500 mL beaker glass upside down on the bottom of the 2 L beaker glass. Avoid air inside the 500 mL beaker glass.

NOTE: The 500 mL beaker glass is meant to raise the weighing boat containing the egg content closer to the PBS surface. By substituting the beaker for objects with other sizes, the distance between the transducer and the egg content can be varied.

2.4.2.2. Place the filled 2 L beaker glass with the 500 mL beaker glass inside in a 37 °C water bath.

2.4.2.3. Take out the egg content as described in section 1.2, 1.3, or 1.4.

2.4.2.4. Wet the egg content with 37 °C PBS and cover the embryo with clear cling film. This can be done to keep the embryo in the same position and prevent it from rotating or floating away.

NOTE: By wetting the egg content with PBS, it will become less sticky which makes it easier to cover the egg content with clear cling film.

2.4.2.5. Place the weighing boat with the egg content in a 90 mm diameter Petri dish and slowly submerge the Petri dish in the PBS (**Figure 5B**).

NOTE: Using two clamps on the sides of the Petri dish opposite to one another makes it easier to submerge the Petri dish.

2.4.2.6. Position the ultrasound transducer with the desired standoff distance.

2.4.3. Ultrasound imaging of the chicken embryo and CAM vessels with a movable transducer

2.4.3.1. Take out the egg content as described in section 1.2, 1.3, or 1.4.

2.4.3.2. Prepare a 2% agarose solution in demi water by heating the solution up to between 80-95 °C in a small glass beaker. Cool the glass beaker with the dissolved agarose solution under a running cold water tab.

2.4.3.3. Pour the agarose solution into a flat container to create an about 1 mm thick agarose pad. When completely cooled down and set, cut the agarose pad to the desired size using a scalpel.

NOTE: The thickness of the agarose pad can be changed to obtain the desired focal distance necessary for correct functioning of the ultrasound transducer.

2.4.3.4. Place the agarose pad on top of the embryo and CAM (**Figure 5C**). Add a few drops of ~30  $\mu$ L of 37 °C PBS onto the top of the agarose pad to create a thin PBS layer between the agarose pad and the transducer.

NOTE: Using PBS will prevent the transducer from sticking to the agarose pad. This is beneficial when, for example, using a motor to mechanically move a two-dimensional transducer to make a three-dimensional scan (**Figure 9B**)<sup>11</sup>. When the transducer does not need to be moved, the PBS can also be substituted with ultrasound gel.

2.4.3.5. Position the desired ultrasound transducer.

## REPRESENTATIVE RESULTS

In this protocol, we describe three methods to take the egg content out of the shell at day 5-8 of incubation (HH 26-35<sup>22</sup>). **Figure 6** shows the egg content in weighing boats after it was taken out of the shell. The 5-day old embryo and CAM (**Figure 6A**) was taken out using the method described in section 1.2. The 6 and 7-day old embryos and CAM (**Figure 6B,C**) were taken out using the method described in section 1.3. The 8-day old embryo and CAM (**Figure 6D**) was taken out using the method described in section 1.4. No bleeding or damage to the embryo or CAM can be observed, indicating that these methods can be used to safely get the egg content out of the shell without harming the embryo or the CAM vessels. When executed correctly, the method for the 5-day old embryos will provide a viable embryo and intact CAM in 90% of all procedures. The viability rate is based on the total number of fertilized eggs successfully extracted from the eggshell. With the second method, for 6 and 7-day incubated eggs, the chance of a viable embryo and intact CAM is around 75% for 6-day old and around 50% for 7-day old. With the third method described for 8-day old embryos, the chance of a viable embryo and intact CAM is around 60%. Differences in developmental stages between the 5 and 8-day old embryos can be observed which concurs with Hamburger and Hamilton<sup>22</sup>. Both the size of the embryo and the complexity of the CAM vessels increase during development (**Figure 6A-D**). **Figure 6C** shows a thin patch of agarose on top of the egg content that allows the embryo and CAM to be imaged using the ultrasound setup shown in **Figure 5C**. After the egg content is taken out of the shell the heartbeat of the embryo is visible with the naked eye. The heart rate of these ex ovo embryos is similar to in ovo embryos at 183 beats per minute (bpm) at day 5 up to ~208 bpm at day 8<sup>30</sup>. When kept humidified and at 37 °C, the embryo will maintain this heart rate for ~5 h in the experimental ultrasound setups.

Multiple complications can occur during the previously described three methods. **Figure 7A**

shows trapped air under the CAM which makes the embryo unsuitable for ultrasound imaging and the pressure of the air bubble(s) can also damage the embryo and/or CAM. This problem arises when the air sac inside the shell does not make contact with the air outside the shell when taking the egg content out of the shell. **Figure 7B** shows a small leakage of yolk from the yolk sack in the top right of the image. This can occur while taking the egg content out of the shell when the yolk sack gets damaged by sharp edges of the shell or when the yolk sack is penetrated by the tweezers. Leakage of the yolk can affect the visibility of the embryo and the CAM vessels. **Figure 7C** shows an embryo in which an air bubble is trapped under the CAM. This sometimes occurs in the embryonic development. Another complication which can occur is damage to the vessels. This damage can be created while taking the egg content out of the shell or when performing an injection (**Figure 7D**). Besides this, the embryo and vessels can also dry out over time (**Figure 7E**). This occurs when the egg content is not sprinkled with PBS. The drying out of the embryo can result in massive capillary obstructions (**Figure 7F**) which affects the viability of the embryo. The massive capillary obstructions can also occur during development or when the heartbeat of the embryo is not stable.

After the egg content is taken out of the shell without any complication, the embryo can be injected with, for example, ultrasound contrast agents such as microbubbles (**Figure 3C**). **Figure 8** shows circulating microbubbles in the lumen of the blood vessel upon injection. These microbubbles are carried along with the blood flow and stay present in the blood circulation for several hours (**Supplemental Video 1**). The presence of these microbubbles in the circulation creates the possibility to perform different types of CEUS and drug delivery experiments<sup>7, 11, 12</sup>. The CAM is ideal to investigate novel ultrasound contrast detection methods for which we show three examples. **Figure 9A** shows high frequency ultrasound subharmonic imaging of a 6-day old chicken embryo in B-Mode and CEUS before and after microbubble injection. Here, the CAM vessels were injected with 5  $\mu$ L of ultrasound contrast agent and imaging was performed with a preclinical animal ultrasound machine with a MS250 probe (30 MHz transmit and 15 MHz receive frequency, 10% power). Before microbubble injection, contrast can already be seen inside the embryonic heart in the B-Mode images (**Figure 9A-I**). This phenomenon is due to the presence of a nucleus in the avian red blood cell which increases the contrast of blood in ultrasound imaging<sup>5, 31</sup>. The addition of the microbubbles increased the contrast and the visibility of the embryo, both in the B-Mode and CEUS imaging. **Figure 9B** shows an optical and a high frequency 3D subharmonic image of a 6-day old embryo and the surrounding vessels. The CAM was injected with 5  $\mu$ L of ultrasound contrast agent and imaging was performed with a preclinical animal ultrasound machine with MS550s probe (transmission frequency of 40 MHz, peak negative pressure  $\sim$ 300 kPa). These results show that the CEUS imaging combined with a contrast agent can also be used to create high frequency 3D subharmonic images and to image the blood vessels outside the embryo. **Figure 9C** shows an optical image and an ultraharmonic intravascular ultrasound (IVUS) image made with a custom probe of CAM microvessels of a 6-day old embryo (26 MHz transmit and 39 and 65 MHz receive frequency). CAM vessels were injected with  $4 \pm 1$   $\mu$ L ultrasound contrast agent. The optical image and IVUS image are from the same embryo and same region of interest showing corresponding vessel networks.

The chicken embryo and CAM vessels can also be used to investigate ultrasound-mediated drug

delivery for which we show one example. Since the yolk obstructs the light path during imaging, the removal of the yolk sack is necessary to optically investigate drug delivery in the embryo and CAM vessels. For this study, the embryo and CAM were prepared for microscopic imaging as explained in section 2.2 by separating the membrane containing the embryo and CAM from the yolk sack (**Figure 4C**). In these embryos, the heart rate is stable around 80 bpm and the embryos stay alive for up to 2 h when kept at a 37 °C<sup>7</sup>. **Figure 10** shows an ultrasound and microbubble mediated drug delivery study in endothelial cells of the CAM vessels. Lipid-coated microbubbles, targeted to the vessel wall using  $\alpha v\beta_3$ -antibodies and stained with the fluorescent dye Dil<sup>7</sup>, were injected into the CAM vessels (**Figure 10A,C**). CAM vessel endothelial cell nuclei were stained with Hoechst 33342 (**Figure 10B**) and the model drug Propidium Iodide (PI) was used to visualize sonoporation<sup>7</sup>. Both these dyes were injected simultaneously with the microbubbles. Upon ultrasound treatment (1 MHz, 200 kPa peak negative pressure, single burst of 1000 cycles), PI uptake was observed in the nuclei closest to the targeted microbubbles (**Figure 10D**). This shows that the ultrasound-induced oscillations of the targeted microbubbles were able to create a pore in the endothelial cell membrane.

## FIGURE AND TABLE LEGENDS

**Figure 1. Embryo preparation equipment.** (A-B) top and side view of the metal egg holder and (C-D) top and side view of the metal weighing boat holder. (E-F) tweezers needed to take the egg content out of the shell. Scale in cm.

**Figure 2. Embryo removal procedure.** (A) Small indent on top of the egg, indicated by the black circle. (B) Small indent 2/3 down the egg, indicated by the black circle. (C) Withdrawing ~2 mL of egg white. (D) Sealed gap on the side with tape. (E) Enlarging the small opening on the top of the egg. (F) The embryo becomes visible after removing part of the shell. (G-H) After rotating the egg 180°, the embryo floats up and will become invisible (arrows indicate moving direction of the embryo). After 1-2 min, the embryo is invisible from the bottom. (I) After scratching the membrane, the egg content drops into the weighing boat.

**Figure 3. Injection of microbubbles into the CAM vessels.** (A) Glass capillary needle. Scale in cm. (B) Propidium iodide (PI) solution (left drop) and microbubbles (right drop) prior to aspiration before injection. Needle (outlined in black) can be seen in the top right corner (C) Microbubble injection. The capillary needle tip is positioned inside the lumen of one of the veins (left). Microbubbles, the white cloud indicated with an arrow, are injected and disperse following the blood stream (right). Scale bar represents 1 mm.

**Figure 4. Removing embryo and CAM from yolk sack and placing on holder with acoustic membrane.** (A) Holder with acoustic membrane filled with agarose layer. (B) Chicken embryo and CAM vessel in weighing boat before cutting. Dotted line indicates the cutting line around the CAM. (C) Chicken embryo and CAM separated from yolk and pinned down on acoustic membrane. (D) Pinned down chicken embryo with an acoustically and optically transparent membrane in a holder (blue) placed on top of the CAM. The holder can be filled with demi water



so a water dipping objective can be used. All scale bars represent 1 cm.

**Figure 5. Different setups for chicken embryo and CAM ultrasound imaging. (A)** Setup for ultrasound imaging from the side. Chicken embryo was placed in a custom-adjusted weighing boat with one acoustically transparent wall and placed in a 37 °C water bath. The ultrasound transducer was positioned on the left (a) side next to the acoustically transparent wall and the laser (b) for photoacoustic imaging on top. **(B)** Setup for ultrasound imaging from the top. Embryo and CAM were submerged in a beaker of PBS that was placed in a 37 °C water bath. Dashed outline shows the 2 L glass beaker (a) with the 500 mL glass beaker (b) inside. **(C)** Setup for ultrasound imaging from the top with a movable transducer. A thin agarose pad (dotted line) was placed on top of the embryo with a thin layer of PBS as coupling between the transducer and the agarose surface.

**Figure 6. Egg content outside the shell. (A)** Egg content taken out of the shell after 5 days of incubation. The chorioallantoic membrane (CAM), embryo body (EB), anterior and posterior vitelline veins (\*), and appropriate sites for injection (arrowheads) are indicated. **(B)** Egg content taken out of the shell after 6 days of incubation. The anterior and posterior vitelline veins (\*) and appropriate sites for injection (arrowheads) are indicated. **(C)** Egg content taken out of the shell after 7 days of incubation. A patch of agarose is placed on top to allow for ultrasound imaging. The corners of the agarose patch are indicated with black circles. **(D)** Egg content taken out of the shell after 8 days of incubation. All scale bars represent 1 cm.

**Figure 7. Complications which can occur during the procedures with the chicken embryo and CAM model. (A)** Air bubbles trapped under the CAM when taking the egg content out of the shell using method 1.2 (5-day old embryo) or 1.3 (5 to 6-day old embryo). **(B)** Small leakage of yolk indicated with an arrow on the top right (6-day old embryo). **(C)** Air trapped under the CAM, indicated by the black dotted circle (7-day old embryo). **(D)** Bleeding, indicated with the black arrows (5-day old embryo). **(E)** Dried out embryo and CAM (5-day old embryo). **(F)** Massive capillary obstructions (5-day old embryo). All scale bars represent 1 cm.

**Figure 8. Microbubbles in CAM blood vessel.** The vessel wall is indicated with a dotted line and single microbubbles are indicated with arrows. Scale bar represents 20 µm. The corresponding microscopy recording can be found in Supplemental Video 1.

**Figure 9. Contrast-enhanced ultrasound imaging in chicken embryos and CAM vessels. (A)** Maximum-intensity projection of B-Mode (I, III) and real-time subharmonic (II, IV) images (preclinical animal ultrasound machine with MS250 probe, 30 MHz transmitting and 15 MHz receiving frequency, 10% power) of a 6-day old embryo with a patch of agarose on top. Top images (I, II) show results before and bottom (III, IV) after injection of 5 µL ultrasound contrast agent. Scale bar represents 1 mm. This image has been modified with permission from Daeichin et al. 2015<sup>11</sup> **(B)** Optical (left) and 3D subharmonic imaging (right) of a 6-day old chicken embryo with a patch of agarose on top. CAM vessels were injected with 5 µL ultrasound contrast agent and imaging was performed with a high-frequency probe (preclinical animal ultrasound machine with MS550s probe, transmission frequency of 40 MHz, peak negative pressure ~300 kPa,

rendered in preclinical animal ultrasound machine 3-D mode). Scale bar represents 5 mm. This image has been modified with permission from Daeichin et al. 2015<sup>11</sup>. **(C)** Optical image (left) and mean intensity projection of ultraharmonic intravascular ultrasound (IVUS) (right) of the CAM microvasculature of a 6-day old embryo. CAM vessels were injected with  $4 \pm 1 \mu\text{L}$  contrast agent. Ultraharmonic IVUS imaging was performed with a custom IVUS probe (transmission frequency 35 MHz, peak negative pressure 600 kPa). Both images are made from the same embryo and region of interest. Arrows indicate corresponding vessels in the two images. Scale bar represents 1 mm. This image has been modified with permission from Maresca et al. 2014<sup>12</sup>.

**Figure 10. Drug delivery to CAM vessel endothelial cells in 6-day old embryo. (A)** Brightfield image of six  $\alpha_v\beta_3$ -targeted microbubbles, indicated with white arrows, adhering to the vessel wall before ultrasound treatment. **(B)** Endothelial cell nuclei fluorescently stained before ultrasound treatment. **(C)** Fluorescent image of the stained targeted microbubbles, indicated with white arrows, before ultrasound treatment. **(D)** Uptake of the model drug propidium iodide (PI) into the cell nuclei underneath the targeted microbubbles after ultrasound treatment (1 MHz, 200 kPa peak negative pressure, single burst of 1000 cycles). Scale bar represents 10  $\mu\text{m}$  and applies to all images. This image has been modified with permission from Skachkov et al. 2014<sup>7</sup>.

## SUPPLEMENTARY FILES

**Supplemental Video 1. Microbubbles in CAM blood vessel.** Scale bar represents 20  $\mu\text{m}$ .

## DISCUSSION

This protocol describes three methods on how to obtain and use 5 to 8-day old chicken embryos and their CAM as an in vivo model to study contrast-enhanced ultrasound imaging and microbubble-mediated drug delivery. The most critical steps for taking 5-day old (section 1.2) and 6 to 7-day old (section 1.3) embryos out of the shell are: 1) make the small hole in the top of the egg to go through the entire eggshell into the air sac before withdrawing egg white; 2) create smooth edges for the large opening in the shell. For the method for taking 8-day old embryos out of the shell (section 1.4) the most critical steps are: 1) Make a sufficient number of indents to create a nice crack along the egg; 2) Keep the egg submerged in PBS. To ensure embryo viability for all methods it is important to keep the egg and its contents at 37 °C. In addition, avoid injecting into a CAM artery. Visually monitoring the heart rate of the embryo during the studies is recommended to ensure embryo vitality. To confirm the exact developmental stage of the embryo, the indication of Hamburger & Hamilton<sup>22</sup> can be used.

It is important to prevent damage to the embryo, CAM, and yolk sack. This damage can affect the viability, blood flow, and visibility of the embryo and CAM. In addition, damage to the yolk sack and consequently a low rigidity of the membrane makes an injection into the CAM vessels impossible. A 5-day old embryo has a relatively small air sac so to be able to make a sufficiently large hole in the shell through which the egg content can be removed, 2 mL of egg white needs to be withdrawn. As a result, more space between the eggshell and embryo is created. After withdrawal of the egg white, a piece of tape needs to close off the hole where the needle went in. If egg white still leaks out, apply another piece of tape. Beside this, the application of tape on the hole on the side creates a vacuum inside the egg which prevents the egg content from falling

out due to its own weight when the large hole is created in step 1.2.2.8. Damage to the embryo or CAM can also occur when the edge of the eggshell was too sharp or when the egg content is dropped into the weighing boat too rigorously, so the eggshell should be kept very close to the weighing boat. Between day 5 and 6 of development, the CAM starts to attach to the shell membrane<sup>32</sup>. This attachment increases the risk of damaging the embryo and CAM when taking the egg content out of the eggshell. By opening the egg after injection of PBS into it for a 6 to 7-day incubated egg or in a PBS-filled container as described for an 8-day incubated egg, the risk of damage is reduced. Regarding an injection into a CAM vein: if the first injection fails, a second injection can be done further upstream in the same vein if the damage was minor or in another CAM vein. Separation of the embryo and CAM from the yolk makes the embryo and CAM vessels optically transparent. As a consequence, the embryo loses its primary source of nutrients<sup>33</sup>. This loss of nutrients could be an explanation for the observed lower heart rate of 80 bpm in comparison to ~190 for a 6-day old embryo that is still in contact with the yolk<sup>30</sup> and the reduced survival time of 2 h after this separation procedure. Another factor that can play a role in the reduced heart rate and survival time is the challenge to keep the yolk-separated embryo and CAM vessels at 37 °C. A microscope stage incubator may be of aid. In addition to this, the detachment of the CAM from the yolk likely leads to mechanical changes in the tissue since the membrane tension becomes less. The lower membrane tension may cause an increased inner vessel shear rate which leads to a lower heart rate.

The ex ovo chicken embryo and CAM vessels have some limitations as in vivo model, including short time observations only, for contrast enhanced ultrasound imaging and microbubble-mediated drug delivery studies. Due to the small blood volume of  $100 \pm 23 \mu\text{L}$  at day 5 and  $171 \pm 23 \mu\text{L}$  at day 6<sup>34</sup>, a maximum volume of ~5  $\mu\text{L}$  can be injected. In the later stages of development (day 7 and older), the vessel stiffness increases and the yolk elasticity decreases. This can complicate a successful injection in older embryos. Once the microbubbles are injected, they circulate for hours because the chicken embryo does not have a fully developed immune system at this stage<sup>35</sup>. Therefore, microbubbles are not cleared within ~6 min as in humans<sup>36, 37</sup> making typical ultrasound molecular imaging studies with a 5-10 min waiting period for non-bound targeted microbubbles to be cleared<sup>38</sup> not feasible. In order to target microbubbles, suitable ligands able to bind to avian endothelial cells need to be used such as previously described for the angiogenesis marker  $\alpha_v\beta_3$ <sup>7</sup>. Other aspects to consider for this model are the increased difficulty of separating the embryo and CAM vessels from the yolk in older embryos (> 8 days) and lower hematocrit of ~20%<sup>39</sup> in comparison to humans. The latter may affect microbubbles oscillations because it is known that microbubble oscillations are damped in a more viscous environment<sup>40</sup>. CAM arteries are less oxygenated than CAM veins<sup>41, 42</sup>. This difference should be taken into account when for example studying photoacoustic imaging of blood oxygenation.

The methods described here allow the egg content to be taken out of the eggshell on the day of the ultrasound imaging or drug delivery study, typically at day 5 to 8 of incubation. This is different to existing methods where the egg content is taken out of the shell after a 3-day incubation and further developed as ex ovo culture<sup>18, 20, 21</sup>. The advantages are a higher survival rate of 90% for 5-day, 75% for 6-day, 50% for 7-day, and 60% for 8-day old incubated eggs in comparison to ~50% for 3-day old embryos taken out of the eggshell and further incubated ex

ovo<sup>1,18</sup> the avoidance of antibiotics during culture<sup>18, 20</sup> and large sterile incubator for the ex ovo culture. The survival of the 6-to-8-day old embryos is lower because the CAM starts to attach itself to the shell<sup>21</sup> which leaves the CAM membrane more prone to rupture upon extraction. The separation of the embryo with the CAM from the yolk is also described making the embryo and CAM optically transparent.

By placing the egg content in different setups, the chicken embryo and CAM can be used for a multitude of ultrasound imaging studies, like IVUS, photoacoustic, without or with ultrasound contrast agents in 2D and 3D. The focus can be on developing new ultrasound pulsing schemes or testing out novel transducers. Besides this, the model can also be used to investigate novel ultrasound contrast agents and their behavior in blood vessels under flow. Since the mechanism of microbubble-mediated drug delivery is still unknown<sup>43</sup>, the use of the in vivo CAM model may help in elucidating the mechanism by studying the microbubble behavior in relation to the cellular response. Finally, the CAM vessels have proven to be a suitable system to investigate xenograft tumor transplantation<sup>44</sup>. This creates the possibility to use the CAM vessel as a model to investigate tumor imaging using ultrasound and to investigate the blood flow inside the tumor using CEUS. The tumors are typically grafted on the CAM vessels of 8 or 9-day old embryos<sup>1, 14, 45</sup>, for which the embryos are taken out of the eggshell at day 3 of incubation and further developed ex ovo. The methods described in this protocol could be used to grow embryos in ovo until the day of tumors grafting.

The authors trust that this paper will be helpful for researchers that want to use chicken embryos and their chorioallantoic membrane (CAM) as an in vivo model for applications of contrast agents and flow studies.

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## DISCLOSURES

The authors have nothing to disclose.

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Figure 1

[Click here to access/download;Figure;Figure 1.pdf](#)

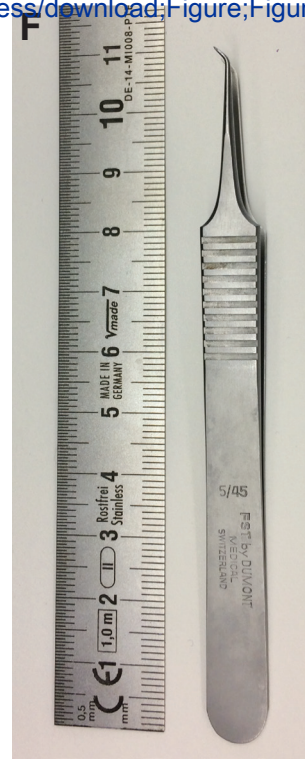
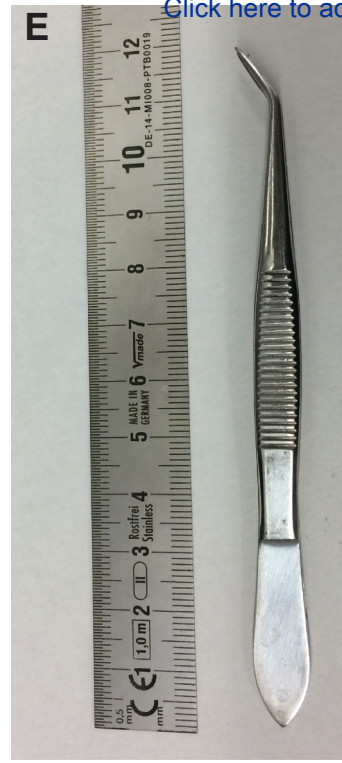
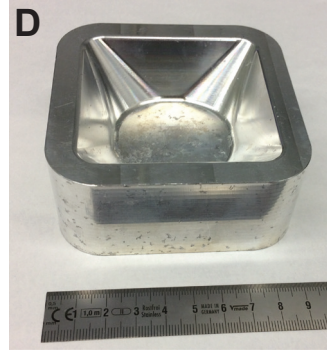
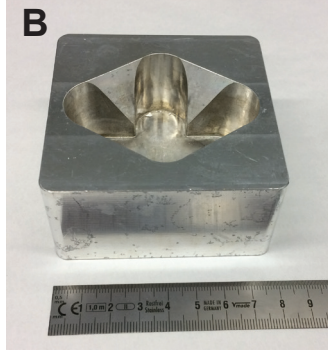
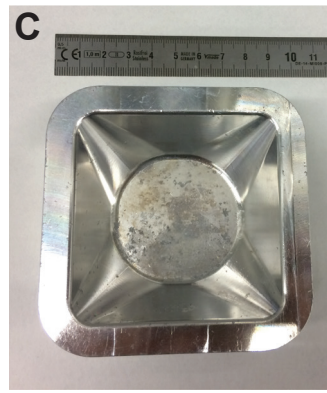




Figure 2

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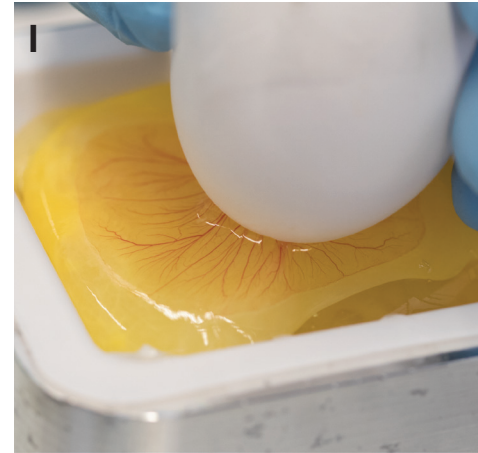
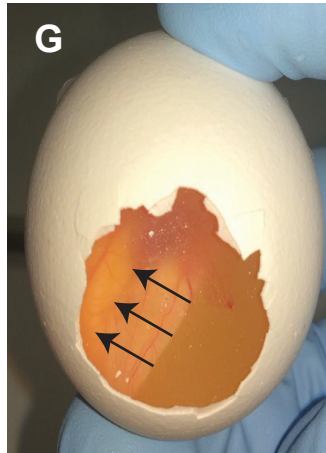
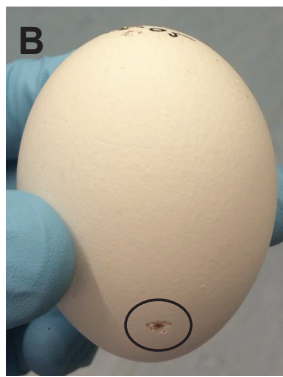
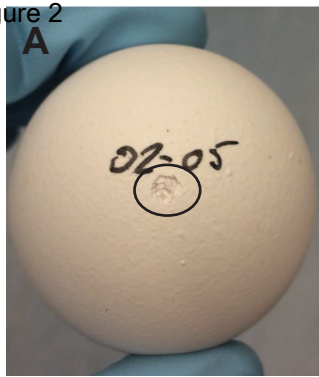


Figure 3

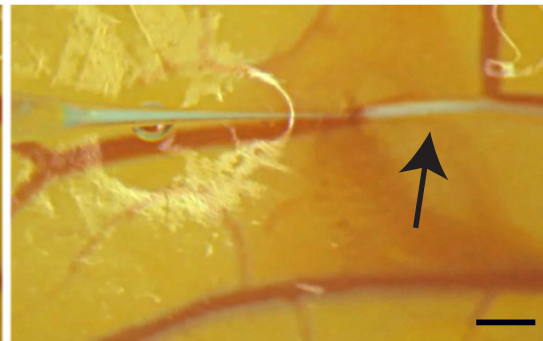
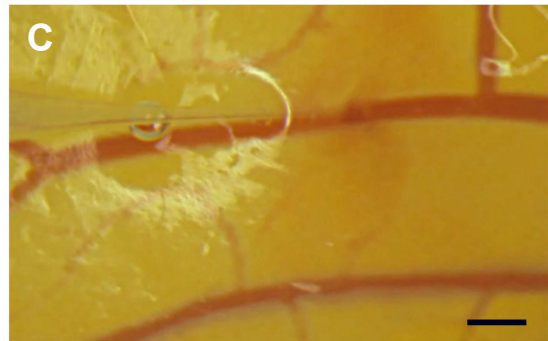
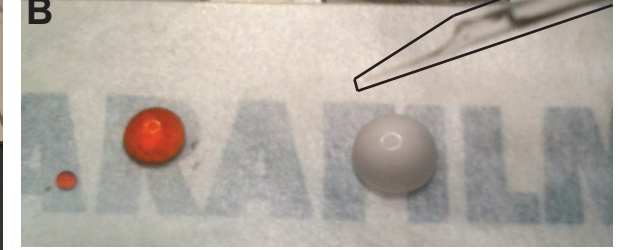


Figure 4

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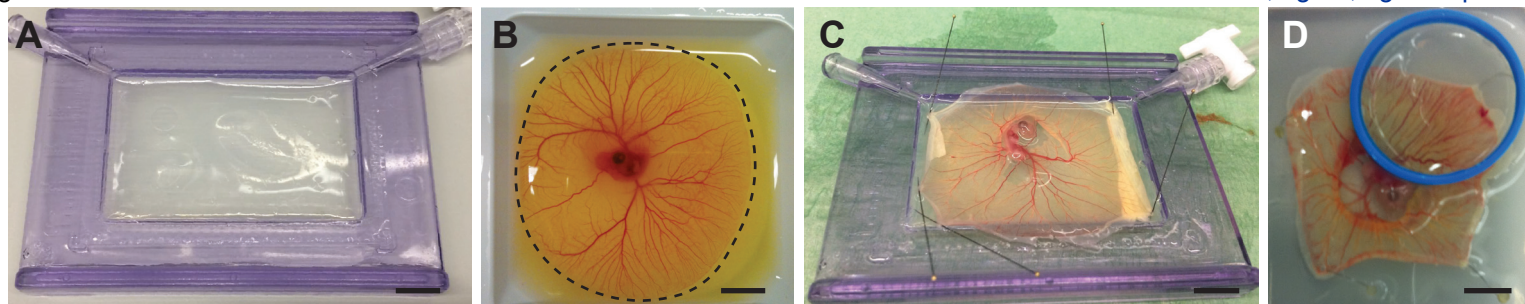
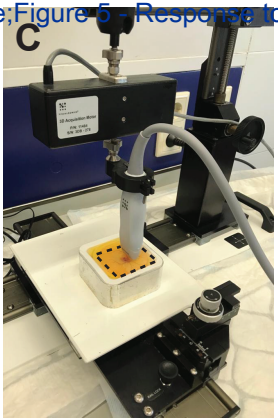
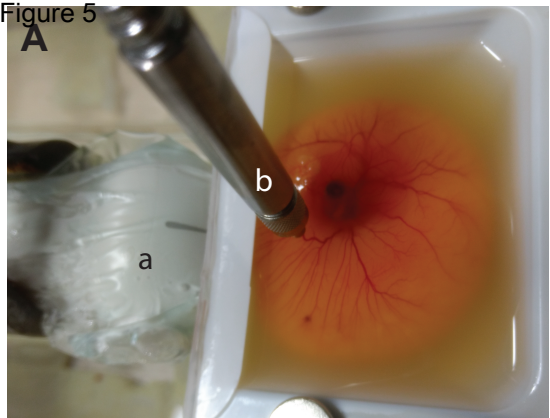


Figure 5



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Figure 6



Figure 7

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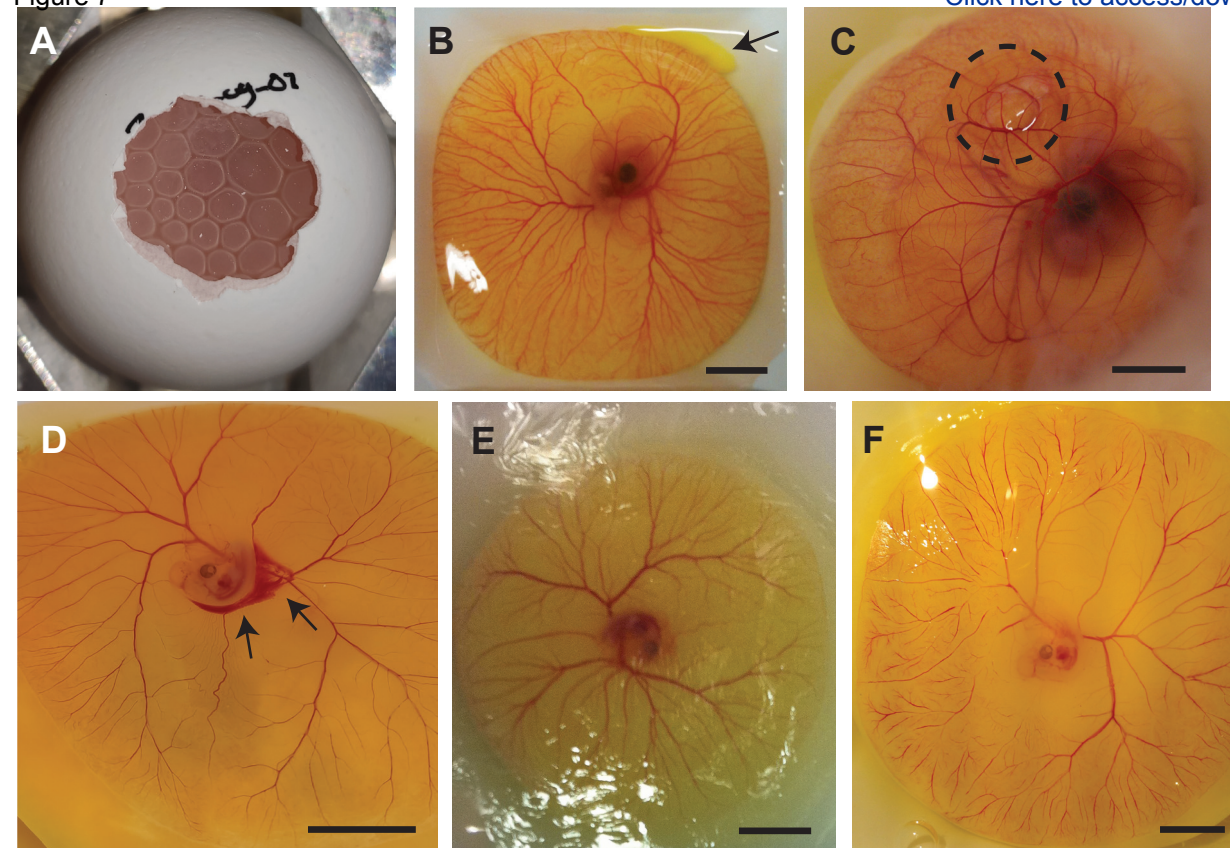
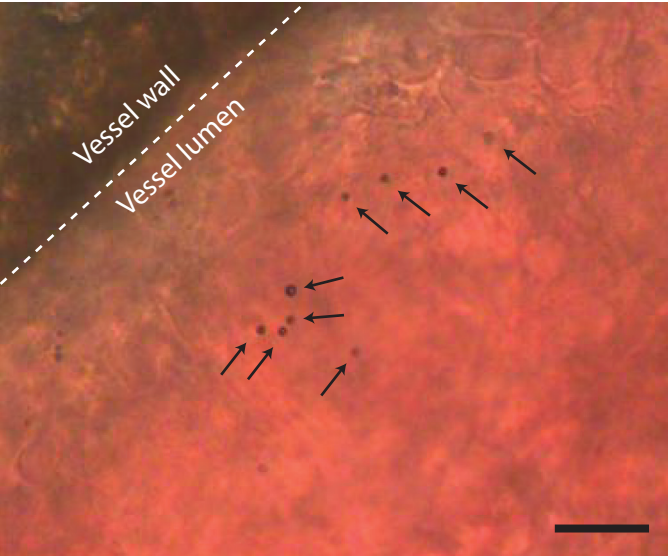


Figure 8



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Figure 9

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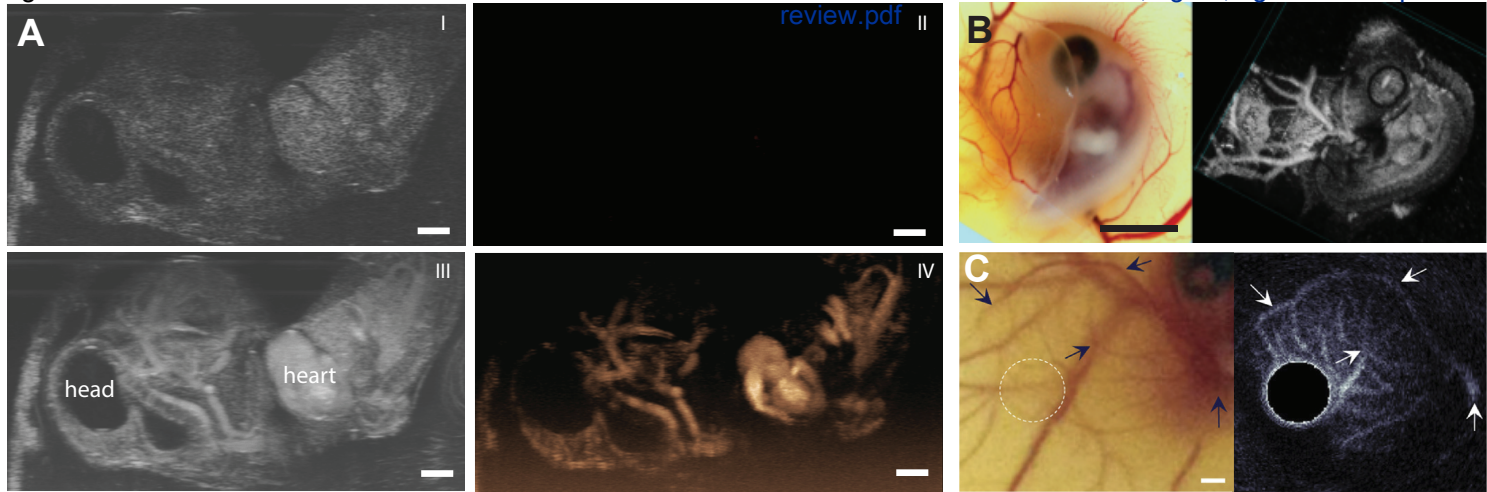
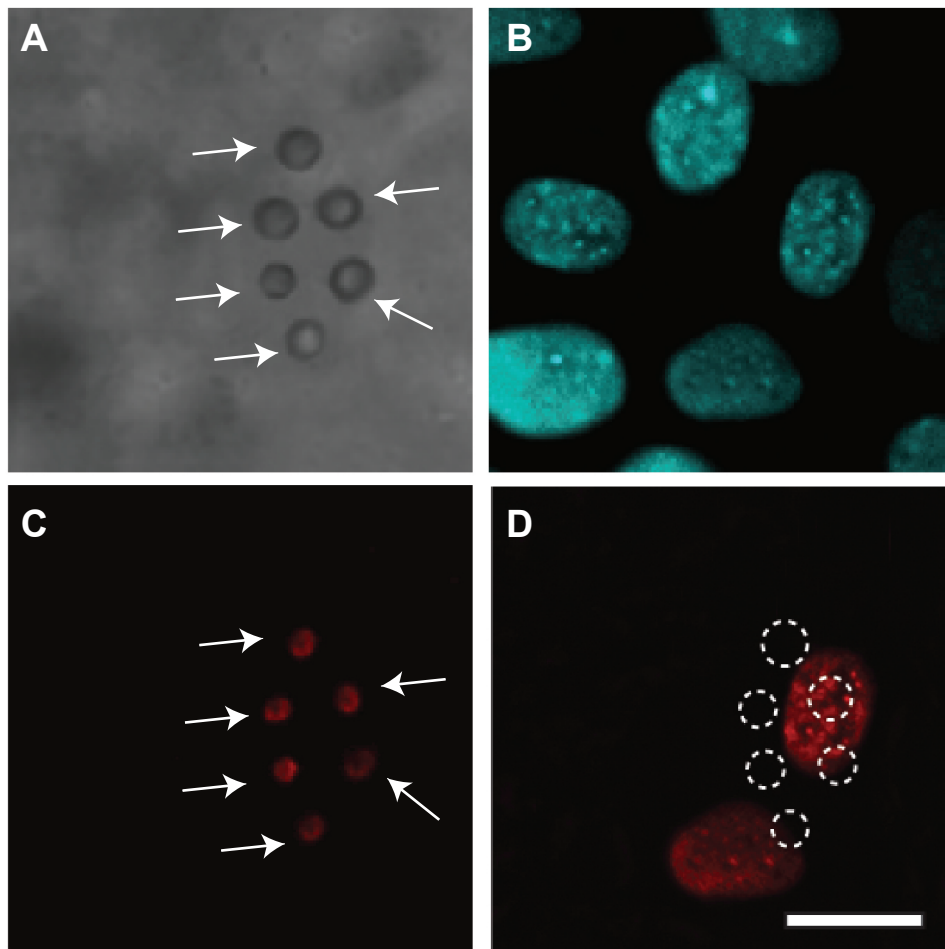
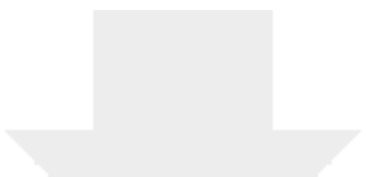




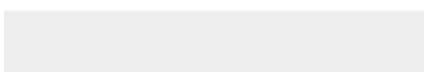
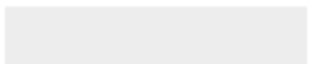
Figure 10

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**Video or Animated Figure**  
Supplemental Video 1.mp4



| <b>Name of Material/ Equipment</b>                            | <b>Company</b>                                   | <b>Catalog Number</b>      |
|---|--|----------------------------|
| Agarose   | Sigma-Aldrich                                    | A9539                      |
| Clamp (Kocher clamp)  |  |                            |
| Cling film  |  |                            |
| Holder with acoustic membrane (CLINicell 25 cm <sup>2</sup> ) | MABIO, Tourcoing, France                         | CLINicell25-50-T FER 00106 |
| Demi water  |  |                            |
| Disposable plastic Pasteur pipets                             | VWR  | 612-1747                   |
| Eggs  | Drost Pluimveebedrijf Loenen BV, the Netherlands |                            |
| Fridge 15 °C  |  |                            |
| Glass capillary needles                                       | Drummond   | 1-000-1000                 |
| Heating plate 37 °C   |  |                            |
| Humidified incubator 37 °C                                    |  |                            |
| Insect specimen pins  |  |                            |
| Metal egg holder  |  |                            |
| Metal weighing boat holder                                    |  |                            |
| Microinjection system   | FUJIFILM VisualSonics                            |                            |
| Mineral oil   | Sigma-Aldrich                                    | M8410-100ML                |
| Needle, 19 G  | VWR (TERUMO)                                     | 613-5392                   |
| Phosphate-buffered saline (PBS), 1x                           | ThermoFisher                                     | 10010023                   |
| Petri dish, 1 L   |  |                            |
| Petri dish, 90 mm diameter                                    | VWR  | 391-0559                   |
| Preclinical animal ultrasound machine (Vevo 2100)             | FUJIFILM VisualSonics                            |                            |
| Probe (MS250)   | FUJIFILM VisualSonics                            |                            |
| Probe (MS550s)  | FUJIFILM VisualSonics                            |                            |
| Scalpel   | VWR (SWANN-MORTON)                               | 233-5363                   |
| Scissors, small   | Fine Science Tools (FST)                         | 14558-09                   |
| Syringe, 5 mL   | VWR (TERUMO)                                     | 613-0973                   |
| Table spoon   |  |                            |
| Tape (Scotch Magic tape)                                      | Scotch   |                            |
| Tissue paper  | Tork   |                            |
| Tweezers large  | VWR (USBECK Laborgeräte)                         | 232-0107                   |

|  |   |           |
|--|---|-----------|
| Tweezers small                                 | DUMONT Medical, Switzerland             | 0103-5/45 |
| Ultrasound contrast agent (custom made F-type) |   |           |
| Ultrasound contrast agent (MicroMarker)        | FUJIFILM VisualSonics, Inc.             |           |
| Ultrasound contrast agent (Definity)           | Lantheus medical imaging, United States |           |
| Ultrasound gel                                 | Aquasonic                               |           |
| Waxi film (Parafilm)                           | Parafilm                                |           |
| Weighing boats (85 × 85 × 24 mm)               | VWR                                     | 611-0094  |

## Comments/Description

Freshly fertilized

Inside diameter: 0.0413 inch

Custom made by Experimental Medical Instrumentation at Erasmus MC. See figure 1 A,B  
Custom made by Experimental Medical Instrumentation at Erasmus MC. See figure 1 C,D

Glass

30 MHz transmit and 15 MHz receive frequency  
transmission frequency of 40 MHz

See figure 1E

See figure 1F

Produced as described by: Daeichin, V. et al. Microbubble Composition and Preparation for Imaging : In Vitro and In Vivo Evaluation. IET



IEEE TRANSACTIONS ON ULTRASONICS. 64 (3), 555–567 (2017).



**Manuscript Reference Number: JoVE62076R1**

**Title: The preparation of chicken *ex ovo* embryos and chorioallantoic membrane vessels as *in vivo* model for contrast-enhanced ultrasound imaging and microbubble-mediated drug delivery studies**

**Authors: Bram Meijlink, Ilya Skachkov, Antonius F.W. van der Steen, Nico de Jong, Klazina Kooiman.**

*The authors thank the editor and reviewers for carefully reviewing the manuscript and providing constructive feedback, which has helped the authors improve the manuscript. We have revised the manuscript accordingly. Please find a point-by-point address to all reviewers' comments below. The reviewer's comments are listed in grey below, with our responses in black italic text. Corresponding changes in the original manuscript text have been highlighted in grey. All page and line numbers in the responses refer to the revised manuscript.*

**Response to editorial comments:**

**Editorial comments:**

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

**Response:** *We carefully and thoroughly went through the manuscript and have removed multiple spelling or grammar issues from the text. For example:*

*Revised text:*

Line 36: "...new ultrasound pulsing schemes, or novel..."

Line 65: "...ultrasound imaging (CEUS)<sup>11-16</sup>. In addition, the model ..."

Line 75: "...content out of the shell<sup>18, 20, 21</sup>. However, to ..."

Line 86: "...three methods to take the egg content out ..."

Line 92: "...and labor. We also detail a method to separate ..."

Line 118: "Warm up a heating plate to 37 °C."

Line 178: "...bottom after 2 min, turn the egg ..."

Line 192 & 193: "...Put one side of the tissue paper on the embryo, drag the tissue paper to..."

Line 195: "...Visually check whether the embryo ..."

Line 211: "...position and leave for another 1 h ..."

Line 243: "Warm up a heating plate to 37 °C."

Line 255: "...and leave for another 1 h ..."

Line 265: "...egg in the 37 °C PBS and keep it submerged for 5 min. After 5..."

Line 268 & 269: "...pull the two egg shell pieces apart ..."

Line 270: "Then, gently drop the egg content into the weighing boat."

Line 277 & 278: "...weighing boat holder and move to the desired ..."

Line 331: "...forward along the vessel to reposition the needle."

Line 416: "...added to the top of the embryo or CAM vessels, like a..."

Line 486: "...the visibility of the embryo and ..."

Line 490 – 494: "...performing an injection (figure 7D). Besides this, the embryo and vessels can also dry out over time (figure 7E). This occurs when the egg content is not sprinkled with PBS. The drying out of the embryo can result in massive capillary obstructions (figure 7F) which ..."

Line 527: "...explained in section 2.2 by separating ..."

Line 593: "...black dotted circle (7-day old embryo). **(D)** Bleeding ..."

Line 622 & 623: "...treatment. **(B)** Endothelial cell nuclei fluorescently stained before ultrasound treatment. **(C)** Fluorescent image of the stained targeted microbubbles ..."

Line 638: "...withdrawing egg white; 2) create ..."

Line 641: "...along the egg; 2) Keep the egg ..."

*Removed text:*

Line 209, Removed: "... (144-168 h)..."; New text: "...experiment, take a 6 to 7-day old incubated egg and rotate..."

Line 253, Removed: "... (192 h)..."; New text: "...take an 8-day old incubated egg ..."

Line 258, Removed: "... (192 h)..."; New text: "...an 8-day old incubated ..."

Line 306, Removed: "...be...". New text: "...microbubbles will concentrate..."

Line 635, Removed: "...chorioallantoic membrane ...". New text: "...their CAM as an *in vivo* ..."

Line 706, Removed: "...to..."; New text: "...CAM can be used for a ..."

*References:*

11. Daeichin, V., Bosch, J.G., Needles, A., Foster, F.S., van der Steen, A., de Jong, N. Subharmonic, non-linear fundamental and ultraharmonic imaging of microbubble contrast at high frequencies. *Ultrasound in Medicine and Biology*. **41** (2), 486–497 (2015).
12. Maresca, D. *et al.* Imaging microvasculature with contrast-enhanced ultraharmonic ultrasound. *Ultrasound in Medicine and Biology*. **40** (6), 1318–1328 (2014).
18. Yalcin, H.C., Shekhar, A., Rane, A.A., Butcher, J.T. An ex-ovo Chicken Embryo Culture System Suitable for Imaging and Microsurgery Applications. *Journal of Visualized Experiments*. (44), 4–7 (2010).
20. Cloney, K., Franz-Odenaal, T.A. Optimized Ex-ovo Culturing of Chick Embryos to Advanced Stages of Development. *Journal of Visualized Experiments*. (95), 6–10 (2015).
21. Dohle, D.S. *et al.* Chick ex ovo Culture and ex ovo CAM Assay: How it Really Works. *Journal of Visualized Experiments*. (33), 2–7 (2010).

*Added references:*

13. Lindsey, B.D. *et al.* High Resolution Ultrasound Superharmonic Perfusion Imaging: In Vivo Feasibility and Quantification of Dynamic Contrast-Enhanced Acoustic Angiography. *Annals of Biomedical Engineering*. **45** (4), 939–948 (2017).
14. Paproski, R.J., Jovel, J., Wong, G.K.S., Lewis, J.D., Zemp, R.J. Enhanced detection of cancer biomarkers in blood-borne extracellular vesicles using nanodroplets and focused ultrasound. *Cancer Research*. **77** (1), 3–13 (2017).
15. Huang, C. *et al.* Short Acquisition Time Super-Resolution Ultrasound Microvessel Imaging via Microbubble Separation. *Scientific Reports*. **10**, 1–13 (2020).
16. Lowerison, M.R., Huang, C., Kim, Y., Lucien, F., Chen, S., Song, P. In Vivo Confocal Imaging of Fluorescently Labeled Microbubbles: Implications for Ultrasound Localization Microscopy. *IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control*. **67** (9), 1811–1819 (2020).

2. Please avoid abbreviations in your title: Contrast-Enhanced Ultrasound Imaging and Microbubble-Mediated Drug Delivery in Chicken Ex Ovo Embryo and Chorioallantoic Membrane Vessels

**Response:** *We have changed the abbreviation 'CAM' to 'chorioallantoic membrane'. Following the suggestion of reviewer 2, we have also added 'preparation of' to the title. The new title is:*

*Revised text:*

Title: "The preparation of chicken ex ovo embryos and chorioallantoic membrane vessels as in vivo model for contrast-enhanced ultrasound imaging and microbubble-mediated drug delivery studies"

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (<sup>™</sup>), registered symbols (<sup>®</sup>), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: CLINicell; MicroMarker (VisualSonics); Vevo 2100 (VisualSonics); etc

**Response:** *We have changed all the words 'Vevo 2100' to 'preclinical animal ultrasound machine'. We have removed all the words 'MicroMarker', 'Definity', 'Kocher', 'parafilm', and 'scotch' (as in scotch tape). We have changed all the words 'CLINicell' to 'holder with acoustic membrane' and introduced the description of it as follows:*

*Revised text:*

Line 346 – 348: "Take a cell culture chamber consisting of a square plastic holder with two parallel 50  $\mu$ m thick acoustically transparent polycarbonate membranes<sup>26</sup>, further referred to as holder with acoustic membrane. Close both ports with a lid."

*Reference:*

26. Beekers, I., van Rooij, T., van der Steen, A.F.W., de Jong, N., Verweij, M.D., Kooiman, K. Acoustic characterization of the CLINicell for ultrasound contrast agent studies. *IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control*. **66** (1), 244–246 (2019).

4. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

**Response:** *We have added more details in the protocol and have added more references on the injection.*

*Revised text:*

Line 136: "Use the pointy back of the tweezers to make a second indent..."

Line 153 & 154: "NOTE 1: Make sure the needle is pointing down towards the bottom of the egg to prevent the chance of damaging the embryo and CAM. This step creates..."

Line 184: "...the yolk sack. Continue until the egg bulges out of the bottom opening."

Line 185: "...holding the bottom of the egg close to the ..."

Line 195: "Visually check whether the embryo is alive and CAM is intact..."

Line 227: "...PBS than the withdrawn volume during step ..."

Line 233: "...and quickly reseal the gap with a piece of tape (figure 2D)..."

Line 264: "...small indents use the pointy back of the large tweezer (figure 1E)..."

Line 294: "...mixed before pipetting<sup>7</sup>."

Line 337: "...glass needle is positioned correctly<sup>17</sup>."

Line 378: "...from the weighing boat and visually inspect..."

Line 531: "... $\alpha_v\beta_3$ -antibodies and stained with the fluorescent dye Dil<sup>7</sup>..."

Line 533 & 534 - "...was used to visualize sonoporation<sup>7</sup>. Both these..."

#### References:

7. Skachkov, I., Luan, Y., van der Steen, A.F.W., De Jong, N., Kooiman, K. Targeted microbubble mediated sonoporation of endothelial cells in vivo. *IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control*. **61** (10), 1661–1667 (2014).
17. Faez, T., Skachkov, I., Versluis, M., Kooiman, K., de Jong, N. In Vivo Characterization of Ultrasound Contrast Agents: Microbubble Spectroscopy in a Chicken Embryo. *Ultrasound in Medicine and Biology*. **38** (9), 1608–1617 (2012).

5. In the protocol, please include some of the steps mentioned in the representative results; if these will not be included in the video, you could cite references to help readers replicate the protocol: injecting fluorescent dye Dil, Hoechst 33342, PI,  $\alpha_v\beta_3$ -antibodies

**Response:** We have added more references on how to prepare the solution before the injection and how to correctly position the needle for injection. Besides this, we have added an extra reference on how to prepare stained and  $\alpha_v\beta_3$ -antibody targeted microbubbles and how to inject fluorescent dyes (like Hoechst 33342 and PI) into the CAM vessels.

#### Revised text:

Line 294: "...mixed before pipetting<sup>7</sup>."

Line 337: "...glass needle is positioned correctly<sup>17</sup>."

Line 531: "... $\alpha_v\beta_3$ -antibodies and stained with the fluorescent dye Dil<sup>7</sup>..."

Line 533 & 534: "...was used to visualize sonoporation<sup>7</sup>. Both these..."

#### References:

7. Skachkov, I., Luan, Y., van der Steen, A.F.W., De Jong, N., Kooiman, K. Targeted microbubble mediated sonoporation of endothelial cells in vivo. *IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control*. **61** (10), 1661–1667 (2014).
17. Faez, T., Skachkov, I., Versluis, M., Kooiman, K., de Jong, N. In Vivo Characterization of Ultrasound Contrast Agents: Microbubble Spectroscopy in a Chicken Embryo. *Ultrasound in Medicine and Biology*. **38** (9), 1608–1617 (2012).

6. After including a one line space between each protocol step, make sure that up to 3 pages of protocol text are highlighted for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

**Response:** *By adding some extra 'how to' steps as suggested in point 4, we have had to delete some text and replaced some other text into a note to make sure the yellow highlighted text in the protocol steps fit onto 3 pages.*

*Moved to note:*

Line 151 & 152, Moved and rephrased to note: "Make sure the needle is pointing down towards the bottom of the egg to prevent the chance of the embryo and CAM getting damaged."

New note, Line 153-154: "NOTE 1: Make sure the needle is pointing down towards the bottom of the egg to prevent the chance of damaging the embryo and CAM. This step creates..."

Line 165 – 168, Moved to note: "...Make sure the edge of the opening in the shell does not go lower than the CAM. Besides this, do not penetrate the inner membrane and prevent sharp edges."

New note, Line 169 – 172: "NOTE: Keep on moving the egg to maintain maximum visibility on the position of the embryo and CAM inside the shell. Make sure the edge of the opening in the shell does not go lower than the CAM. Besides this, do not penetrate the inner membrane and prevent sharp edges."

Line 185 – 188, Moved to note: "...If the egg content does not come out, use the needle on the syringe to puncture the side opening again with the needle pointing upwards."

New note, Line 189 & 190: "NOTE: If the egg content does not come out, use the needle on the syringe to puncture the side opening again with the needle pointing upwards."

*Removed text:*

Line 130, Removed: "... (120 h) ..."; New text: "...a 5-day old incubated ..."

Line 130, Removed and replaced: "...the egg..."; New text: "...and transfer it to the..."

Line 131, Removed: "...during transfer..."; New text: "...same orientation with the date ..."

Line 164, Removed: "...top of the..."; New text: "...egg white to the ultrasound gel ..."

Line 188, Removed: "Keep the bottom of the egg close to weighing boat so the egg content...";

New text: "... (figure 1F) and gently drop the egg content into the weighing ..."

Line 218, Removed "to prevent the chance of the embryo and CAM getting damaged." New text: "...bottom of the egg."

Line 222, Removed: "...top of the..."; New text: "...egg white to the ultrasound gel ..."

Line 276: Removed: "...from the weighing boat."; New text: "...excess PBS."

7. Please sort the Materials Table alphabetically by the name of the material.

**Response:** *We have put the Materials Table in alphabetical order.*

**Response to reviewer 1:**

**Reviewer #1:**

#### Manuscript Summary:

Overall the manuscript was well written and easy to follow, particularly the protocol steps. As the authors correctly state, this manuscript would prove useful for researchers looking to utilize chick embryo and CAM as an in vivo model. The figures also did a good job to complement the text. There were only a few discussion points to expand upon or offer better clarity on (detailed under Major Concerns) as well as minor proofreading changes to make (detailed under Minor Concerns).

#### Major Concerns:

1. Regarding protocol step 1.2.2.3, if a hole is accidentally created and necessarily sealed with scotch tape, should another indentation be made instead? Step 1.2.2.5 utilizes this indent for the egg white withdrawal. If the accidental hole was made, this needle stick could be accomplished by puncturing the scotch tape, or it could be accomplished through any other indent made following 1.2.2.3. Any way should work as long as the egg white is withdrawn effectively and the penetrated shell is sealed again afterward. Is there any insight the authors could offer regarding this situation? Would the authors recommend another indentation or using the accidental (sealed) hole?

**Response:** *No, if in step 1.2.2.3 a hole is accidentally created instead of an indent, another indent should not be made instead. In step 1.2.2.5 the same accidental hole sealed with scotch tape could indeed be used to withdraw the egg white by sticking the needle through scotch tape and then resealing the hole with scotch tape. We have included a note to step 1.2.2.5. to make this clear:*

Added text:

Line 138 & 139: "... the indent too big and create a hole. If by accident a hole is created, seal the hole with tape and do not make another indent."

Line 153 - 158: "NOTE 1: Make sure the needle is pointing down towards the bottom of the egg to prevent the chance of damaging the embryo and CAM. This step creates..."

NOTE 2: If you accidentally created a hole instead of an indent in step 1.2.2.3, puncture the tape with the needle for the withdrawal of the egg white. Reseal the puncture with another piece of tape."

2. For step 1.4.2.3, at what height should the small indents be made? Other indentation steps included this information (top or 2/3 down the egg, for example). And for clarification, by "entire width", does this mean only one 180 deg of the egg shell is indented, not an entire ring of 360 deg? A visual depiction in a figure might be helpful here.

**Response:** *The small indents should be made at 1/2 down the egg. We meant that the small indents should be made in a ring of 360° around the egg. We have incorporated this into the text of step 1.4.2.3. We did not include a visual depiction in a figure since this will become clear from the video:*

Revised text:

Line 259 – 261: "Hold the egg horizontally and use the pointy back of the large tweezer (figure 1E) to make a small indent 1/2 down the egg. Continue making small indents in a ring pattern 360° round the egg shell. Use a spacing of ~10 mm between the indents."

3. Microbubble concentration tends to be a very important aspect of an experiment to report. How do the waiting/concentration steps of 2.1.1.4 and 2.1.1.5 affect the concentration of the solution? As in, for a given prepared concentration of solution placed on the parafilm, what tends to be the resulting concentration within the glass tip needle tip? Further, if possible, could the authors comment on what working concentration this translates to post-injection, within the vasculature?

**Response:** *The waiting/concentration steps increase the concentration of microbubbles with ~30%. We experimentally determined this for the F-type custom-made ultrasound contrast agent (Daeickin et al. 2017) by using a Multisizer 3 to assess the microbubble concentration. The in-vial concentration was around  $6,0 \times 10^8$  microbubbles / mL. After the waiting/concentration steps of 2.1.1.4 and 2.1.1.5, the concentration was increased to around  $8,0 \times 10^8$  microbubbles / mL. The concentrations in the embryo is dependent on total blood volume of the chicken embryo which are described in the discussion in line 664 & 665. Once injected into the embryo, the working concentration in the blood will be between  $32 \times 10^3$  microbubbles /  $\mu\text{L}$  for 5-day old embryos and  $19 \times 10^3$  microbubbles /  $\mu\text{L}$  for 6-day old embryos. We have added these numbers into the text:*

*Revised text:*

Line 297- 300: "...For the F-type custom-made ultrasound contrast agent<sup>28</sup>, this concentration step will increase the to-be-injected microbubble concentration with ~30%. The post-injection concentration in the chicken embryo blood will be between  $32 \times 10^3$  microbubbles /  $\mu\text{L}$  for 5-day old embryos and  $19 \times 10^3$  microbubbles /  $\mu\text{L}$  for 6-day old embryos."

*Added reference:*

28. Daeichin, V. et al. Microbubble Composition and Preparation for Imaging: In Vitro and In Vivo Evaluation. *IEEE TRANSACTIONS ON ULTRASONICS*. **64** (3), 555–567 (2017).

4. Step 2.2.1.4 mentions cooling down the agarose/demi water solution to 37 deg C but step 2.2.1.3 does not state what the solution was originally heated to. Could the authors offer a temperature range used in the solution preparation step?

**Response:** *We have included the temperature range to which the solution was original heated to in step 2.2.1.3. Since the agarose preparation is also described in step 2.3.3, we have also added the information of the temperatures in steps 2.3.3.2:*

*Revised text 2.2.1.3:*

Line 352 – 356: "Prepare ~15 mL of 2% agarose in demi water solution by heating to between 80-95 °C in a small glass beaker. Cool the glass beaker with the dissolved agarose solution under a running cold water tab.

NOTE: If the agarose is too hot it will melt the acoustic membrane which will create an uneven surface.

*Revised text 2.3.3.2:*

Line 440 – 442: "2. Prepare a 2% agarose solution in demi water by heating the solution up to between 80-95 °C in a small glass beaker. Cool the glass beaker with the dissolved agarose solution under a running cold water tab."

5. Protocol 2.3 detailing all the ultrasound imaging all reference acquiring the egg content by section 1.3. Is it correct to understand that to mean these techniques can only be applied to 6-7 day old embryos? In contrast, section 2.2.2.1 explicitly states that the egg content can be removed by section 1.2, 1.3, or 1.4. If it is true that 2.3 only applies to 6-7 day old embryos, could the authors comment on why this is the case?

**Response:** *All the ultrasound imaging methods described in the protocol in 2.3 can be used with embryos taken out with the methods described in section 1.2, 1.3, or 1.4 and we have adjusted the text to reflect this:*

*Revised text:*

Line 406: "...as described in section 1.2, 1.3, or 1.4. However, do..."

Line 427: "...described in section 1.2, 1.3, or 1.4."

Line 439: "...as described in section 1.2, 1.3, or 1.4."

6. For this embryo/CAM model overall, what vessel sizes could be expected, if known? How does this size distribution change, if at all, from 5 days versus 8 days old?

**Response:** *The overall diameter size of the small capillaries is between 10-15  $\mu\text{m}$  (Ribatti et al. 2001). The diameter size of the larger vessels is between 115-136  $\mu\text{m}$  (Ribatti et al. 2001; DeFouw et al. 1989). We have implemented this in manuscript:*

*Revised text:*

Line 88 - 90: "...day of the experiment. The CAM vessel size ranges from 10-15  $\mu\text{m}$  in diameter, in the smaller capillaries of an 8-day old embryo<sup>24</sup> to 115-136  $\mu\text{m}$  in diameter in the larger vessel of 6 and 8-day old embryos<sup>24, 25</sup> The three described methods only require..."

*Added references:*

24. Ribatti, D., Nico, B., Vacca, A., Roncali, L., Burri, P.H., Djonov, V. Chorioallantoic Membrane Capillary Bed: A Useful Target for Studying Angiogenesis and Anti-Angiogenesis In Vivo. *Anatomical Record*. **324**, 317–324 (2001).
25. DeFouw, D.O., Rizzo, V.J., Steinfeld, R., Feinberg, R.N. Mapping of the Microcirculation in the Chick Chorioallantoic Membrane during Normal Angiogenesis. *Microvascular research*. **38**, 136–147 (1989).

*We have extensively searched the literature and to the best of our knowledge the vessel size distribution change is not described for 5-day to 8-day old embryos.*

*Minor Concerns:*

7. Line 134 - "... the indent too\* big and..."
8. Line 138 - "... penetrate the shell too\* deep."
9. Line 206 - the only Note not stylized NOTE\*
10. Line 218 - "... as possible but make\* sure the edge..."



11. Line 254 - "... procedure, small cracks\* can start to..."
12. Line 259 - "...the large hole\* and gently open..."
13. Line 277 - "... beveled, break off\* a small part..."
14. Line 337 - "... agarose is too\* hot it will..."
- 15, Line 644 - "... reduced heart\* rate and survival..."

**Response to minor concerns:** *We have incorporated all the suggested changes.*

**Response to reviewer 2:**

**Reviewer #2:**

Manuscript Summary:

This manuscript described three methods for taking the content out of the egg shell between day 5 to 8 of incubation, how to inject cavitation nuclei into the CAM vascular system, how to separate the membrane containing the embryo and CAM from the rest of the egg content for optically transparent studies, and how to use the chicken embryo and CAM in a variety of short-term ultrasound experiments. The step-by-step protocol is clear to understand. The article is well-written and well-organized.

Major Concerns:

NA

Minor Concerns:

1. Suggestion: adding words like "preparation" into the title, for instance, "The preparation of chicken ex ovo embryo and CAM vessels as in vivo model..."

**Response:** *We agree with the reviewer and have incorporated the word 'preparation' into the title. We have also changed the abbreviation 'CAM' to 'chorioallantoic membrane' according to the suggestion of the editor. The new title is:*

*Revised text:*

Title: "The preparation of chicken ex ovo embryos and chorioallantoic membrane vessels as in vivo model for contrast-enhanced ultrasound imaging and microbubble-mediated drug delivery studies"

2. Please indicate the full version for "HH" when using it for the first time.

**Response:** *We have changed the first HH in line 77 and 78 to Hamburger & Hamilton.*

*Revised text:*

Line 77: "... after 3 days of incubation, i.e. Hamburger & Hamilton (HH) stage 19-20<sup>22</sup>, and continue the culture ex ovo ..."

*Reference:*

22. Hamburger, V., Hamilton, H. A Series Of Normal Stages In The Development Of The Chick Embryo. *Journal of Morphology*. **88** (1), 231–272 (1951).

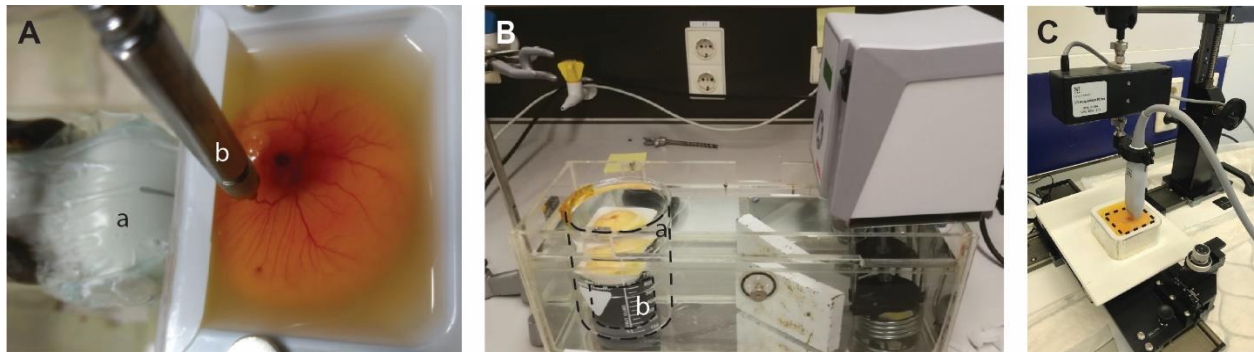
3. Please indicate in Figure 5B which is the 2L beaker and which is the 500mL beaker. It's hard to tell from the image.

**Response:** We have indicated the 2 L and 500 mL beaker using dashed lines in Figure 5B. We have also incorporated the small letter a & b to make a distinction between the two beakers.

*Revised text:*

Line 574 & 575: "... water bath. Dashed outline shows the 2 L glass beaker (a) with the 500 mL glass beaker (b) inside. (C) Setup ..."

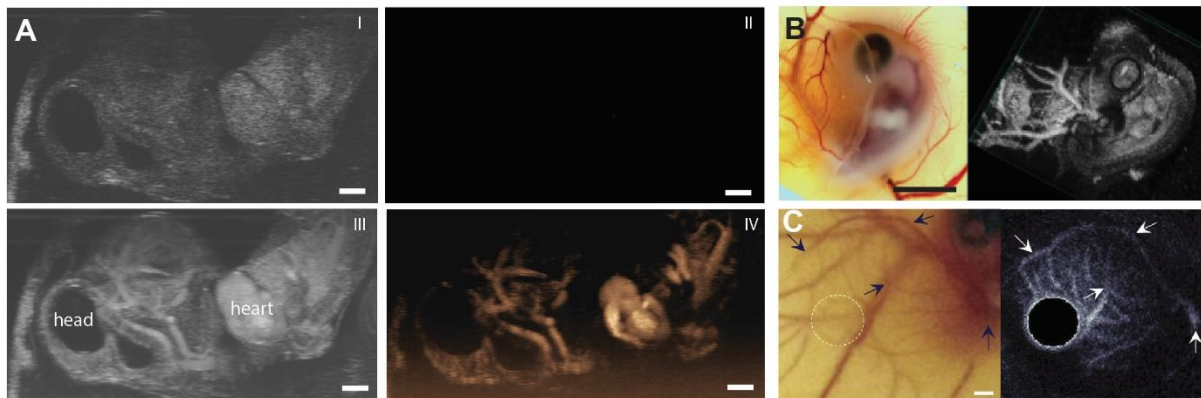
*Revised image:*



4. What do the arrows indicate in Figure 9?

**Response:** The arrows in figure 9A were meant to indicate some vessels. The arrows in figure 9C indicate corresponding vessels in the two images of figure 9C. Since the use of arrows can be confusing between the A and B images, we have removed the arrows in figure A, also because there are more vessels present in the image than indicated with the arrows.

*Revised image:*



**Response to reviewer 3:**

**Reviewer #3:**

Manuscript Summary:

The manuscript, entitled: "The chicken ex ovo embryo and CAM vessels as in vivo model for contrast-enhanced ultrasound imaging and microbubble-mediated drug delivery studies," is an interesting manuscript to demonstrate how chicken embryo can be used in ultrasound imaging research. As a manuscript which is written particularly for the method, the manuscript is clear and easy to follow. I have some minor editing errors as shown below.

**Major Concerns:**

it is puzzling to me that how the method will be useful for general ultrasound imaging. Researchers in the field of microscopy may be a better audience. Particularly, high resolution microscopy (such as photoacoustic microscopy) for microvessel imaging field may find the method more attractive than field of contrast-enhanced ultrasound imaging and drug delivery. You may get a little more interest there although the overall interest from imaging field will not thrilling.

**Response:** *We are convinced the chicken embryo model is useful for contrast-enhanced ultrasound imaging and drug delivery studies because we and others in the field have been and are using this in vivo model. At the moment we are working on two manuscripts for which this model was detrimental for the ultrasound imaging research questions we had. After having been approached by three different internally renowned ultrasound imaging groups to share our expertise on this model and teach them how to work with this model, we took the effort to write this manuscript so others can benefit from it too. Also, we now have incorporated more references of published contrast-enhanced ultrasound imaging articles using the chicken embryo model into our manuscript to reflect the broad interest in this model.*

*Revised text:*

Line 74: "...and 14 to 18-day old embryos<sup>13-16</sup>. Multiple ..."

*Added references:*

13. Lindsey, B.D. *et al.* High Resolution Ultrasound Superharmonic Perfusion Imaging: In Vivo Feasibility and Quantification of Dynamic Contrast-Enhanced Acoustic Angiography. *Annals of Biomedical Engineering*. **45** (4), 939–948 (2017).
14. Paproski, R.J., Jovel, J., Wong, G.K.S., Lewis, J.D., Zemp, R.J. Enhanced detection of cancer biomarkers in blood-borne extracellular vesicles using nanodroplets and focused ultrasound. *Cancer Research*. **77** (1), 3–13 (2017).
15. Huang, C. *et al.* Short Acquisition Time Super-Resolution Ultrasound Microvessel Imaging via Microbubble Separation. *Scientific Reports*. **10**, 1–13 (2020).
16. Lowerison, M.R., Huang, C., Kim, Y., Lucien, F., Chen, S., Song, P. In Vivo Confocal Imaging of Fluorescently Labeled Microbubbles: Implications for Ultrasound Localization Microscopy. *IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control*. **67** (9), 1811–1819 (2020).

**Minor Concerns:**

Line 138: "to deep"

Line 337: "to hot"

Line 423: "pour the agarose..."

Line 431: "example for"

**Response:** *We have incorporated the suggested changes.*

*Revised text:*

Line 142: "...too deep..."

Line 355: "...too hot..."

Line 443: "Pour the agarose..."

Line 451: "...for example..."

Line 431: not sure what is a 3D scanning? How are you going to do a 3D scanning

**Response:** *We have modified the text and added a reference to clarify 3D scanning:*

*Revised text:*

Line 451 & 452: "...when, for example, using a motor to mechanically move a two-dimensional transducer to make a three-dimensional scan (**Figure 9B**)<sup>11</sup>. When the ..."

*Reference:*

11. Daeichin, V., Bosch, J.G., Needles, A., Foster, F.S., van der Steen, A., de Jong, N. Subharmonic, non-linear fundamental and ultraharmonic imaging of microbubble contrast at high frequencies. *Ultrasound in Medicine and Biology*. **41** (2), 486–497 (2015).

#### **Response to reviewer 4:**

##### **Reviewer #4:**

Manuscript Summary:

This manuscript describes three techniques for removing the egg contents from fertilized chicken eggs at different stages of development (5-day, 6- to 7-day, and 8-day old) while keeping the chorioallantoic membrane (CAM) intact, along with a variety of techniques suited to contrast-enhanced ultrasound and optical-acoustic imaging of the chicken embryo. Specifically, the authors describe a microinjection technique for CAM vessels, the removal of the yolk sack, and use of a CLINicell for optical imaging, and also present several examples of ultrasound imaging of the embryo. Furthermore, they also present some of the common pitfalls of CAM imaging and potential solutions to those issues.

In my opinion, the submitted manuscript has merit, particularly for the description of the variety of acoustic and optical imaging platforms. The protocols are described well, would benefit from a video presentation, and seem reasonable to implement. However, the advantage of the techniques for removing egg contents from older (greater than 3-day old) fertilized chicken eggs needs some more justification and/or clarification. For instance, the authors cite Martowicz et al. as an example of the ~50% attrition rate for ex ovo cultured chicken embryos. That study used 9-day old chicken embryos, which are a day older than the oldest chicks in this manuscript, and it is reasonable to assume that the attrition rate will increase with age. In conjunction with this, some clarification is required for the survival rates presented by the authors. In the Representative Results section, they list the survival for 8-day old embryos as 60%, which is scarcely better than the ~50% for ex ovo cultured chicks. In the

Discussion section however, the survival for 8-day old embryos is listed as 80%. Furthermore, the limitations of this protocol can be better contextualized with other research in the field. For example, is the use of the CAM for tumor xenograft transplantation compatible with the methods presented in this manuscript?

**Response:** *In theory, the methods presented in this manuscript can also be used for tumor xenograft transplantation. The grafting of the tumor is usually performed at day 8-9 of development (Martowicz et al. 2015; Paproski et al 2017; Sys et al. 2013). If the egg content is sterilely taken out of the shell as described in section 1.2, 1.3, or 1.4, a tumor could be grafted at this stage and the embryo and CAM could further be developed ex ovo. We have incorporated this in the text.*

*We have addressed all other points below.*

*Revised text:*

Line 716 – 719: "...The tumors are typically grafted on the CAM vessels of 8 or 9-day old embryos<sup>1, 14, 45</sup>, for which the embryos is taken out of the egg shell at day 3 of incubation and further developed ex ovo. The methods described in this protocol could be used to grow embryos in ovo until the day of tumors grafting."

*Added references:*

14. Paproski, R.J., Jovel, J., Wong, G.K.S., Lewis, J.D., Zemp, R.J. Enhanced detection of cancer biomarkers in blood-borne extracellular vesicles using nanodroplets and focused ultrasound. *Cancer Research*. **77** (1), 3–13 (2017).
45. Sys, G.M.L. et al. The In ovo CAM-assay as a Xenograft Model for Sarcoma. *Journal of Visualized Experiments*. **77**, 1–7 (2013).

*References:*

1. Martowicz, A., Kern, J., Gunsilius, E., Untergasser, G. Establishment of a Human Multiple Myeloma Xenograft Model in the Chicken to Study Tumor Growth, Invasion and Angiogenesis. *Journal of Visualized Experiments*. (99), 1–9, doi: 10.3791/52665 (2015).

This manuscript can be considered for publication in JoVE if the following comments can be addressed.

Major Concerns:

Introduction:

Page 1, lines 71-75:

"The majority of the previous chicken embryo studies involving ultrasound contrast agents used 5 to 6-day old incubated eggs, since at this stage the amount and variety of vessels in the CAM are abundant and the vessel size and structure allows for easier ultrasound contrast agent and cavitation nuclei injection 7, 11-13, 15."

This is too general of a statement, as other research groups have performed contrast-enhanced ultrasound imaging on older chicken embryos. For example, the Dayton group used 14-day old chicken embryos for their superharmonic perfusion imaging (PMID: 27832421), the Zemp group used day 14+

embryos for their nanodroplet cavitation experiments (PMID: 27793845), the Chen group (PMID: 32265457) and Song group (PMID: 32305910) used 18-day old chicken embryos for their super-resolution ultrasound microbubble imaging, and Schellpfeffer and Kolesari (PMID: 22266227) performed CEUS on in ovo chicken embryos ranging from Day 8 to Day 19.

This statement should be rewritten to reflect that citations 7, 11-13, and 15 in this manuscript all reference work from the same group (de Jong and Kooiman).

**Response:** We agree with the reviewer and have modified the text. We did not include the Schellpfeffer and Kolesari paper since they used an in ovo model and our manuscript focusses on ex ovo models:

*Revised text:*

Line 72 - 74: "...egg shell<sup>18</sup>. Previous chicken embryo studies involving ultrasound contrast agents used 5 to 6-day old embryos<sup>7, 11, 12, 17, 19</sup> and 14 to 18-day old embryos<sup>13-16</sup>. Multiple approaches..."

Line 83 & 84: "...beneficial especially for experiments on 5 to 8-day old embryos."

*References:*

7. Skachkov, I., Luan, Y., van der Steen, A.F.W., De Jong, N., Kooiman, K. Targeted microbubble mediated sonoporation of endothelial cells in vivo. *IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control*. **61** (10), 1661–1667 (2014).
11. Daeichin, V., Bosch, J.G., Needles, A., Foster, F.S., van der Steen, A., de Jong, N. Subharmonic, non-linear fundamental and ultraharmonic imaging of microbubble contrast at high frequencies. *Ultrasound in Medicine and Biology*. **41** (2), 486–497 (2015).
12. Maresca, D. *et al.* Imaging microvasculature with contrast-enhanced ultraharmonic ultrasound. *Ultrasound in Medicine and Biology*. **40** (6), 1318–1328 (2014).
17. Faez, T., Skachkov, I., Versluis, M., Kooiman, K., de Jong, N. In Vivo Characterization of Ultrasound Contrast Agents: Microbubble Spectroscopy in a Chicken Embryo. *Ultrasound in Medicine and Biology*. **38** (9), 1608–1617 (2012).
18. Yalcin, H.C., Shekhar, A., Rane, A.A., Butcher, J.T. An ex-ovo Chicken Embryo Culture System Suitable for Imaging and Microsurgery Applications. *Journal of Visualized Experiments*. (44), 4–7 (2010).
19. Kokhuis, T.J.A. *et al.* Intravital microscopy of localized stem cell delivery using microbubbles and acoustic radiation force. *Biotechnology and Bioengineering*. **112** (1), 220–227 (2015).

*Added references:*

13. Lindsey, B.D. *et al.* High Resolution Ultrasound Superharmonic Perfusion Imaging: In Vivo Feasibility and Quantification of Dynamic Contrast-Enhanced Acoustic Angiography. *Annals of Biomedical Engineering*. **45** (4), 939–948 (2017).
14. Paproski, R.J., Jovel, J., Wong, G.K.S., Lewis, J.D., Zemp, R.J. Enhanced detection of cancer biomarkers in blood-borne extracellular vesicles using nanodroplets and focused ultrasound. *Cancer Research*. **77** (1), 3–13 (2017).
15. Huang, C. *et al.* Short Acquisition Time Super-Resolution Ultrasound Microvessel Imaging via Microbubble Separation. *Scientific Reports*. **10**, 1–13 (2020).

16. Lowerison, M.R., Huang, C., Kim, Y., Lucien, F., Chen, S., Song, P. In Vivo Confocal Imaging of Fluorescently Labeled Microbubbles: Implications for Ultrasound Localization Microscopy. *IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control*. **67** (9), 1811–1819 (2020).

Page 1, line 79:

Is the increased risk of fatality for ex ovo culture really a substantial barrier to chicken embryo imaging research, considering the low cost of developing the model?

**Response:** *The reviewer is correct that the cost of an egg itself is low. At the same time additional costs are needed for the ex ovo culture since this requires extra materials and equipment to culture the embryos under sterile conditions, such as antibiotics and large sterile incubators, and a person to check on the embryos during the days of ex ovo culture. Differences in the total vessel length between embryos grown in ovo and ex ovo have also been reported with the in ovo grown embryos having a larger total vessel length (Ribatti D 1995). We have added the difference in vessel length to the manuscript:*

*Revised text:*

Line 79 & 80: "...culture (~50%)<sup>1,18</sup>, the use of antibiotics<sup>18, 20</sup>, and decreased total vessel length in comparison to *in ovo* growth<sup>23</sup>."

*References:*

1. Martowicz, A., Kern, J., Gunsilius, E., Untergasser, G. Establishment of a Human Multiple Myeloma Xenograft Model in the Chicken to Study Tumor Growth, Invasion and Angiogenesis. *Journal of Visualized Experiments*. (99), 1–9 (2015).
18. Yalcin, H.C., Shekhar, A., Rane, A.A., Butcher, J.T. An ex-ovo Chicken Embryo Culture System Suitable for Imaging and Microsurgery Applications. *Journal of Visualized Experiments*. (44), 4–7 (2010).
20. Cloney, K., Franz-Odenaal, T.A. Optimized Ex-ovo Culturing of Chick Embryos to Advanced Stages of Development. *Journal of Visualized Experiments*. (95), 6–10 (2015).

*Added reference:*

23. Ribatti, D. A morphometric study of the expansion of the chick vasculosa in shell-less culture. *Journal of Anatomy*. **186**, 639–644 (1995).

Page 2, line 90:

"... making the CAM optically transparent for microscopy studies."

Is this step necessary for reflected microscopy imaging and/or optically tagged microbubble imaging?  
What about photoacoustic imaging?

**Response:** *It is possible to perform reflected microscopy imaging and photoacoustic imaging as long as the light path and or laser path are not affected by the yolk, for example when all the imaging is performed from above. For this application, it is not needed to separate the membrane containing the embryo and CAM from the yolk. However, when the setup requires imaging from the bottom, the embryo can be made transparent with the described method in the protocol.*

*Revised text.*

Line 96 & 97: ...the setup can also be made acoustically transparent<sup>26</sup> allowing the combination of microscopy and ultrasound studies when the light path will be affected by the yolk.

*Reference:*

26. Beekers, I., van Rooij, T., van der Steen, A.F.W., de Jong, N., Verweij, M.D., Kooiman, K. Acoustic characterization of the CLINicell for ultrasound contrast agent studies. *IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control*. **66** (1), 244–246 (2019).

*Protocol:*

I think it would be worthwhile, given that the proceeding protocols depend on the embryo age, to add a statement to this section on the methods for estimating the embryonic development stage of chicken eggs. Many researchers who are using fertilized chicken eggs do not have control of the precise development stage of their egg delivery, which leads to a disperse cohort age.

**Response:** *The exact stage of embryonic development can be confirmed using the paper of Hamburger and Hamilton (Hamburger & Hamilton, 1951). We have added this to the manuscript*

*Revised text:*

Line 469 & 470: "...between the 5 and 8-day old embryos can be observed which concurs with Hamburger and Hamilton<sup>22</sup>. Both the size of the..."

Line 644 & 645: "...embryo vitality. To confirm the exact developmental stage of the embryo, the indication of Hamburger & Hamilton<sup>22</sup> can be used."

*Reference:*

22. Hamburger, V., Hamilton, H. A Series Of Normal Stages In The Developent Of The Chick Embryo. *Journal of Morphology*. **88** (1), 231–272 (1951).

Page 2, line 107:

"Turning the eggs during incubation is not necessary."

This statement is surprising to me, as the conventional wisdom is that turning eggs during incubation improves hatchability. Further evidence is required to support this statement.

**Response:** *We agree with the reviewer that turning the eggs improves hatchability and this is supported by literature, for example Elibol et al. Length of Egg Storage, and Frequency of Turning During Storage on Hatchability of Broiler Hatching Eggs. *Poultry Science*. **81** (7), 945–950, doi: 10.1093/ps/81.7.945 (2002). However, we take the egg content out of the shell well before hatching. Others have also not rotated the eggs for their experiments, for example Ribatti D, (1995) A morphometric study of the expansion of the chick area vasculosa in shell-less culture. *J. Anat.* 186 ( Pt 3):639-44. Besides, it is common for eggs not to be turned when a window has been made in the shell for the requirement of the study. For example for up to 14-day old embryos as published by Shumko et al., (1988) Vascular histodifferentiation in the chick chorioallantoic membrane: a morphometric study. *Anat Rec* 220:179-89.*



Page 4, line 189-192:

"Check whether the embryo is alive and whether the CAM is intact by ensuring the heartbeat is still present, the vessels are intact and there is no bleeding, and there is no leakage of yolk. If one of these things is not correct, discard the embryo and CAM because it will not be viable"

Slight clarification needed: Is the viability rate, listed elsewhere, based on the total number of eggs per cohort? Or the total number of fertilized eggs?

**Response:** *The viability rate listed elsewhere is based on the total number of fertilized eggs successfully extracted from the egg shell. We have added this to the manuscript.*

*Revised text:*

Line 464 - 466: "...procedures. The viability rate is based on the total number of fertilized eggs successfully extracted from the egg shell. With..."

Page 4, line 221:

I'm curious as to why an injection of PBS is required in the protocol for 6-day and 7-day old embryos.

**Response:** *Because the egg white (~160 cP) has a higher velocity than PBS (~1 cP) (Lang, E.R. & Rha, C., 1982), substituting egg white with PBS will reduce both tension and stress on the embryo and CAM while taking the egg content out of the shell. We have added this to the manuscript.*

*Revised text:*

Line 230 – 232: "NOTE: Since egg white has a higher velocity (~160 cP)<sup>27</sup> than PBS (~1 cP), substituting the egg white with PBS reduces both tension and stress on the embryo and CAM while taking the egg content out of the shell."

*Added reference:*

27. Lang, E.R., Rha, C. Apparent shear viscosity of native egg white. *Journal of Food Science and Technology*. **17**, 595–606 (1982).

Page 6, line 278:

Some explanation should be provided as to why the mineral oil is needed in this step.

**Response:** *The mineral oil is used as this is according to the manufacturer's instruction of the needle injection system. We have added the following text to reflect this.*

*Revised text:*

Line 287 & 288: NOTE: The mineral oil is added as per instruction of the manufacturer of the injection system we used.

Page 7, line 298-299:

"Always inject into one of the veins of the embryo. These are the vessels in which the blood flow moves towards the embryo."

It is also possible to identify the veins of the CAM based on the coloration of the blood in the vessels. It

may be useful to the reader to demonstrate some low magnification images of the CAM surface with the appropriate injection sites identified.

**Response:** The reviewer is correct that it is possible to identify the veins of the CAM based on the coloration because veins are lighter in color than arteries due to the oxygenated blood. In addition, veins are always on top of arteries in the CAM, unless it is the anterior or posterior vitelline vein since this does not have any artery in its neighborhood. The text in the manuscript has been revised accordingly:

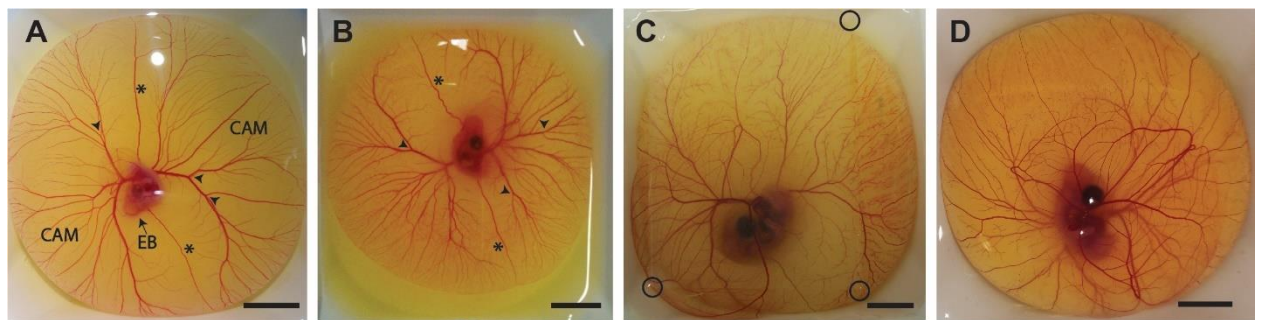
*Revised text:*

Line 311 -314: "... flow moves towards the embryo. Veins are lighter in color than the arteries due to the oxygenated blood<sup>29</sup>. In addition, veins are always on top of the artery with two exceptions namely the anterior and posterior vitelline veins (i.e. the less branched veins, indicated with asterisks in **Figure 6A,B**) which do not have an artery in their surroundings."

*Added reference:*

29. McFerrin, H.E., Olson, S.D., Gutschow, M. V, Semon, J.A., Sullivan, D.E., Prockop, D.J. Rapidly Self-Renewing Human Multipotent Marrow Stromal Cells (hMSC) Express Sialyl Lewis X and Actively Adhere to Arterial Endothelium in a Chick Embryo Model System. *PLoS ONE*. **9** (8), 1–11 (2014).

**Response:** We agree with the reviewer that it is useful for the reader to demonstrate appropriate sites for injection and have added this to **Figure 6A** and **Figure 6B**. Besides this we have added the anterior or posterior vitelline veins with asterisks.



*Revised text:*

Line 316: "...injection. Good injection sites have been indicated with arrowheads in **Figure 6A,B**. It..."

Line 581 – 584: "...The chorioallantoic membrane (CAM), embryo body (EB), anterior and posterior vitelline veins (\*), and appropriate sites for injection (arrowheads) are indicated. **(B)** Egg content taken out of the shell after 6 days of incubation. The anterior and posterior vitelline veins (\*) and appropriate sites for injection (arrowheads) are indicated. **(C)** Egg content taken ..."

Page 8, lines 349-352:

Does cutting into the membrane of the yolk sack cause bleeding (e.g.: from the vitelline circulation) and/or reduce the viability of the embryo?

**Response:** *No, cutting the yolk sack membrane will not create any bleeding as long as the embryo and vessels are not damaged in the process. Removing the membrane containing the embryo and CAM from the yolk sack will eventually affect the viability of the embryo, since the embryo is removed from its nutrition source. As described in the result and discussion section (line 456-459 & line 645-650), the viability time (from ~5 h down to ~2 h) and the heart rate (from ~190 bpm down to ~80 bpm) is reduced after separating the membrane containing the embryo and CAM from the yolk sack.*

Page 8, lines 362-364:

Given that the CAM is the main site of gas-exchange for the embryo, is there a risk that submerging the CAM in PBS in this step will suffocate the embryo?

**Response:** *Submerging the embryo in PBS will affect the gas-exchange of the embryo. However, in the time period in which we have used the embryos in our experiments (up to maximum 5 hours), we did not observe major changes in viability based on the heart beat.*

Discussion:

Page 15, line 655:

"[Later stages of development] can prevent a successful injection in older embryos"

This is too strong of a statement, given the aforementioned research groups that performed contrast-enhanced ultrasound on day-14 and above embryos. Instead, this claim could be rephrased, or clarified, by stating that the vessel stiffness can complicate injections (or equivalent).

**Response:** *We rephrased the sentence:*

*Revised text:*

Line 681: "This can **complicate** a successful injection in older embryos."

Page 16, line 671-672:

"The advantages are a higher survival rate of 90% for 5-day, 75% for 6-day, 50% for 7-day, and 80% for 8-day old incubated eggs..."

It was mentioned above that some clarification was needed for this statement, given that it conflicts somewhat with the numbers listed in the results section. Assuming these are the correct numbers, can the authors speculate as to why the 6- and 7-day old embryos have such a relatively poor survival rate? Furthermore, what are the approximate N numbers used by the authors to calculate these survival percentages?

**Response:** *We have unfortunately made a textual mistake in Lines 44 and 698 in the abstract and discussion. The survival rate of the 8-day old embryos should have been 60%, which was mentioned correctly in the results section. We have adjusted this in the text. The survival of the 6-to-8-day old embryos is lower because the CAM starts to attach itself to the shell which leaves the CAM membrane more prone to rupture upon extraction. The approximate N numbers for the survival percentages were 150 for the 5-day old embryos and 10 for the 6-to-8 day old embryos.*

*Revised text:*

Line 44: "...7-day, and 60% for 8-day old incubated eggs in comparison ..."

Line 698: "...of 90% for 5-day, 75% for 6-day, 50% for 7-day, and 60% for 8-day old incubated..."

Line 701 & 702: "...The survival of the 6-to-8-day old embryos is lower because the CAM starts to attach itself to the shell<sup>21</sup> which leaves the CAM membrane more prone to rupture upon extraction."

*Reference:*

21. Dohle, D.S. *et al.* Chick ex ovo Culture and ex ovo CAM Assay: How it Really Works. *Journal of Visualized Experiments*. (33), 2–7 (2010).

Minor Concerns:

Protocol:

Page 3, line 138: Typo. This should be "... do not penetrate the shell too deep".

**Response:** *We have revised the text according to the suggestion of the reviewer.*

*Revised text:*

Line 142: "...too deep..."

Page 4, line 211: Would this be multiple shell penetrations to withdraw 6mL of egg white using a 5mL syringe? If so, how many penetrations, and how should the hole be sealed between withdrawals?

**Response:** *It is possible to withdraw up to 6 mL with the 5 mL syringe were using. This means that the total volume of 6 mL can be withdrawn in one penetration. We have added a note to this step to clarify this.*

*Added text:*

Line 219 & 220: "NOTE: With the 5 mL syringe we used, it is possible to withdraw up to 6 mL so only one penetration is needed."

Page 6, line 282: "...of the mineral oil and the to-be-injected solution" should be hyphenated to make the meaning clearer.

*We have hyphenated the text according to the suggestion of the reviewer:*

*Revised text:*

Line 291 & 292 - "...and the to-be-injected solution."

Page 10, line 423: Typo. This should be "Pour the agarose solution..."

**Response:** *We have revised the text accordingly.*

*Revised text:*

Line 443: "Pour the agarose..."

Representative Results:

Page 11, line 469: Approximately what volume of PBS should be sprinkled on the egg contents?

**Response:** *One drop of PBS is ~30  $\mu$ L. We have added this into the protocol:*

*Added text:*

Line 194: "...a few drops of ~30  $\mu$ L of PBS (37 °C) using a plastic Pasteur pipet."

Line 201: "...put small drops of ~30  $\mu$ L of 37 °C PBS on the embryo ..."

Line 447 & 448: "...Add a few drops of ~30  $\mu$ L of 37 °C PBS ..."