

# Journal of Visualized Experiments

## Organotypic Culture Method to Study Developmeny of Embryonic Chicken Tissues

--Manuscript Draft--

<b>Article Type:</b>	Methods Article - Author Produced Video
<b>Manuscript Number:</b>	JoVE57619R1
<b>Full Title:</b>	Organotypic Culture Method to Study Developmeny of Embryonic Chicken Tissues
<b>Keywords:</b>	Culture; developmental biology; tissue; embryo; microscopy; manipulations; development
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<b>Author Comments:</b>	Response to reviewers will be uploaded as an attached file. Video was submitted via separate link sent by editor.
<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	05-31-2018

**TITLE:**

Organotypic Culture Method to Study the Development of Embryonic Chicken Tissues

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**KEYWORDS:**

Culture, developmental biology, tissue, embryo, microscopy, manipulations, development

**SHORT ABSTRACT:**

Here, we present an organotypic culturing protocol to grow embryonic chicken organs *in vitro*. Using this method, the development of embryonic chicken tissue can be studied, while maintaining a high degree of control over the culture environment.

**LONG ABSTRACT:**

The embryonic chicken is commonly used as a reliable model organism for vertebrate development. Its accessibility and short incubation period makes it ideal for the experimentation. Currently, the study of these developmental pathways in the chicken embryo is conducted by applying inhibitors and drugs at localized sites and at low concentrations using a variety of methods. *In vitro* tissue culturing is a technique that enables the study of tissues separated from the host organism, while simultaneously bypassing many of the physical limitations present when working with whole embryos, such as the susceptibility of embryos to high doses of potentially lethal chemicals. Here, we present an organotypic culturing protocol for culturing the embryonic chicken half head *in vitro*, which presents new opportunities for the examination of developmental processes beyond the currently established methods.

**INTRODUCTION:**

The embryonic chicken (*Gallus gallus*) is an excellent model organism commonly used in the field of biology. Its incubation period is roughly 21 days and many eggs can be incubated simultaneously, making experimentation quick and efficient. Perhaps most importantly, the embryo is also easily manipulated, enabling the extensive study of key developmental processes and of the genes and proteins that drive these processes.

The embryonic chicken eye is a complex organ that develops *via* the interaction of a number of different tissues similar to many other body systems. This method enables the study of the development of these tissues, particularly at advanced stages of the development. For example,

the multi-layered retina may be of particular interest to those studying the development of the nervous system. Alternative methods that enable the study of other eye tissues such as the cornea, the vitreal body, the lens, the sclera, and the eyelids are of benefit to researchers. The chicken embryonic eye also contains a series of flat bones, the scleral ossicles, which can be used as a model for the study of intramembranous bone induction and ossification in vertebrates<sup>1</sup>.

Currently, there are a number of methods used to study embryonic development. Microinjections of inhibitory antibodies or other inhibitory molecules<sup>2-3</sup>, surgically implanted microbeads soaked in inhibitor<sup>4</sup>, and electroporation<sup>5</sup> are all methods that can be used to downregulate genes or proteins of interest in an embryo. Similar methods are used to upregulate proteins. These methods are not without their limitations. For example, when using chemicals to alter the embryonic development, the lethal effects on the embryo must be evaluated, and this limits the use of the aforementioned methods to localized sites of application at doses low enough to ensure the survivability of the embryo.

*In vitro* tissue culturing has been used in a wide range of organisms to study development and can be used to bypass some of the aforementioned limitations. For example, the femora<sup>6</sup>, feather buds<sup>7-8</sup>, and limbs<sup>9</sup> of the chicken have all been studied using tissue culturing methods, as have the testes of the mouse<sup>10</sup> and the roots and stems of plants<sup>11</sup>. These methods grant scientists a high degree of control over the tissue development, such as the ability to fluctuate the temperature and alter the nutrient availability. The isolation of the tissue from the whole embryo also makes it far less susceptible to the lethal effects of chemicals, thus enabling manipulation studies on a global scale at higher concentrations. Another notable advantage of *in vitro* culturing is the preservation of the tissue's cellular environment; the arrangement of tissues remains relatively unchanged, making it possible to study the interactions between different tissue types<sup>9</sup>. Thus, *in vitro* culturing opens doors to additional experimental approaches not available in *in vivo* or *in ovo* models.

Currently, studying the development of the embryonic chicken eye using chemicals is particularly challenging. A number of extraembryonic membranes cover the embryo, making it difficult to apply microbeads or chemicals; the embryo is also very active within the egg as it gets older, further complicating an already difficult method. This protocol enables easy access to the eye and its surrounding tissues, eliminating these barriers, while also providing new opportunities to examine the developmental processes within the eye. This protocol was established to study the induction of the scleral ossicles within the embryonic eye.

## **PROTOCOL:**

Note: For embryo stages, utilize the Hamburger and Hamilton<sup>12</sup> (HH) staging table.

### **1. Embryo Incubation**

1.1. Incubate fertilized chicken eggs in a sterile, temperature-controlled incubator at 37 °C ± 1 °C and ~40% humidity.

1.2. Turn the eggs 1x per day and allow them to incubate to HH34 (8 days post-fertilization).

## **2. Preparation and Sterilization of the Materials**

2.1. For 12 embryos, autoclave 2 L of distilled water, 1 L of 0.85% chick saline, 12 glass pipettes, 1 box of paper tissue, 24 2.5 cm x 2.5 cm squares of semi-porous filter paper, and 24 2.5 cm x 2.5 cm squares of steel wire mesh with the edges curved downwards. To sterilize the materials, autoclave them at 121 °C for at least 15 min.

Note: The autoclaving of the materials can be done in advance.

2.2. Sterilize the incubator with 70% ethanol by wiping the sides, shelves, and door. Place 2 plastic containers containing autoclaved distilled water inside the incubator to maintain humidity. Prewarm the culture incubator to  $37\text{ °C} \pm 1\text{ °C}$  and ~40% humidity. Ensure the incubator is completely dark; if the door is transparent, cover it with tinfoil.

2.3. Prewarm 100 mL of nutrient medium and 1 mL of penicillin by placing them in the culture incubator.

2.4. Cover the workspace with a paper towel and sterilize the bench by spraying it with 70% ethanol. Sterilize 2 pairs of forceps, dissection scissors, a razor blade, and a plastic spoon with 70% ethanol in a similar manner.

2.5. Place 12 sterile 100 mm Petri dishes and 24 sterile 35 mm Petri dishes on the bench for use. Ensure the dishes remain in a sterile bag until use.

## **3. Embryo Preparation**

Note: From this point onwards, wear a protective dust face mask to avoid contaminating the cultures. A bacterial or fungal infection will ruin the culture and there is a risk of it spreading quickly to all cultures in the incubator.

3.1. Crack open the egg and transfer the embryo to a 100 mm Petri dish containing 0.85% chick saline. Stage the embryo according to the anatomical features described in the Hamburger and Hamilton staging guidelines<sup>12</sup> and confirm the embryo is at HH34.

3.2. Cut the neck and then bisect the head of the embryo at the midline using a sterilized razor blade. Using sterile forceps, remove the brain. Leave the beak intact.

## **4. Culture Setup**

4.1. Place 2 of the 35 mm Petri dishes inside one 100 mm Petri dish.

133 4.2. Place a steel wire mesh inside each 35 mm Petri dish.

134  
135 4.3. Using forceps, transfer 1 of the bisected heads to a piece of 2.5 cm x 2.5 cm semi-porous  
136 filter paper with the eye facing up. Ensure the tissue is secure and does not slip off by tilting it  
137 slightly.

138  
139 4.4. Using forceps, carefully place the eye tissue and the filter paper on top of the steel wire mesh  
140 in 1 of the 35 mm Petri dishes, creating a raised stage with the eye tissue on top.

141  
142 4.5. Repeat steps 4.3 and 4.4 with the other half head.

143  
144 4.6. Using a sterile glass pipette, carefully add nutrient medium directly into each 35 mm Petri  
145 dish until it reaches the level of the filter paper. Do not submerge the half head in the nutrient  
146 medium.

147  
148 4.7. Add 50  $\mu$ L of 10,000 U/mL penicillin-streptomycin to the nutrient medium in each Petri dish.

149  
150 4.8. Fold a piece of tissue paper into a small square and place it inside the 100 mm Petri dish.  
151 Moisten it with autoclaved distilled water.

152  
153 4.9. Place the culture dish into the sterile, dark incubator at  $37\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$  and ~40% humidity for  
154 up to 4 days.

155  
156 4.10. Repeat steps 3 and 4 for each embryo.

157  
158 Note: The number of embryos needed will depend on the specific experiment; here, we describe  
159 the protocol for 12 embryos.

## 160 161 **5. Culture Maintenance**

162  
163 5.1. Once per day, top up the plastic containers in the incubator with fresh, autoclaved distilled  
164 water.

165  
166 5.2. Daily check all cultures for bacterial or fungal infections. Cultures that have disintegrated or  
167 in which the medium has changed color are infected. Discard all cultures that are infected.

## 168 169 **6. Fixation**

170  
171 6.1. Following culturing, remove all dishes from the incubator.

172  
173 6.2. Using a pair of forceps, gently remove the eye from the filter paper, taking care not to tear  
174 the tissue.

175  
176 6.3. Fix the eye tissue in 4% paraformaldehyde in 1x phosphate-buffered saline overnight at  $4\text{ }^{\circ}\text{C}$

or in 10% neutral-buffered formalin overnight at room temperature.

6.4. Store the tissue at 4 °C in 1x phosphate-buffered saline.

#### REPRESENTATIVE RESULTS:

Using this method, an embryonic chicken eye can be cultured from day 8 of its development (HH34) *in vitro* for 4 days. Four days of *in ovo* development corresponds to HH38.

This culturing method supports the development of feather buds surrounding the eye and, on the eyelids (**Figure 1B**). These feather buds are not present *in ovo* at HH34 prior to the culturing (**Figure 1A**), indicating that feather bud development is initiated during the culturing period, as it is *in ovo*<sup>13</sup> (**Figure 1C**). The eyelids and nictitating membrane grow to cover the eye during the culturing (**Figure 1B**). The eyelids appear more mature after the *in vitro* culturing than at the start of the culturing at HH34 (**Figure 1A**) but are less developed than at HH38 (**Figure 1C**). This data shows that the key aspects of embryonic development are supported, albeit with some small delay.

After culturing, we dissected the anterior portion of the eye to show the conjunctival papillae (a transient ring of 13 - 14 epithelial protrusions), which act as yet another landmark indicating that the developmental events are supported in culture. Typically, these papillae are fully formed by HH34 (**Figure 1D**), and degenerate over time as they induce skeletogenic condensations in the underlying mesenchyme; they disappear by HH38<sup>1</sup> (**Figure 1F**). Our data shows that this degeneration occurs *in vitro* (**Figure 1E**) and is preceded by a flattening of the papillae following the *in ovo* pattern of development. Unstained condensations, however, are not visible in the *in vitro* samples, unlike at HH38 (**Figure 1F**). We used alkaline phosphatase staining, which detects early osteoblast activity to further assess the induction of these condensations<sup>14</sup>; this process occurs alongside the degeneration of the conjunctival papillae *in ovo*. The induction of the skeletogenic condensations, which begins at HH34 (**Figure 1G**), does proceed *in vitro* as evidenced by the alkaline phosphatase staining (**Figure 1H**). These condensations, however, are not as large as they are at HH38<sup>14</sup> (**Figure 1I**), again indicating that there is a small delay in the development. We estimate that the 4 days of culturing represent approximately 2 days of embryonic development; hence a delay of about 50%. Collectively, these results show that the induction and development of the intramembranous bones, the scleral condensations, is supported *in vitro* and that the developmental processes such as eyelid growth and feather bud formation proceed *in vitro*.

#### FIGURE AND TABLE LEGENDS:

**Figure 1. Morphological characteristics of *in vitro* cultured eyes, HH34 eyes, and HH38 eyes.** Panels A - F are unstained and panels G - I are stained for the alkaline phosphatase (AP) enzyme to distinguish active osteoblasts within the scleral condensations. A, D, and G show eyes at the start of the culturing period at HH34. B, E, and H show eyes after 4 days of *in vitro* culturing. C, F, and I show eyes after 4 days of *in ovo* culturing, at HH38. All eyes are viewed laterally. (A) In this panel, the conjunctival papillae are visible in a ring surrounding the cornea. An asterisk indicates

papilla #12, the first one to form. The arrow indicates the edge of the eyelid in the nasal region of the eye. **(B)** The conjunctival papillae have flattened and begun to degenerate. The arrow indicates a growing feather bud. **(C)** The conjunctival papillae have completely degenerated and are covered by eyelids and the nictitating membrane. The open arrow indicates the edge of the eyelid in the nasal region of the eye. The solid arrow indicates a growing feather bud. **(D)** This panel shows a dissected anterior portion of the HH34 eye. The asterisk indicates papilla #12. **(E)** This panel shows a dissected anterior portion of an HH34+4 *in vitro* eye showing flattened papillae. The papillae in the temporal region (solid arrow) have begun to degenerate; these are the oldest papillae in the ring. **(F)** This panel shows a dissected anterior portion of an HH38 eye with the eyelids and nictitating membranes removed, showing that all papillae have degenerated. The dashed lines outline the position of two adjacent unstained condensations. **(G)** This panel shows an HH34 eye stained for AP showing very small skeletogenic condensations indicative of the start of induction. **(H)** This panel shows an HH34+4 eye stained for AP showing enlarged, growing skeletogenic condensations. **(I)** This panel shows an HH38 eye stained for AP showing large skeletogenic condensations. All scale bars are 1 mm.

#### DISCUSSION:

This protocol makes use of established tissue culturing techniques to achieve the growth of a chicken eye from embryonic day 8 (HH34) *in vitro* for 4 days. This grid-culturing method was originally described by Trowell<sup>15</sup>. We optimized a protocol from Pinto and Hall's 1991 study<sup>16</sup> utilizing a semi-porous membrane with the grid to study inductive signals between separated tissue layers of the embryonic chicken eye<sup>15</sup>. Using this method, Roach cultured 14-day old chicken femur<sup>6</sup>. This method has also proven effective in culturing mouse tissues<sup>6,17</sup>. The use of the filter paper is critical to the success of the protocol as it helps the tissue to take up nutrients from the medium effectively without saturating or submersing the tissue.

Nutrient medium at a proper pH is critical for optimal growth. The medium should be at or near the physiological pH, and the use of phenol red in the medium as an indicator provides an advantage in that pH changes can be easily detected. Serum-free media can be used, and changing the media daily is not necessary and does not affect the results. The protocol was developed using a commercially available medium (see **Table of Materials**), which contains high levels of amino acids and glucose; no further supplementation of the media is needed. Most occurrences of poor tissue growth using this protocol occurred when the nutrient media was either at an improper pH or contaminated.

The eye can survive and grow for up to 4 days *in vitro*, starting at embryonic day 8, when the protocol is performed under optimal conditions. One limitation of this protocol is that the survivability is impacted beyond 4 days of culturing. Thus, the developmental processes that span a longer timeframe will require modifications to the protocol. Another limitation of the protocol is the absence of an active blood flow and vascularization. Blood vessels are important for many developmental processes, not just for the delivery of nutrients to the tissue<sup>9</sup>, which is achieved *in vitro* via capillary action through the filter paper on which the tissue rests.

This protocol can be used to study the development of entire organs or large pieces of tissue *in*

*vitro*. Vertebrate organs are highly complex, and their development is regulated by many distinct spatiotemporal patterns. The study of these developmental pathways relies on the ability to manipulate the signals that regulate them. Current methods limit this manipulation to relatively small, localized areas, and to low enough concentrations to avoid embryonic lethality. For example, the *ex ovo* method of culturing the entire chicken embryo in culture<sup>18</sup> or the method to window eggs<sup>19</sup> to conduct manipulations are limited in that either the entire embryo must be treated, which can cause lethality, or only a small localized area is treated. The advantage of the protocol presented here is that the effect of these signals can be studied on entire organs or large amounts of tissue, without affecting the embryonic viability. This protocol does not require any supplementation with carbon dioxide as in earlier protocols<sup>15</sup>. The delay in development that we observed is an advantage in that rapid developmental processes can be studied.

#### **ACKNOWLEDGMENTS:**

The authors would like to thank Gregory Haller (Mount Saint Vincent University) for his preliminary work in the development of the protocol. The authors would also like to thank Nicholas Jones (Mount Saint Vincent University) for his technical expertise and assistance with the filming and production of the audio/visual portion of the manuscript. Daniel Andrews was supported by funding from MSVU and the Natural Sciences and Engineering Research Council of Canada (NSERC) *via* an Undergraduate Student Research Award. Tamara A. Franz-Odenaal is supported by an NSERC Discovery Grant.

#### **DISCLOSURES:**

The authors have nothing to disclose.

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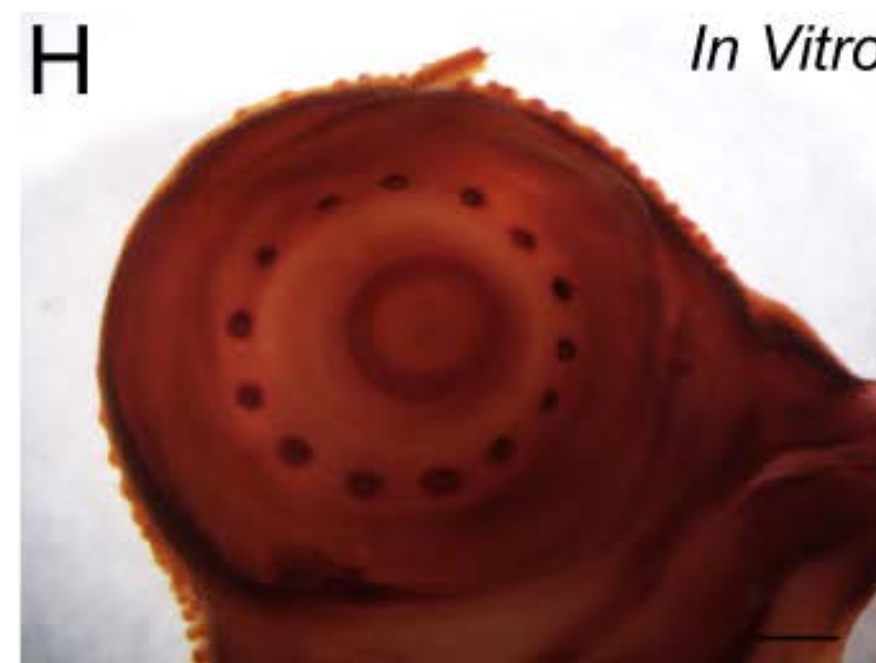
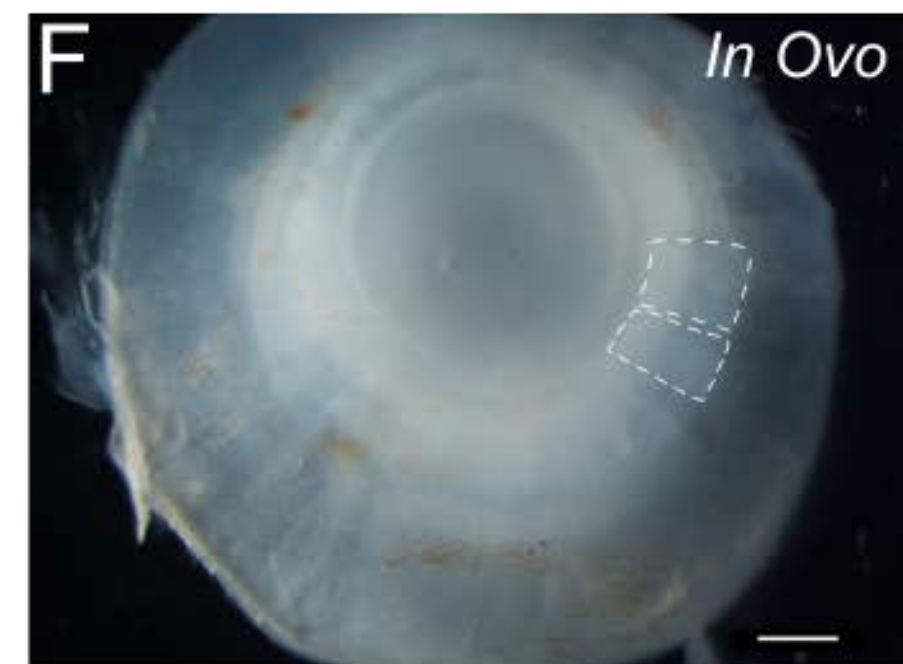
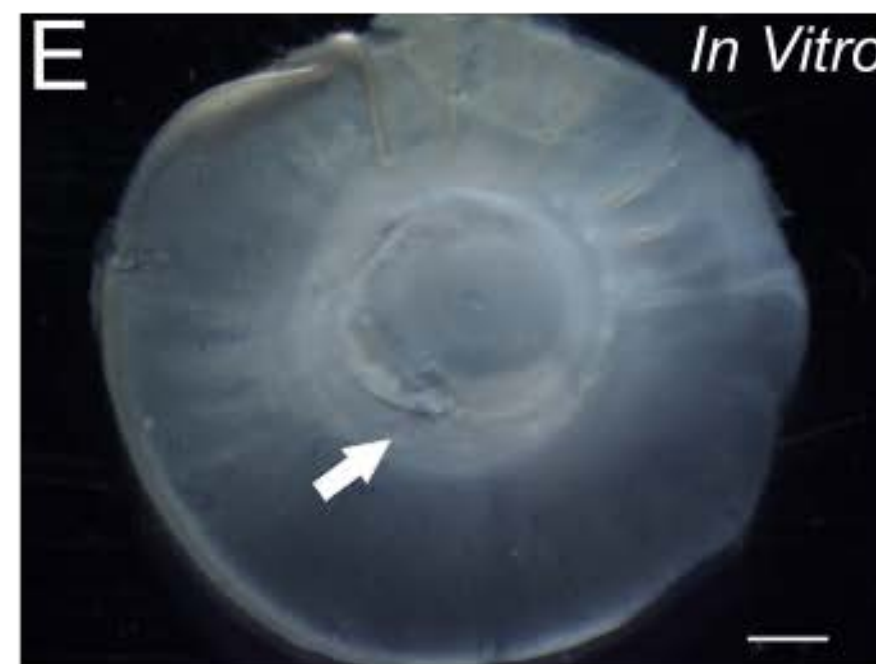
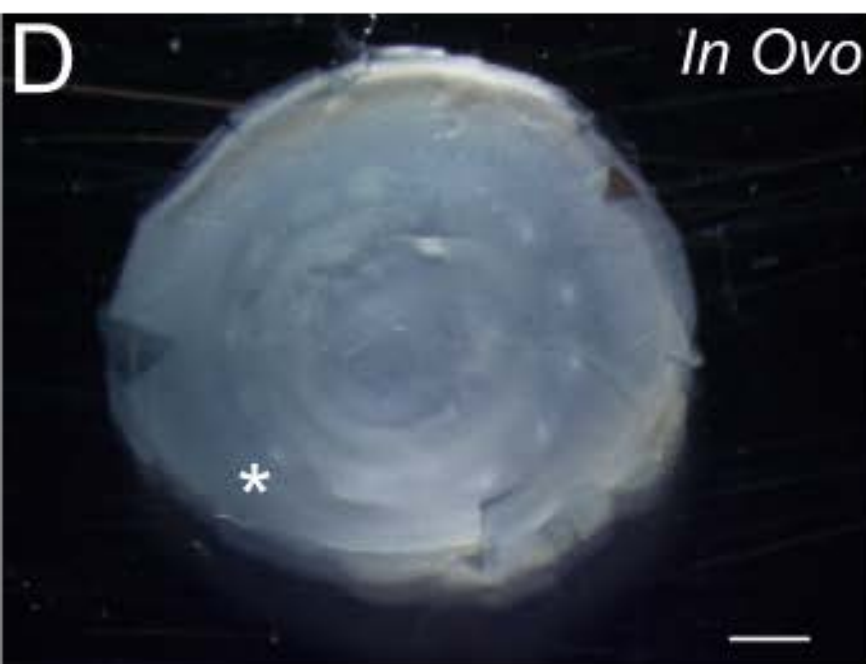
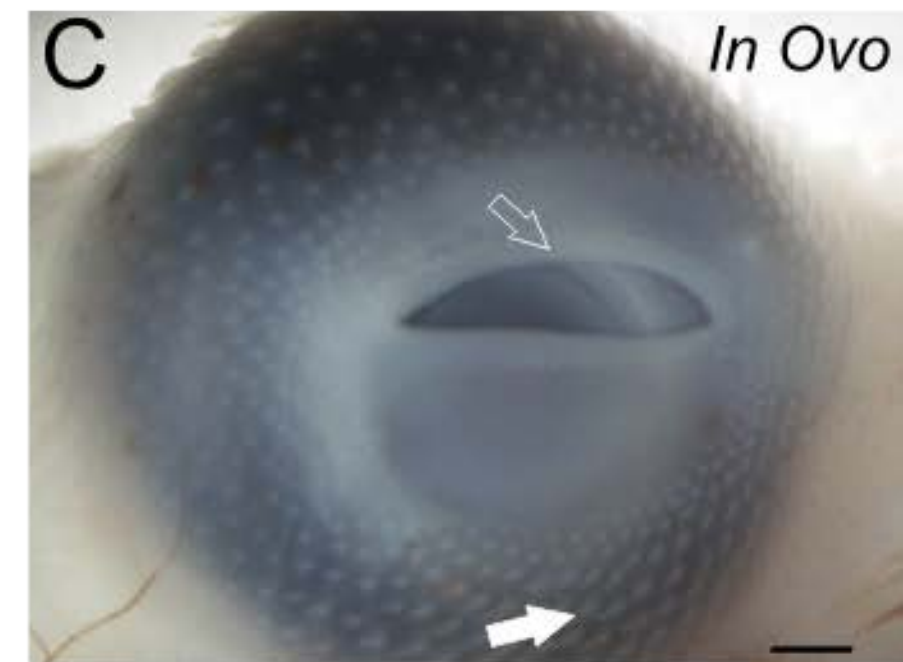
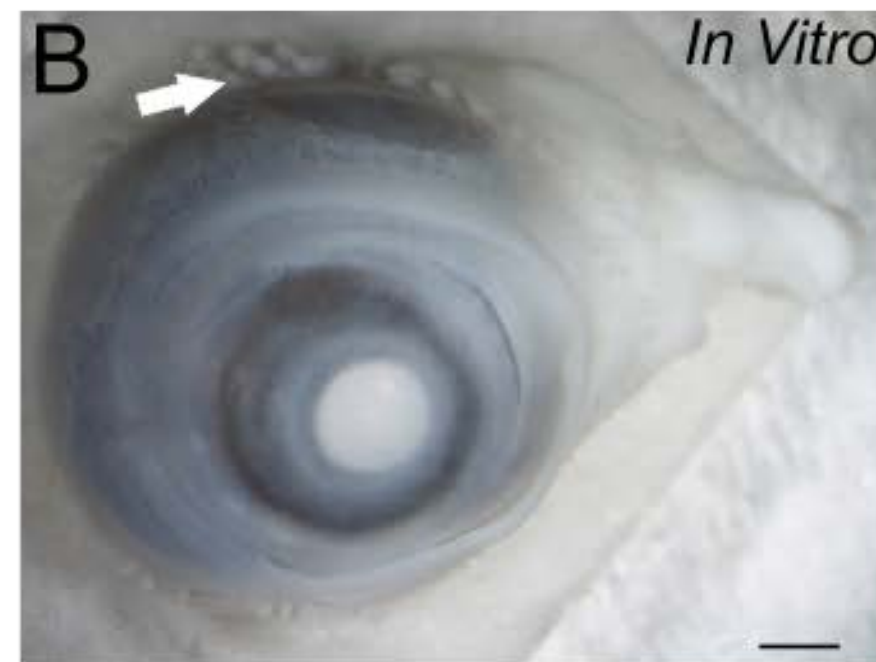
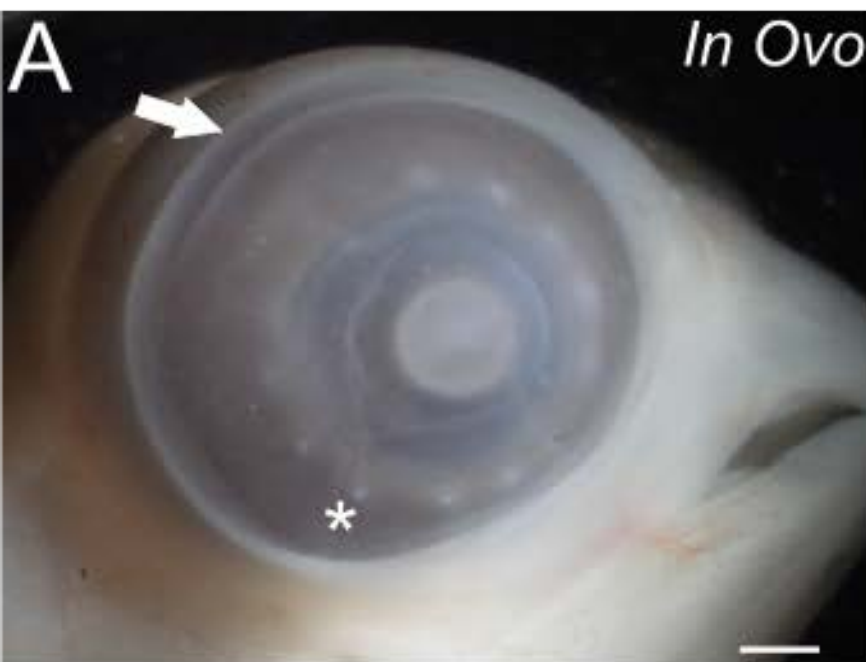


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HH34

HH34+4d

HH38



Name of Material/ Equipment	Company	Catalog Number
35 mm cell culture petri dishes	Corning	353001
100 mm cell culture petri dishes	Corning	353003
paper tissue	Kimtech	34155
wire mesh	n/a	n/a
disposable glass pipettes	VWR	14673-010
nutrient medium	Gibco	12591-038
penicillin-streptomycin	Sigma-Aldrich	P4458
filter paper	Whatman	1454 090
fertilized chicken eggs	Dalhousie University Agricultural College	n/a
sodium chloride (NaCl)	EMD	SX0420-3
1 L glass bottle	VWR	89000-240
ethanol	Fisher Scientific	BP82011
tupperware containers	n/a	n/a
disposable razor blades	VWR	55411-050
plastic spoons	n/a	n/a
dust mask	3M	n/a
paraformaldehyde	Sigma-Aldrich	P6148
neutral-buffered formalin	Fisher Scientific	72210
phosphate buffered saline (PBS)	n/a	n/a

15 ml falcon tubes	VWR	21008-216
forceps	FST	n/a
chick saline	n/a	n/a
tinfoil	n/a	n/a
paper towel	n/a	n/a

### **Comments/Description**

easy grip tissue culture dish, polystyrene, non-pyrogenic

tissue culture dish, polystyrene, non-pyrogenic

Kimtech Science Brand Task Wipers, 280 per carton

stainless steel wire mesh (grid size 0.7 mm)

Borosilicate glass disposable 5 3/4"

Fitton-Jackson Modification, [+] L-glutamine with phenol red (BGJB)

10000 units/mL penicillin streptomycin solution stabilised

semi-porous filter paper 90mm

can be obtained from local farms

sodium chloride crystals, reagent grade

1 L pyrex autoclavable glass bottle

70% molecular biology grade

store-bought and sterilized with EtOH

single edge industrial razor blades (surgical carbon steel)

store-bought and sterilized with EtOH

3M 8500 Comfort Mask

paraformaldehyde, reagent grade, crystalline

10% neutral buffered formalin

10X phosphate buffered saline pH 7.4 (137mM NaCl, 2.5mM KCl, 4.3mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4mM KH<sub>2</sub>PO<sub>4</sub>)

presterilized centrifuge tubes

fine forceps

0.85% NaCl

store-bought

store-bought





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**Response to Editorial comments:**

1. Manuscript has been proofread
2. Reference format has been checked
3. All abbreviations are defined before use
4. Table of supplies have been revised to include all essential supplies, reagents and equipment
5. All commercial language has been removed from the text
6. Short abstract has been revised as suggested
7. Text has been changed to imperative tense
8. Details to some steps have been added
9. How staging is confirmed as been added to 3.1
10. Clarification on numbers of embryos has been provided in 4.10 and 2.1
11. A description in 5.2 has been added to clarify this point
12. Substeps have been created in #6
13. All figures are primary figures obtained by the authors
14. On discussion with the editor, we were told we could ignore this comment (the information requested is in the discussion)

Video: All video edits have been made as suggested. The VO was re-recorded. Commercial language has been removed, imperative tense is used, inserted video section added to show VO filling water container, arrows are now animated to appear as VO speaks.

Animal use concern: our university ethics board does not require approval of research using embryos.

**Response to Reviewer #1:**

1. The distinction between this protocol and others has been provided
2. This has been explained in the discussion

**Response to Reviewer #2:**

Title has been changed as suggested

#1.2a this has been added to the table of supplies

#3.2a This has been corrected

#3.2b this is more fully described now

#3.2c this edit has been made

#4.5 this has been revised throughout the text

Representative results: we have clarified the delay observed

Table: dimensions of petri dishes have been revised

Other comments:

This additional information has been added as suggested

**Response to Reviewer 3:**

The comments made by this reviewer were confusing and unclear. On discussion with the editor, it was agreed that many of the comments were statements with no actions required. References to the grid method have been included.