

## Protocol

# In Ovo Live Imaging of Avian Embryos

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## INTRODUCTION

Vertebrate development is best studied in an intact embryo model, but a robust interface between time-lapse microscopy and in vivo embryo health and maintenance can be difficult to achieve in model systems that rely on external factors for life support. This protocol presents a system for in ovo culture and time-lapse imaging of fluorescently labeled cells within living avian embryos, using a Teflon membrane that is oxygen-permeable and liquid-impermeable. The protocol describes the Teflon membrane assembly (the assembly size can be changed to fit smaller eggs, such as those of the quail), its interface with the egg window, and the use of an upright microscope and heated chamber. The use of the system is demonstrated in chick embryos by following individual fluorescently labeled neural crest cells, a multipotent stem cell-like population that differentiates into a wide range of derivatives and travels extensively throughout the embryo. By combining in ovo culture with confocal or two-photon four-dimensional time-lapse imaging, embryo health can be maintained for up to 5 d, and neural crest cell behaviors can be visualized for long periods of time (~36 h). This technique has been adapted to study somitogenesis.

## RELATED INFORMATION

Figure 1 summarizes the techniques described in this protocol. A protocol is also available for **Multi-Position Photoactivation and Multi-Time Acquisition for Large-Scale Cell Tracing in Avian Embryos** (Steen et al. 2010). Additional protocols are available for **Construction of a Heated Incubation Chamber around a Microscope Stage for Time-Lapse Imaging** (Kulesa and Kasemeier-Kulesa 2007) and **Photoactivation Cell Labeling for Cell Tracing in Avian Development** (Stark et al. 2008).

## MATERIALS

**CAUTIONS AND RECIPES:** Please see Appendices for appropriate handling of materials marked with <I>, and recipes for reagents marked with <R>.

### Reagents

Eggs, fertile, chick, White Leghorn (VWR WL51475)

*Quail eggs can be used as an alternative.*

Ethanol (70%)

India Ink, Fount (Pelikan) (10% in Ringer's solution)

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<R>Mix 1 mL of India Ink and 9 mL of Ringer's solution in a 15-mL tube.

Reagents for fluorescent labeling of cells

## Equipment

Beeswax, white (Fisher Scientific W25-500)  
Egg candler  
Egg incubator (G.Q.F. Manufacturing 1550)  
Equipment for fluorescent labeling of cells  
Filter paper, Grade 1 (Whatman 1001-185)  
Forceps, Dumont #5 (Fine Science Tools 11252-30)  
Hot plate  
Marker for drawing ring on eggshell  
Microscope chamber, heated

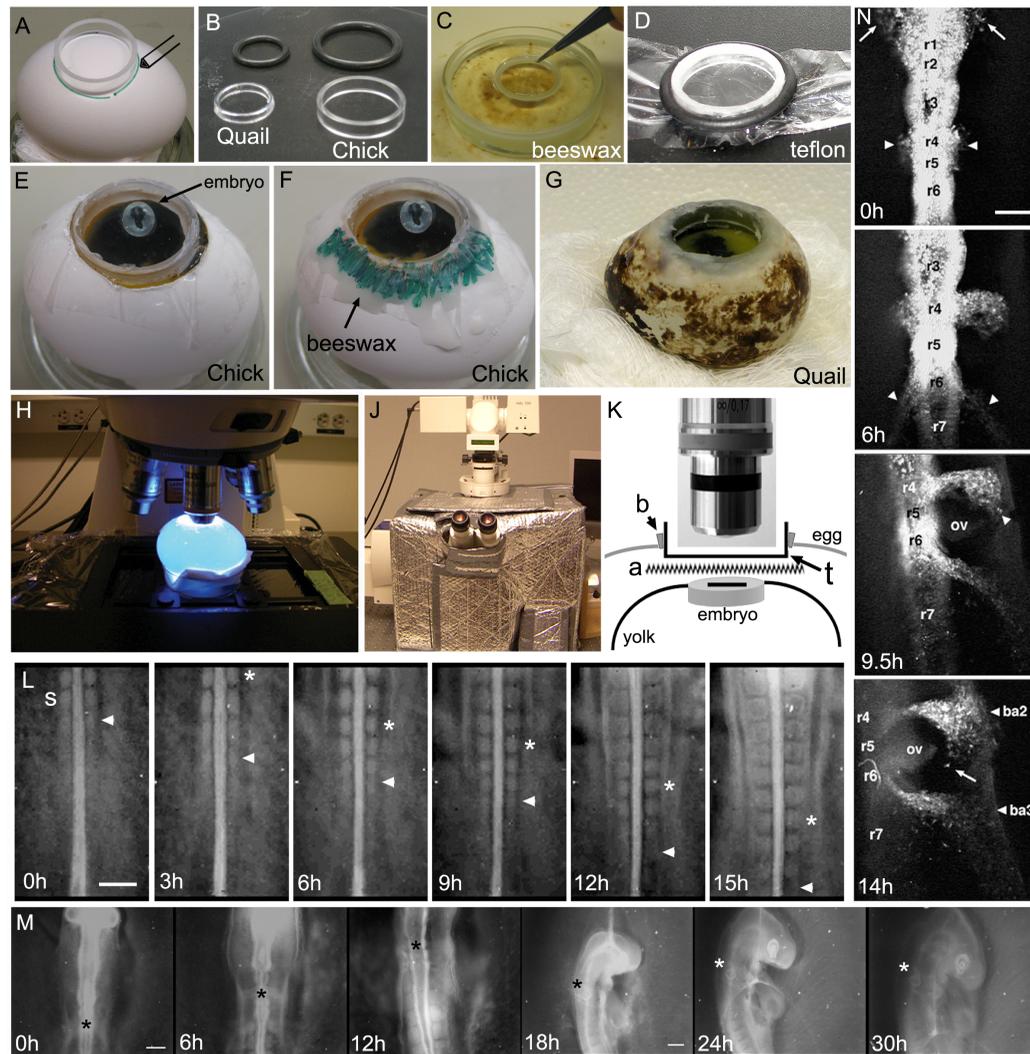
*The heated chamber (Fig. 1J) can be built in-house (see **Construction of a Heated Incubation Chamber around a Microscope Stage for Time-Lapse Imaging** [Kulesa and Kasemeier-Kulesa 2007]) or purchased commercially (e.g., PeCon XL LSM S).*

Microscope, upright, confocal, laser-scanning (e.g., Zeiss LSM 710 or equivalent)  
Needles, 18- and 25-gauge  
O-ring, rubber, 17 mm OD, 15 mm ID (for quail eggs) (Fig. 1B)  
O-ring, rubber, 24 mm OD, 21 mm ID (for chicken eggs) (Fig. 1B)  
Objectives  
Plan-Neofluar 10X/numerical aperture [NA] 0.3 objective for global field-of-view events  
Higher-magnification long-working-distance objective for high-resolution imaging  
Petri dish, glass, Kimax, 100 × 15 mm  
Petri dishes, 35 × 100 mm  
Ring, acrylic, 19 mm OD, 16 mm ID, 2.5 mm tall (for quail eggs) (Fig. 1B)  
Ring, acrylic, 26 mm OD, 22 mm ID, 5 mm tall (for chicken eggs) (Fig. 1B)  
Scissors, iris (Fine Science Tools 14063-09)  
Spatula, metal  
Syringes, 1- and 5-mL  
Tape, transparent  
Teflon membrane, high-sensitivity (YSI, Inc. 5794)  
Thermometer, digital (Fisher Scientific 15-077-17A)  
Transfer pipettes, plastic

## METHOD

### Egg Preparation

1. Rinse eggs with 70% ethanol.
2. Remove albumen:
  - From chicken eggs*
    - i. Using a 5-mL syringe and an 18-gauge needle, remove 3 mL of albumen from the caudal part of the egg.
  - From quail eggs*
    - ii. Using a 1-mL syringe and a 25-gauge needle, remove 1 mL of albumen from the caudal part of the egg.
3. Place the egg against the egg candler to locate the position of the blastoderm/embryo.



**FIGURE 1.** In ovo culture and imaging of chick embryogenesis. (A-G) In ovo imaging begins with marking the position of the presumptive window in the eggshell and creating a Teflon membrane that will fit into the window to provide an optical pathway to the embryo. (A) An acrylic ring is placed over the embryo (as determined by candling), and its position is marked. (B) The ring diameter can vary depending on the size of the egg or embryo. Acrylic rings and rubber O-rings appropriate for quail and chick eggs are shown. (C) An acrylic ring is dipped into melted beeswax before (D) being placed onto a Teflon membrane and secured in place with a rubber O-ring around its circumference. (E) After stretching and cutting the Teflon to fit tightly over the acrylic ring, the ring is placed into the hole in the eggshell over the embryo (the embryo contrast has been adjusted to better visualize its position). (F) Warmed beeswax (highlighted in green) is spread around the ring to seal it into the eggshell. (G) The same method applied to a quail egg. (H) The egg is placed on the microscope stage under the objective (highlighted with blue excitation light), which is (I) surrounded by a heated chamber. (K) The optical pathway through the Teflon window includes the Teflon membrane (t), laid above the albumen and Ringer's solution (a) that sits over the embryo on top of the yolk surface; (b) beeswax. (L) A typical in ovo time-lapse imaging session of somitogenesis shows somites (s) being added at a consistent rate of two somites/3 h. (Arrowhead) The last-forming somite; (asterisk) the two somites formed between each frame. The embryo was unlabeled, and the images were created by opening the shutter of a widefield microscope at 3-min intervals. The reflected light image was enhanced by a low-intensity light source shone onto the Teflon surface. (M) A typical in ovo time-lapse of head morphogenesis reveals a tremendous amount of growth and movement (rotation) of the early chick embryo (cf. the head size at 0 and 30 h). (Asterisk) The axial level of rhombomere 4. After 12 h, embryo growth requires a change to a lower-magnification objective (from 5X to 2.5X). The embryo was not labeled, and images were collected in the same manner as in L. (N) In ovo time-lapse imaging of an embryo labeled fluorescently with DII revealing migration of cranial and post-otic neural crest cells emerging from both sides of rhombomere 1 (r1, arrows) and r4 (arrowheads) at 0 h. By 6 h, neural crest cells emerge from r6 (arrowheads), and, as the embryo rotates (9.5 h), neural crest cells begin to reach the peripheral branchial arches near the front of the migratory stream (arrowhead). At 14 h, the embryo has rotated to reveal the position of the branchial arches (ba2 and ba3) and otic vesicle (ov). Individual migratory neural crest cells are seen moving at the migratory front (arrow). (L-N) Scalebars, 200  $\mu$ m.

4. Place an acrylic ring on top of the eggshell over the center of the blastoderm. Draw a circle around the ring (Fig. 1A).
5. Use transparent tape to cover the circled portion of the eggshell.
6. Using iris scissors, cut a hole in the eggshell along the line drawn around the acrylic ring. Remove most of the air bubbles on the exposed surface.
7. Inject the India Ink solution under the blastoderm with a 25-gauge needle to visualize and stage the embryo.
8. Perform cell labeling or manipulation of the embryo, as desired.

### Teflon Membrane Assembly

*The goal is to stretch the Teflon membrane across the acrylic ring, forming a taut drumhead. Prepared rings can be used for more than one imaging session if the Teflon membrane and beeswax are removed and replaced.*

9. Use the hot plate to melt the beeswax in a glass Petri dish.  
*Adjust the temperature of the hotplate to be high enough to melt beeswax, but low enough to touch when manipulating the acrylic ring on the surface of the plate.*
10. Holding the acrylic ring horizontally with forceps, submerge it such that three-fourths of the ring is covered with warmed beeswax for ~10 sec (Fig. 1C).  
*See Troubleshooting.*
11. Place the ring on a rectangular piece of Teflon membrane such that the beeswax is in contact with the Teflon.
12. Flip the assembly over so that the acrylic ring lies underneath the membrane.
13. Place a rubber O-ring on top of the Teflon. Push it onto the ring such that the Teflon membrane is taut and the O-ring is around the circumference of the ring (Fig. 1D).  
*See Troubleshooting.*
14. Let the ring assembly cool for ~5 min. After 5 min, remove the O-ring. Cut the excess Teflon membrane away.

### In Ovo Imaging Preparation

15. After finishing any manipulations or labeling of the embryo (see Step 8), place the ring assembly (from Step 14) Teflon-side down into the hole cut in the eggshell such that it lies directly over the embryo (Fig. 1E).  
*Be careful not to allow an excess air space between the embryo and Teflon surface.*  
*See Troubleshooting.*
16. Dip a metal spatula into the warmed beeswax. Gently spread the beeswax into the gap between the eggshell and the ring assembly. Continue to place the beeswax around the circumference of the ring and eggshell border, checking to make sure there are no leaks of the egg contents (Fig. 1F).  
*The egg is now ready to position on the microscope stage (Fig. 1H) such that the embryo is visible through the Teflon membrane with a low-magnification (e.g., 5X or 10X) objective or long-working-distance objectives.*  
*See Troubleshooting.*

### Heated Chamber Calibration

*Unless the system is changed (temperature or components), calibration need only be performed once.*

17. Insert the probe of a digital thermometer in the heated chamber. Tape the probe onto the microscope stage.
18. Turn on the heated chamber until the temperature at the stage is maintained at 38°C.  
*Record the time to reach equilibrium. This should be factored into the in ovo culture preparation to limit the time the egg is out of the incubator.*
19. Allow the system to equilibrate for 30 min before imaging.

## Imaging Parameters

20. Use a Plan-Neofluar 10X/NA 0.3 objective for global field-of-view events.  
*See Troubleshooting.*
21. Use a higher-magnification long-working-distance objective for high-resolution imaging (Fig. 1K).  
*See Troubleshooting.*
22. Use appropriate laser lines to excite the fluorescent compounds with which cells were labeled. For example, use 488-nm and 543-nm lasers to excite enhanced green fluorescent protein (EGFP) and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), respectively.  
*Photoactivation of GFP or GFP variants can be performed in ovo (see **Photoactivation Cell Labeling for Cell Tracing in Avian Development** [Stark et al. 2008]).*
23. Collect single-track z-sections at appropriate time and space intervals to produce a three-dimensional time-lapse movie.  
*Refocus to center the embryo as necessary.*  
*See Troubleshooting.*

## TROUBLESHOOTING

**Problem:** The acrylic ring is deformed after it is removed from the warmed beeswax.

[Step 10]

**Solution:** The acrylic ring might have contacted the bottom of the glass dish, causing the ring to deform. When holding the ring in the beeswax, do not let it touch the bottom of the dish.

**Problem:** The Teflon surface is wavy and not taut across the surface on the acrylic ring.

[Step 13]

**Solution:** The Teflon should be stretched circumferentially around the acrylic ring. If parts of the Teflon membrane do not adhere to the sides of the ring, roll the ring vertically along the hotplate such that the beeswax between the ring and the Teflon melts. Pull on the Teflon membrane so that it is taut around the ring. There is a bit of finesse to holding the ring with one hand and pulling the membrane tighter with the thumb and forefinger to stretch it over the ring. Gently warming the ring in the region of interest will help to melt the beeswax locally, allowing the membrane to be stretched across the ring with ease.

**Problem:** The embryo is not visible when the ring is placed over the vitelline membrane.

[Step 15]

**Solution:** There is an air bubble between the embryo and the Teflon surface. Remove the Teflon membrane assembly and reposition it over the embryo.

**Problem:** There is wet albumen around the circumference of the Teflon ring assembly after the ring has been sealed in place.

[Step 16]

**Solution:** There is a gap in the beeswax seal of the Teflon ring assembly with the eggshell. Reheat the beeswax, and seal the hole using the spatula to apply the beeswax to the eggshell and ring.

**Problem:** The objective is not compatible with the Teflon ring assembly.

[Steps 20, 21]

**Solution:** The circumference of the Teflon ring limits the diameter of the objective that can be used, unless the objective has a long working distance. Consider the following:

1. Change to a long-working-distance objective if one is not being used already.
2. Alter the height of the acrylic ring by using a hacksaw or similar, to cut the ring in half, through its height, before the Teflon is attached.

**Problem:** The embryo drifts dramatically during time-lapse imaging.

[Step 23]

**Solution:** There could be a hole or leak in the eggshell. This causes the surface of the vitelline membrane and embryo to drop away from the Teflon surface. Alternatively, an air bubble within the egg could have burst and changed the depth of the embryo surface. Refocus on the embryo, and remember to remove nearly all air bubbles before placing the Teflon membrane in the egg.

## DISCUSSION

We present here a technique developed for in ovo time-lapse imaging of live avian embryos. The technique uses a Teflon membrane window in the egg and is adaptable to upright widefield, confocal, and two-photon microscopy (Fig. 1E,K). The basic technique can be used with smaller-diameter rings to observe smaller eggs (e.g., quail). Previously, it was difficult to image cell dynamics in living avian embryos in their natural, intact environment because of the inability to prevent the embryo surface from drying after exposure to air through a window in the egg. The use of a Teflon membrane that is oxygen-permeable, liquid-impermeable, and optically transparent solves this problem.

We have used our technique to study chick somite formation (Fig. 1L) and neural crest cell migratory behaviors (Fig. 1N; Kulesa and Fraser 2000), including analysis of cell-cell and cell-environment interactions. Time-lapse imaging was begun in HH Stage 9 embryos (Hamburger and Hamilton 1951) and continued for 36 h (Fig. 1M). Embryo health was monitored separately by assessing somite development. Using this method, chick embryos can be re-incubated and imaged periodically through the Teflon window for up to 5 d.

Monitoring avian development in ovo allows for more accurate assessment of morphogenesis because cell-tissue microenvironment interactions and physical forces within the embryo remain intact. In contrast, a typical embryo in ex ovo culture on a membrane insert or paper ring will only grow for ~24 h, and embryo health must be maintained by external factors. Ex ovo avian embryo growth is typically complicated by embryo turning and pH changes in the culture medium. In ovo imaging circumvents these roadblocks.

One remaining complication of in ovo imaging is that the embryo, having more freedom of movement to grow, becomes rapidly three-dimensional, and its heartbeat makes it challenging to maintain a focused field of view. This is especially true as the decrease in yolk volume (resulting from normal embryo growth) lowers the surface of the embryo from the dorsal surface of the eggshell. Future designs might extend the time window without limiting resolution by providing a means to maintain the embryo near the surface of the egg and Teflon membrane. Nevertheless, the use of the Teflon membrane assembly and in ovo imaging techniques, when combined with fluorescently labeled cells and molecular perturbation, offers a powerful tool to gain insight into developmental events of the avian embryo in its natural setting.

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