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Avian Models in Teratology and Developmental Toxicology

Susan M. Smith, George R. Flentke, and Ana Garic

Department of Nutritional Sciences, University of Wisconsin-Madison, 1415 Linden Drive, Madison WI 53706

Abstract

The avian embryo is a long-standing model for developmental biology research. It also has proven utility for toxicology research both *in ovo* and in explant culture. Like mammals, avian embryos have an allantois and their developmental pathways are highly conserved with those of mammals, thus avian models have biomedical relevance. Fertile eggs are inexpensive and the embryo develops rapidly, allowing for high throughput. The chick genome is sequenced and significant molecular resources are available for study including the ability for genetic manipulation. The absence of a placenta permits the direct study of an agent's embryotoxic effects. Here we present protocols for using avian embryos in toxicology research including egg husbandry and hatch, toxicant delivery, and assessment of proliferation, apoptosis, and cardiac structure and function.

Keywords

chick embryogenesis; teratology; developmental toxicology; whole embryo culture; apoptosis; echocardiography

1. INTRODUCTION

Chicken and quail are long-established models for developmental biology research; much of what we know regarding vertebrate morphogenesis was first established with avian models and its popularity continues. Avian embryos and their cultured tissues also have proven utility for mechanistic studies into diverse toxicants including pharmaceuticals, environmental contaminants, industrial chemicals, heavy metals, and dietary components.

Avian models offer several advantages for toxicology research. Fertile eggs are inexpensive, commercially available, and require only an inexpensive incubator to develop. The shell is windowed to directly view or manipulate the embryo, and is easily resealed to continue development. The developmental stages are well documented and standardized (1, 2). The egg's self-containment and uniform size allows precise control of dose and exposure. Its ease of use, low cost, and rapid development lends it for high-throughput screens of developmental toxicity. Although birds are oviparous, the embryos develop an allantois and thus are developmentally closer to mammals than are reptiles, amphibians or fish. Avian developmental pathways are highly conserved with mammals and thus have direct

biomedical relevance. For toxicity work, the egg and embryo are directly treated, thus permitting direct study of the toxicant's embryonic effects and circumventing the contributions of maternal metabolism and placental transport. With respect to genetics, although avian strains are not highly inbred, commercial layer and broiler strains show limited genetic variability and high stability as most flocks have been closed for over four decades. Many strains are maintained as four-way grandparental crosses and are selected for uniform egg and growth characteristics. The chicken, quail and zebrafish genomes have been sequenced and significant genetic and molecular resources are available for its analysis (www.chicken-genome.org; www.ncbi.nlm.nih.gov/projects/genome/guide/chicken) including commercial microarrays. The avian embryo can be manipulated using electroporation of plasmid and retroviral vectors for transgenic study and knockdown can be achieved using morpholinos or siRNA (for protocols see 3, 4). Additionally most commercial antibodies directed against mammalian proteins have excellent specificity for the avian homologue. The non-profits AddGene (www.AddGene.org) and the Developmental Studies Bank (www.dshb.biology.uiowa.edu) are excellent resources for avian cDNA and antibody reagents, respectively. This review emphasizes the chick embryo (*Gallus gallus*); quail (*Coturnix japonica*) and zebra finch (*Taeniopygia guttata*) models also have excellent utility but can be more difficult to locate.

2. MATERIALS

2.1 Egg Handling

- 1 Unincubated fertile chicken eggs (*see* Note 1).
- 2 A refrigerator set at 15°C for egg storage.
- 3 Forced air egg incubator (e.g., Humidaire, New Madison OH; Model 1502, G.Q.F. Manufacturing Co., Savannah, GA) set to 38°C and 95% relative humidity, and ventilated to ambient air (*see* Note 2). Humidity is maintained using a pan of distilled water.
- 4 Incubator racks that hold eggs horizontal. These can be made inexpensively from egg cartons, egg packing trays or 1" thick Styrofoam sheets. They should not be so large as to impair air circulation.
- 3 Stereoscopic microscope (e.g. Wild M5A) equipped with ×1 objective, ×10 eyepieces, and low intensity fiber optic illumination to visualize the *in ovo*

¹Fertile eggs can be obtained from commercial vendors (e.g. SPAFAS, Hyline), or from a local facility that supplies fertile layer or broiler strains. Most studies utilize layer strains due to high egg productivity, but broiler strains are also acceptable. Always indicate the strain used, as their toxicant responses can vary. Store eggs at 15°C because colder temperatures kill the embryo; conversely, warmer temperatures will activate embryo development. Use eggs within 1 wk of arrival. For studies of early embryogenesis, the freshest eggs are best. Careful attention should be paid to storage and incubation temperatures to assure synchronous development. High quality eggs have ~95% fertility. Additional details on egg handling are found in several excellent reviews (17, 20).

²Cell culture or closed air incubators are unsuitable because the embryos must receive fresh, circulating air to prevent suffocation. Unopened eggs should be rotated using an automated rocker, or manually twice daily, to prevent embryos from adhering to the overlying shell. Opened eggs should not be rotated due to leakage. Because temperature dictates the rate of development, do not overcrowd the incubator to assure even air circulation. Distribute eggs randomly throughout the interior, rather than clustered by treatment. Maintain humidity using a tray of distilled water. Sterilize the tray weekly to prevent bacterial or mold growth, which can kill the embryos. For studies of hatched birds, fumigate the incubator and hatcher before beginning the study. To initiate incubation in the middle of night, start the eggs in an inexpensive styrofoam tabletop incubator and transfer eggs the following morning to a regular incubator having more precise temperature and humidity control.

embryo. The stage should be outfitted with an egg rest, for example a 65 mm Syracuse glass dishes holding a plastic ring, nest of cotton or modeling clay to form an egg rest.

2.2. Toxicant Treatment

There are many possible techniques to expose avian embryos to toxicants. All directly treat the egg or embryo; treatment of the hen has little value because eggs are produced on a 26hr “conveyor belt” and thus exposure is imprecise. The embryo obtains yolk nutrients via endodermal phagocytosis during pre-vascular stages and via yolk abstraction by the capillary bed once the allantois has formed. Thus yolk exposure mimics normal delivery and is less likely to elicit non-specific stressors. However, both untreated embryos and vehicle-only exposure must be included so that the non-specific exposure effects are understood. The exposure method used is driven by the experimental endpoints. *In ovo* exposure is less disruptive than whole embryo culture, embryos can develop to hatch, and high-throughput is feasible. Whole embryo culture is especially useful for mechanistic studies, to precisely control dose or staging, and to periodically assess the developmental progression. A number of avian organs and tissues can be studied in explant culture, e.g. retina, spinal cord, limb chondrogenesis, and liver, and the reader is directed to those publications for methods. We present three methods of toxicant exposure that have worked well in our avian embryology research.

2.2.1. *In Ovo* Toxicant Exposure by Yolk Injection

1. Stock concentration of toxicant dissolved in sterile water or 100% ethanol (USP grade), DMSO (cell culture grade; stored as frozen aliquots) or dimethylformamide (DMF). The final solvent concentration should not exceed 0.1% for ethanol and 0.05% for DMSO and DMF. Stored and handle in glass when possible.
2. Sterile water.
3. 10X phosphate buffered saline (PBS): 30 mM KCl, 1.3 mM NaCl, 20 mM K₂HPO₄, 80 mM Na₂HPO₄, pH 6.4. Sterilize before use.
4. 2.5 ml Luer-tip Hamilton gas-tight, glass syringe (Mfr #81401). The syringe interior can be sterilized using 100% ethanol.
5. 22g sterile needles.
6. Fertile eggs and incubator as detailed in (2.1).

2.2.2. *In Ovo* Targeted Slow Release using Microbead Implants—As an alternative to yolk injection, delivery can be targeted to specific tissues by implanting toxicant-impregnated microbeads or agar gel plugs (5, 6). Exposure is limited to the diffusion radius of the compound and is a function of substrate size and concentration (5). We usually use concentrations that create a 200–300 μ m diffusion radius. Select beads with the appropriate chemical properties, anion or cation exchangers for charged molecules, polystyrene beads for hydrophobic compounds, and Affygel Blue for peptides. We use the carrier solvents DMSO and DMF at final concentrations no greater than 0.05%. Controls

should receive solvent-treated beads because both DMSO and DMF can cause modest developmental delays.

- 1 Column filtration microbeads of 75–150 μm diameter. We use SM-2 (20–50 mesh) for hydrophobic compounds, AG1-X2 (200–400 mesh) for anions, AG50-X2 (200–400 mesh) for cations, and AffyGel Blue for proteins (all from BioRad, Hercules, CA). Beads should be washed extensively according to manufacturer's directions before use and stored in sterile water at 4°C. Compounds can also be impregnated in 1% high-melt agarose plugs (electrophoresis grade, Fisher).
- 2 Stock solution of toxicant dissolved in sterile water or 100% ethanol, DMSO or DMF. Generally the bead loading concentration is 100–1000-fold greater than that used for direct exposure and is determined experimentally (5, 6).
- 3 Medium 199 (Gibco/BRL, Bethesda, MD) or 0.05% Neutral Red in 0.9% saline, warmed to 38°C.
- 5 Egg white albumin, ~1–2 ml for each egg that is operated upon. This can be taken from the infertile eggs of the clutch.
- 6 Scotch brand Super 33+ black electrical tape (3M, Minneapolis, MN).

2.2.3. Ex Ovo Toxicant Exposure—There are several good techniques for *ex ovo* culture of avian embryos. Most are based on the original method of New (7), although that method requires specialized equipment and is technically challenging. Toxicology studies (8, 9) have typically used the EC embryo culture technique described by Chapman and Schoenwolf (10), and is an excellent reference clearly detailing the materials and protocols, including photos. Here we provide a single modification for toxicology research. Using EC culture, embryos will develop for 24–72hr with only minor developmental delays.

1. Stock solution of toxicant prepared as in (2.2.1) and at a concentration 1000-fold greater than its final concentration.
2. All other reagents and materials are identical to those described in Chapman and Schoenwolf (10).

2.3. Endpoint Assessment

A wide range of processes can be studied in the embryo and hatched chick, from organogenesis to cell differentiation to physiology, and behavior. We focus here on methods used in our laboratory to assess toxicant effects upon apoptosis, proliferation, and cardiac endpoints. We also include a hatch protocol because chicks are born precocious and can be studied for a range of activities within a few days of hatch. We presented detailed protocols for real-time calcium imaging and genetic manipulation of avian embryos in a previous volume of this series (4).

2.3.1. Apoptosis Assessment using Vital Dyes—There are many techniques to apoptosis assessment in avian tissues. The antibody #9661 (Cell Signaling, Danvers MA) recognizes activated cleaved caspase-3 (Asp175) in avian tissues and works well using

standard immunohistology methods on paraformaldehyde-fixed tissue. TUNEL detects cleaved DNA ends and is discussed in (2.3.2). Vital dyes detect apoptosis and phagocytosed cellular particles in the living embryo. These dyes differ in their chemical properties. LysoTracker Red is permanent and can be visualized in fixed tissue (11), whereas acridine orange is impermanent and will not affect subsequent immunostains (12). Both are fluorescent. Vital dye assays are rapid and easy; however, pilot studies using TUNEL or caspase-3 must first confirm that the vital dye detects apoptosis and not autophagy.

1. 1X Tyrode's buffer with calcium (TWC): 137 mM NaCl, 2.7 mM KCl, 1.36 mM CaCl_2 , 0.5 mM MgCl_2 , 0.3 mM Na_2HPO_4 , 12 mM NaHCO_3 , 5.6 mM glucose, pH 7.8–8.2
2. Acridine orange (5 $\mu\text{g}/\text{ml}$ in TWC; Sigma/Aldrich) or LysoTracker Red (0.5 μM in TWC; Invitrogen). Acridine orange is a carcinogen and appropriate handling precautions should be taken.
3. Fluorescent microscope (epi-illumination or inverted) outfitted with FITC (acridine orange) or Texas Red (LysoTracker Red) filter set, $\times 10$ objectives, and a digital camera with imaging software.
4. Paper rings cut from Whatman #1 filter paper, outer diameter 1.5 cm, inner diameter 5–6 mm.
5. Glass slides.
6. Forced air incubator with rocker set at 37°C .

2.3.2. Apoptosis Assessment using TUNEL—Apoptotic cells can be identified using terminal deoxynucleotidyl transferase (TdT) to label cleaved DNA ends using epitope-tagged nucleotide such as BrdU. TUNEL detects apoptosis in both paraffin-embedded sections (13, see below) and in whole chick embryos (see 14 for this protocol).

- 1 Avian tissue sections cut to 5–7 μm thickness and mounted on charged glass slides (Superfrost Plus, Fisher #12-550-15). Frozen sections should also work well.
- 2 Dewaxing solutions: 100% Hemo-De or xylene, 100% and 95% ethanol, 70%, 50% and 30% ethanol in ddH₂O.
- 3 1 x PBS
- 4 10 mg/ml Proteinase K (Promega #V3021) stock in 50 mM Tris-HCl, 5 mM EDTA pH 7.5. Store as 200 μl aliquots at -20°C .
- 5 20% glycine in ddH₂O (unbuffered, sterile filtered). Dilute to 2% in H₂O prior to use.
- 6 10% formalin freshly diluted in 1x PBS.
- 7 5x TdT buffer: 1 M Cacodylic acid-NaOH pH 6.6 containing 1.25 mg/ml BSA (Fraction V cold alcohol precipitated, Fisher #BP1605-100). Adjust the pH prior to BSA addition.

- 7 BrdUTP Reaction Mixture. Make fresh as follows: 64 μ l sterile ddH₂O + 20 μ l 5X TdT buffer + 10 μ l 10 mM CoCl₂ + 4 μ l 2 mM BrdUTP (Sigma B-0631) + 2 μ l TdT enzyme (5 units/ μ l, Promega #M1871). Use 200 μ l per slide, made fresh immediately before use. The final composition is 1x TdT buffer, 1 mM CoCl₂, 80 μ M BrdUTP, 10 units TdT/100 μ l/slide.
- 8 Tris-buffered saline plus Tween (TBST): 140 mM NaCl, 2.7 mM KCl, 25 mM Tris-HCl, 0.1% Tween-20, pH 7.5.
- 9 Heat-inactivated Goat Serum (HIGS): Incubate 100% goat serum (Sigma #G6767) for 30 min at 57°C. Cool to room temperature. Freeze in 10ml aliquots at -20°C.
- 10 G3G4 anti-BrdU antibody (Developmental Studies Hybridoma Bank #G3G4). Usually a 1:50 dilution works well but this should be empirically determined.
- 11 Tagged secondary antibody directed against mouse IgG. We currently use a 1:500 dilution of Alexis 594 donkey anti-mouse (#A-21203, Molecular Probes, Eugene OR).
- 12 50 μ g/ml DAPI stock solution in ddH₂O.
- 13 Super HT™ PAP pen (RPI #195505).

2.3.3. Proliferation Assessment using BrdU—Several approaches work well to detect proliferating cells in avian tissues including anti-PCNA and BrdU incorporation (13). For the latter, detect apoptosis on the same section using a non-BrdU method (e.g. activated caspase-3).

1. Fertile eggs incubated to the desired developmental stage, materials for windowing eggs in ovo as in (2.1).
2. 10 mM BrdU stock solution in 1x PBS or 1x DMEM, stored in frozen aliquots at -20°C. Warm to 37°C immediately prior to use.
3. Scotch Super 33+ electrical tape.
4. Materials for embryo harvest as described in (2.3.1).
5. 4% paraformaldehyde in 1x PBS, freshly prepared and chilled to 4°C prior to use (see Note 3).
6. 5–7 μ m thick paraffin tissue sections of BrdU-labeled chick tissue, mounted on charged glass slides (Superfrost Plus, Fisher #12-550-15).
7. Solutions to dewax and rehydrate sections: Hemo-De (Fisher), 100% and 95% ethanol, 70%, 50% and 30% ethanol diluted in ddH₂O.

³As a tissue fixative 4% paraformaldehyde should always be prepared fresh on the day of use. Paraformaldehyde dissolves poorly in water, at pH<7, and at room temperature. To prepare it, don the appropriate protective personal equipment (face mask, gloves, lab coat) and weigh out 4g paraformaldehyde (96%, pelleted, Acros #41678-5000). Add it to 100 ml of 1xPBS in a loosely capped flask or bottle. In a fume hood, heat the solution to 60°C with gentle stirring, using either a stir plate or microwave. With heating the paraformaldehyde melts into monomers that expand slightly, become translucent and then disappear completely. Cool the solution to 4°C before use; it will not recrystallize once dissolved.

8. 1 x PBS.
9. 4 N HCl in water.
10. Blocking solution: 5% BSA (Fraction V, Sigma) + 0.05% Triton X-100 in 1 x PBS.
11. G3G4 anti-BrdU antibody (Developmental Studies Hybridoma Bank #G3G4). Usually a 1:50 dilution works well but this should be empirically determined.
12. Tagged secondary antibody directed against mouse IgG. We currently use a 1:500 dilution of Alexis 594 donkey anti-mouse (#A-21203, Molecular Probes, Eugene OR).
13. 50 ug/ml DAPI stock solution in ddH₂O.
14. Coverslip mounting medium such as FluoroMount-G (Southern Biotech, Birmingham AL).

2.3.4. Embryo Echocardiography—The heart is a dynamic organ and static morphological measures provide an incomplete portrait of cardiac function across the contraction cycle. Advances in the resolution of ultrasonograph probes allow assessment of embryonic heart function as early as 3.5 days incubation. Ventricular wall thickness and stroke volume can be precisely quantified. Doppler mode also captures blood flow direction and the backflows characteristic of fenestrations and valve failures. Provided access to sufficiently sensitive ultrasonography, the method for avian embryos is actually quite simple. This protocol works well to image toxicant-challenged chick hearts at 5.5 – 7.5 days incubation (15); it is based on McQuinn et al. (16) and the reader is directed there for additional details.

1. Fertile eggs incubated to the desired developmental stage or hatched chicks.
2. Open-top 42°C circulating water bath outfitted with a stable platform immersed 1.5 cm below the water surface.
3. Hexagonal plastic weigh boat, size (Fisher #02-202B): inner diameter bottom 4.7cm, inner diameter top 6.7cm, depth 1.5cm.
4. Vevo 770 ultrasonograph (Visual Sonics, Toronto, Canada) with a 55-mHg transducer, capable of M-mode, B-mode and Doppler imaging.

2.3.5. Heart Fixation—The heart changes its size and shape across the contraction cycle; these contractions alter the heart wall thickness and chamber volume. Any static assessment of heart morphology must fix hearts in uniform contractility. This is achieved by arresting the hearts at end-diastole using a solution of 20% KCl in 1xPBS.

1. Fertile eggs incubated to the desired stage of development.
2. 20% KCl in 1xPBS.
3. 0.9% saline.
4. 4% paraformaldehyde in 1 x PBS, freshly prepared and cooled to 4°C.
5. 5–20 cc syringe fitted with 25g needle.

2.3.6. Hatching of Chicks for Functional Study

1. Hatcher. There are several ways to achieve this, ranging from a lower incubator tray that is held horizontal and not rocked during the last stages of incubation, to a separate incubator having higher humidity and greater air circulation. The same incubator can be used for hatch, provided the rotator is turned off and an additional humidifying tray is provided. It is critical that the tray and eggs both be horizontal, so that the chick is correctly positioned for hatch. The tray should be a wire mesh so that chicks can stand on it without falling and so that feces pass through.
2. Brooders. These are easily located from commercial vendors. The brooder should be temperature controlled with a range from 100°F to 70°F. One side should be unheated so that chicks do not overheat, and covered so they cannot escape. Wire bottoms work well. It should contain ad lib food and water.

3. METHODS

3.1. Egg Handling and Husbandry

1. Remove eggs from 15°C refrigerator and bring to room temperature (2–4 hr). Transfer to flat trays and incubate at 38°C, 95% humidity. At the desired time of development, open 3–4 eggs to confirm the correct stage is achieved. Use the criteria of Hamburger and Hamilton (2) to establish the embryo's developmental stage. Incubate eggs horizontally rather than blunt end up, to position the embryo for developmental manipulations. The embryo resides at the uppermost position directly beneath the shell. Visualize the embryo by cutting a small hole in the shell using small dissecting scissors (3.2.1), or harvest the embryo by carefully cracking the egg into a 100 mm Petri dish (3.2.2). If incubating closed eggs for more than 72hr, gently rock or rotate the eggs 2–3 times daily to prevent the embryo from adhering to the inner shell. Many egg incubators are equipped with a rocker device.

3.2. Toxicant Exposure Techniques

3.2.1. *In Ovo* Toxicant Exposure

1. Prepare solutions just prior to injection. We prefer to deliver toxicants in sterile 1× PBS. The toxicant may be initially dissolved in DMSO or ethanol. However, the final solution should be diluted into 1× PBS such that the carrier solvent does not exceed 1% for ethanol and 0.1% for DMSO. Controls should use the same solvent dilutions without the toxicant. Store and handle all solutions in glass, not plastic. Use a chemical hood when appropriate.
2. Remove 1 dozen eggs from incubator. Open 3–4 eggs to assure that the embryos have reached the desired developmental stage. Position eggs horizontally, blunt end outward on a styrofoam tray or on dishes. Rotate each egg 180° along its horizontal axis to dislodge the embryo from the overlying shell. Clean the injection site by wiping the egg's blunt end with a tissue soaked in 95% ethanol. Using a metal dissecting probe (Fisher #08-965A) or 16g needle, through the shell's blunt end pierce a small hole that is only large enough to accommodate the injection needle. Insert the probe no deeper than 5 mm such that only the air sac is pierced.

3. Using a 2.5 ml glass syringe (e.g. Hamilton) fitted with a 1 ½” 20g sterile needle, insert all but ¼” of the needle into the hole, horizontally and with bevel side down. This places the injection into the approximate yolk center; affirm the technique with test injections of India ink. Slowly (over 3–5sec) inject 250 µl of saline or ethanol solution into each egg. The air sac volume is ~350 µl and larger injection volumes will crush the embryo. Rotate the egg 180° along its horizontal axis. Seal the injection hole with a small piece of cellophane tape, label the treatment on the shell using pencil, and immediately return eggs to incubator. Over 15–90 min, the saline and toxicant diffuse upward to contact the embryo and then diffuse through the embryo, yolk and white. Because the embryo abstracts nutrients from the underlying yolk, this method mimics the normal exposure route.
4. As an alternate *in ovo* exposure route, position the egg vertically and make a small hole in the upward blunt end as above. Inject the solution directly into the air sac without penetrating the embryo or yolk beneath. Seal the hole with tape as above. Because liquids placed directly on the embryo can be damaging, especially at early stages (8), we recommend using the yolk-injection method.
5. As a third method of *in ovo* exposure, make a window above the embryo as described in 3.2.3 below. Make the window no larger than 2–3 mm diameter. Gently drip the solution directly atop the embryo in a volume of 50–100 µl. Reseal the holes with Super 33+ or cellophane tape.

3.2.2. Targeted Slow-Release Delivery using Impregnated Implants

1. For microbeads, load the toxicant onto the substrate by transferring ~5–10 µl of beads to a 1.5 ml Eppendorf tube. Spin briefly, decant. Add 50 µl of the desired agent to beads. Mix vigorously on a benchtop shaker (Vortex Genie 2, speed 5) for 20 min. Pellet briefly in microfuge. Decant. (See Note 4). Add 1 ml 0.05% Neutral Red in 0.9% saline to stain beads for subsequent visualization. Shake for 10 min. Spin. Decant. Add 1 ml 0.9% saline. Shake for 10 min. Spin. Decant; repeat the saline wash twice more. Transfer beads to a 35 mm tissue culture dish.
2. For agarose plugs, prepare a solution of 2% melted agarose in 1xPBS. Cool to 50–55°C and mix with an equal volume of toxicant at twice the desired concentration. Pour into a 35 mm tissue culture dish. Cool. Immediately prior to use, cut out individual plugs with a capillary tube or pasteur pipette to assure uniform size and delivery. Gently aspirate the plug onto or adjacent the tissue of interest. For solvent carriers such as ethanol or DMSO the final solvent concentration should not exceed 0.01% for DMSO and 0.1% for ethanol.
3. Remove from the incubator four eggs at the appropriate stage of development. Rotate each egg 180° horizontally to dislodge the embryo from the overlying shell.

⁴The precise concentration used is determined experimentally, and is generally 100–1000-fold greater than that used for direct exposure. Washed beads release the adsorbed compound following steady-state kinetics for ~18–24 hr; a detailed discussion of the kinetics is found in (5). The bead loading time can be increased to 1 hr, depending on the compound of interest. Beads must be used immediately upon preparation. If the embryo is going to develop longer than 24 hrs, 1–2 drops of antibiotic (penicillin-streptomycin) can be applied to minimize bacterial growth; egg white lysozyme has modest antibacterial properties.

Using a 12 ml syringe, withdraw ~1 ml of albumin through the injection hole at the blunt end; insert the needle downward to avoid damaging the yolk or embryo.

Using a metal probe, chip a small hole directly above the embryo, no larger than 5–10 mm diameter. Gently tease open the underlying membrane. The embryo should immediately drop into the egg; if not, repierce the air sac. Rock the egg slightly to center the embryo beneath the hole. If not immediately using the egg, loosely seal the hole with a small piece of low-tack cellophane tape.

4. Remove the cellophane tape and view the opened egg under the stereomicroscope. Add one drop (30 μ l) of warmed Medium 199 atop embryo to visualize it; wait 10–20 sec for dye uptake.
5. Using a mouth capillary pipettor, hand-held pipettor (5 μ l), or similar device, pick up a bead or an agar plug and place it in the desired location of the embryo. Let the embryo rest for 30–60 sec; a slight drying of the embryo's surface is usually sufficient to hold the bead or plug in place. For larger structures (e.g. limb bud) a small hole can be teased in the structure using a fine tungsten needle, and the bead tucked within it. Discard eggs with bleeding or a pierced yolk as they will not survive.
6. Gently refill the egg with reserved albumin so that the embryo rises to the opening. Seal the egg with a small piece of Super 33+ electrical tape. Rotate the egg 30° along the horizontal axis such that the embryo is under shell rather than tape. Notate the treatment on shell using pencil. Reincubate the egg to the desired developmental stage. Do not rock opened eggs because the surgery hole may leak.
7. Beads can be removed to investigate later development. Gently remove the electrical tape using a fingernail. Remove the bead using gentle aspiration from a hand-held pipettor (5 μ l). Reseal the egg with fresh electrical tape and reincubate. Keep the tape and hole size as small as possible to facilitate the embryo's development.

3.2.3. Ex Ovo Toxicant Exposure—Detailed methods on the EC embryo culture technique are found in Chapman and Schoenwolf (10). We provide here the one modification for toxicology research and it involves the preparation of the culture plates.

1. Prepare a stock solution of the toxicant at 1000-fold excess of the final concentration desired.
2. Prepare the agar-albumen culture media as described (10, 17). While the media is still liquid and immediately after adding the penicillin/streptomycin, add the chemical toxicant. Also prepare plates containing the same concentration of solvent-only as a control for non-specific effects.
3. Pour the warm mixture as directed into 35 mm Petri dishes using 2.5 ml per plate as described in (10).
4. Plates can be used for up to one week following preparation if they are stored at 4°C in an airtight container. However, this time is also dictated by the toxicant's stability.

3.3. Endpoint Assessment

3.3.1. Apoptosis Assessment using Vital Dyes

1. Prewarm all solutions to 38°C.
2. Harvest the embryo by gently pressing the horizontal egg against the bottom of a 100 mm Petri dish to fracture the shell bottom. Insert the thumbs into the fracture and gently pull the bottom shell apart, still holding it against the dish bottom. As the shell lifts away the contents should gently drop, embryo-upward, into the dish. Immediately place a filter paper ring over embryo, centering the open hole over the embryo. Using small scissors quickly cut completely around the ring and free the embryo from its membranes. Using forceps (Dumont #5) or a spatula (e.g. Spoonula™, #14-375-10, Fisher Scientific), transfer the embryo and ring to a Petri dish containing prewarmed TWC.
3. Using a Pasteur pipette or gentle rocking in a 37°C incubator, gently swish solution beneath the embryo to remove any adhering yolk, which has significant autofluorescence and will interfere with the analysis. Hold embryos at 38°C until ready to use. With practice, it should take no longer than 5 min to harvest 1 dozen embryos. Harvest no more embryos than can be processed in one-half hour.
4. Transfer the embryos, with or without their paper rings to a fresh, prewarmed dish containing the vital dye of interest. For acridine orange, incubate embryos in 5 µg/ml acridine orange in TWC for 5 min 38°C with gentle rocking. (*See Note 5*). For LysoTracker Red, incubate embryos in 50 µg/ml LysoTracker Red in TWC for 30 min 38°C with gentle rocking 5. For both dyes, transfer embryos to fresh TWC at 38°C. Destain embryos 15 min 38°C with gentle rocking.
5. Using forceps or a spatula, quickly transfer an embryo to a clean glass slide. Viewing through a stereomicroscope, position the embryo and pull away any membranes using tungsten needles. Transfer the slide to a fluorescence microscope and immediately visualize and photograph the apoptotic cells using the appropriate fluorescence settings; we generally use x10 lenses. For LysoTracker Red use 577 nm excitation and 589 nm emission (Texas Red settings) and for acridine orange use 502 nm excitation and 526 nm emission (FITC settings). Mount only the number of embryos (3–4) that can be imaged in 5 min, and work quickly because autofluorescence increases as the embryo cools. Exclude all yolk and extraembryonic membranes from the view field as their strong autofluorescence will disrupt the exposure.
6. Code the images and quantify the signal using imaging software such as NIH Image or Metamorph. For LysoTracker Red, the embryos can be fixed using standard techniques (e.g. 4% paraformaldehyde in 1xPBS) and the signal detected

⁵Embryos must be maintained at 38°C because cooling reduces their ability to export the vital dye, and background fluorescence will rise significantly. Because avian embryos significantly autofluoresce in the FITC range, LysoTracker Red offers a cleaner background signal. Protect reagents and embryos from light. It may be necessary to tease away the cephalic membranes using fine forceps to fully expose the head for staining and imaging.

in whole mount or in paraffin- or cryopreserved sections. Note that LysoTracker Red signal is lost when antigen-retrieval methods are used on the tissue.

3.3.2. Apoptosis Assessment using TUNEL

1. Dewax the paraffin sections using Hemo-De or xylene, 2 changes for 5 min each.
2. Rehydrate the sections through a graded ethanol series: 100%, 100%, 95%, 70%, 50%, 30%, 2min each, followed by two changes of 1xPBS 5 min each.
3. Incubate the slides in 20 µg/ml proteinase K in 1x PBS at room temperature. Decant. Incubate the slides in 2% glycine for 30 sec to terminate the reaction. The exact time for proteolysis is determined experimentally and ranges from 0.5–20 min depending on the tissue.
4. Re-fix the tissue by incubated 5 min room temperature in 10% formalin in 1xPBS. This is necessary to prevent tissue loss after proteolysis. Wash the slides 3 x 5 min in 1x PBS.
5. Using a Super PAP HT pen, draw a perimeter around the sections to be reacted. Add 100 µl of the TdT/BrdU reaction mixture onto the slide. Incubate in a humidified chamber. Incubate at 37°C for 90 min. Wash the slides 2 x 5 min in 1 x PBS.
6. Block the tissue with 10% heat-inactivated goat serum (HIGS) in TBST buffer for 60 min room temperature. Incubate in a humidified chamber.
7. Add 100 µl of G3G4 anti-BrdU antibody diluted 1:50 in 1% HIGS in TBST. Incubate 90 min at room temperature in a humidified chamber. Wash the slides 3 x 10 min in 1x PBS.
8. Add 100 µl of secondary antibody diluted in 1% HIGS in TBST. Incubate 90 min at room temperature in a humidified chamber. Wash the slides 3 x 10 min in 1x PBS.
9. Counterstain slides with 0.5 µg/ml DAPI in ddH₂O for 20 min. Wash twice with ddH₂O. Coverslip using FluoroMount-G. Let dry overnight and seal cover slip edges with 1:1 permount:xylene or clear nail polish.

3.3.3. Proliferation Assessment using BrdU

1. Window egg as in (3.2.2) to expose the embryo. Viewing embryo under a stereomicroscope, use a tungsten needle to make a small opening in the membrane over the tissue of interest.
2. Pipette 50 µl of 10 mM BrdU in 1xPBS prewarmed to 37°C. Seal the egg with Super 33+ tape. Reincubate embryo for 4hr.
3. Harvest the embryo using the paper ring method of (3.3.1). Rinse in ice cold 1x PBS and incubate at 4°C in fixative (e.g. 4% paraformaldehyde in 1x PBS). Fixation time is experimentally determined and dictated by the embryo's developmental stage and size. Prepare 5–7 µm thick paraffin sections on charged glass slides using standard techniques.

4. Dewax and rehydrate the tissue sections as described in step 1 of (3.4.2).
5. Incubate the slides in 4N HCl for 20 min at room temperature. Wash slides 15min in 1xPBS.
6. Block the tissue with 5% BSA/0.05% Triton X-100 in 1xPBS for 60 min room temperature. Incubate in a humidified chamber.
7. Add 100 μ l of G3G4 anti-BrdU antibody diluted in 5% BSA/0.05% Triton X-100 in 1xPBS. Incubate overnight at 4°C in a humidified chamber. Wash 3 x 10 min in 1x PBS.
8. Add 100 μ l of secondary antibody diluted in 5% BSA/0.05% Triton X-100 in 1xPBS. Incubate 90 min at room temperature in a humidified chamber. Wash 3 x 10 min in 1x PBS.
9. Counterstain slides with 0.5 μ g/ml DAPI in ddH₂O for 20 min. Wash twice with ddH₂O. Coverslip using FluoroMount-G. Let dry overnight and seal cover slips with 1:1 permount:xylene or clear nail polish.

3.3.4. Echocardiography of *In Ovo* Embryos

1. If possible, incubate the eggs in the same room housing the echocardiography equipment. Minimize transport distances because the vibrations can introduce vascular microtears that quickly kill the embryo. Remove one egg at a time from the incubator. Gently crack it into a hexagonal weigh boat and transfer the boat to an immersed platform in a 42°C waterbath. Make sure the embryo is atop the intact yolk and is not twisted as this impedes blood flow.
2. Quickly position the Vevo 770 ultrasonograph probe over the heart. Because of the chick heart's position, one can simultaneously view all four chambers. Adjust the probe position to view the largest diameter across the ventricles. Capture both cross-sectional B-mode images and M-mode images. Good recordings are usually obtained in the first 5–7 minutes of culture; thereafter the heart rate slows as the chick cools.
3. Use the Doppler imaging mode to measure cardiac outflow at the level of the mitral and tricuspid openings and the interventricular foramen. Doppler-mode electrocardiography provides unequivocal identification of dysmorphic foramen in the septa and outflow tract. Preserve the hearts for morphological and histological analysis as described in (3.3.5).
4. From the M-mode images, measure the end diastolic and systolic left ventricular (LV) diameters and anterior wall (AW) and posterior wall (PW) thickness using leading edge-to-leading edge convention. Obtain these values from the mean of at least three consecutive and high-quality cardiac cycles. Calculate LV fractional shortening as $[(LV\ diameter_{diastole} - LV\ diameter_{systole}) / LV\ diameter_{diastole}] \times 100$. Estimate LV mass using the formula $[1.05 \times ((PW_{diastole} + AW\ diastole + LV\ diameter\ diastole)^3 - (LV\ diameter\ diastole)^3)]$.

3.3.5. Cardiac Fixation

- 1 For embryos younger than 7–10 days incubation, dissect the embryo from its surround and directly place it into 20% KCl in 1xPBS. Several minutes after the heart stops beating, transfer the embryo to the desired fixative (e.g. 4% paraformaldehyde in 1 x PBS) for further processing and analysis. It is best to leave the heart in the embryo to achieve the proper orientation for sectioning.
- 2 For older embryos or hatched chicks (euthanized using appropriate institutional care guidelines), open the sternal area with scissors to expose the heart. While the heart is still beating, inject 20% KCl in 1xPBS into left ventricle near the apex until the heart stops in diastole. Then cut the descending aorta to start the blood flow. Insert a 25g needle and syringe into the cardiac apex and push the needle up and into the chick's left ventricle and toward the atrium. Do not confuse your left with the chick's left. Over 1–2 min perfuse with 0.9% saline, using at least 20 ml, until the organs lose their deep red color. Then perfusion fix with 4% paraformaldehyde in 1xPBS. Remove the heart by cutting the pulmonary artery and aorta, leaving ~10 mm attached so that great vessel defects can be observed. Rinse with PBS or saline. Transfer heart to a 35 mm scintillation vial filled with cold fresh fixative (10 vol fixative to tissue). Fix overnight at 4°C with gentle rocking.
- 2 To identify structural malformation we use the right ventricular microdissection approach described by Pexieder (18, 19). Alternately, hearts can be dehydrated through ethanol and embedded in paraffin for histological analysis.

3.3.6. Hatching of Chicks for Functional Study—Hatching requires strong, coordinated motor function and an egg tooth at the beak's maxillary tip. Toxicants can impair those actions and hatch protocols should account for that possibility.

1. Chicks hatch at 21 day incubation. Daily inspect incubated eggs for dead embryos. This is done by holding the egg against a strong light source such as an egg candle or strong flashlight. It may be necessary to perform this in a darkened room. Handle the egg very gently to prevent tearing of the delicate vasculature. The shadow of the healthy vasculature will be seen to spread across the shell's inner surface. In a dead embryo the shadow has collapsed centrally as the embryo drops inward; such eggs should be immediately discarded to prevent bacterial or fungal contamination of healthy eggs.
2. Three days prior to hatch, either shut off the automated turner or transfer the viable eggs to a hatching incubator, positioning the eggs horizontally on a wire mesh surface. Cessation of rocking is essential so that the chicks are properly positioned for hatch. Increase the humidity by adding an additional water pan and open an additional air port to increase air circulation. Peeping is heard from 2hr to 24hr prior to hatch. Young chicks poorly thermoregulate so minimize opening the hatcher to maintain the heat and humidity. Pipping is visible as a hole poked out of the shell. Cracks extend from it as the chick presses against the shell. The time from pip to hatch ranges from 1hr to 24 hr. Some eggs may continue to hatch for 2

days thereafter. Inspect unhatched eggs for presence of pip holes and attempts to split the shell. Note failure-to-hatch as a treatment outcome. Protocols should have predetermined criteria to decide if chicks will be assisted to hatch. This may be necessary for toxicants that impair motor function or beak development or for certain genetic mutants such as *limbless*.

3. Once the chicks have dried and resorbed the yolk, usually 12–24hr post-hatch, transfer them to a brooder; one side of the brooder should be held at 100°C and one side left unheated. Reduce the temperature by 5°C weekly until it reaches 70°C. Provide commercial chick food and water. Chicks are a strongly social species and should be group-housed for normal development. Monitor the birds in case weaker chicks are mistreated by others in the flock. Attach a numbered wingband to each chick for identification and to permit double-blinded study. Legbands are too constrictive for the chick's rapid growth. Thoroughly clean and disinfect the hatcher and incubator prior to reuse.

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Figure 1.

(A) A windowed fertile chick egg. An embryo having 3 somites (arrow) is visible atop the yolk and is surrounded by the transparent area pellucida. (B) Same embryo 24hr later, harvested using the filter ring method and transferred to a tissue culture dish. The embryo, now having 18 somites, is centered within a paper ring cut from Whatman #1 filter paper. The outer ring diameter is 15mm and inner opening is 5mm. The nascent extraembryonic vasculature is clearly visible surrounding the embryo proper. (C) *In ovo* chick embryo having 4 somites (stage 8) viewed dorsally and stained with 30 μ l of 0.05% neutral red. Resting on the right neural fold is an AG50W-X8 resin bead (arrow, BioRad) of 100–200 mesh and having a 100–250 micron diameter. We typically use this bead size for chick embryo work.