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Dissection and Downstream Analysis of Zebra Finch Embryos at Early Stages of Development

--Manuscript Draft--

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Abstract:	The zebra finch (Taeniopygia guttata) has become an increasingly important model organism in many areas of research including toxicology1,2, behavior3, and memory and learning4,5,6. As the only songbird with a sequenced genome, the zebra finch has great potential for use in developmental studies; however, the early stages of zebra finch development have not been well studied. Lack of research in zebra finch development can be attributed to the difficulty of dissecting the small egg and embryo. The following dissection method minimizes embryonic tissue damage, which allows for investigation of morphology and gene expression at all stages of embryonic development. This permits both bright field and fluorescence quality imaging of embryos, use in molecular procedures such as in situ hybridization (ISH), cell proliferation assays, and RNA extraction for quantitative assays such as quantitative real-time PCR (qtRT-PCR.) This technique allows investigators to study early stages of development that were previously difficult to access.
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*Cover Letter

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Dear Editors,

We respectfully submit our article entitled, "Dissection and Downstream Analysis of Zebra Finch Embryos at Early Stages of Development " for publication in JoVE. We feel that this information would provide a valuable addition to the literature. Several years ago when we were just entering the field of early avian embryology using the zebra finch model system, we found not guidelines on how to dissect the embryos from such small eggs. We had to work out this procedure from scratch. Within the past few years zebra finch has become a premier model system, particularly for language acquisition and toxicological studies. However there are few studies employing embryos from the early stages of development. The lack of an appropriate stage guideline as well as the absence of clear guidelines on how to obtain embryos from early stages has surely contributed to this. Our lab has now published a guideline for staging (Murray et al., J. Morph., 2013), and we wish to address the lack of a clear dissection protocol with this submission to JoVE. Obviously JoVE is the perfect venue for such a manuscript given that explanation of the technique is enhanced so much by visualization. Our group has worked with Rachelle Baker for past submission (now published) and we have been in touch with Dr. Kira Henderson as well. As requested, we have included author contributions and a list of six possible reviewers. We thank you in advance for your consideration of our manuscript.

Sincerely,
Margaret Saha
Professor of Biology

Author Contributions:

Murray, Jessica R: developed and implemented the dissection protocol; wrote initial draft of manuscript.

Stanciauskas, Monika E: assisted with implementation and refinement of the protocol; contributed to writing of manuscript.

Aralere, Tejas S: developed EdU protocol and wrote EdU section of manuscript.

Saha, Margaret Ph.D: PI of lab; provided funding; oversaw animal care; project development; reviewed and help write manuscript; oversaw all experiments.

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SHORT ABSTRACT:
The zebra finch (*Taeniopygia guttata*) is a valuable model organism; however, early stages of zebra finch development have not been extensively studied. The protocol describes how to dissect early embryos for developmental and molecular applications.

LONG ABSTRACT:
The zebra finch (*Taeniopygia guttata*) has become an increasingly important model organism in

many areas of research including toxicology[1,2], behavior[3], and memory and learning[4,5,6]. As the only songbird with a sequenced genome, the zebra finch has great potential for use in developmental studies; however, the early stages of zebra finch development have not been well studied. Lack of research in zebra finch development can be attributed to the difficulty of dissecting the small egg and embryo. The following dissection method minimizes embryonic tissue damage, which allows for investigation of morphology and gene expression at all stages of embryonic development. This permits both bright field and fluorescence quality imaging of embryos, use in molecular procedures such as *in situ* hybridization (ISH), cell proliferation assays, and RNA extraction for quantitative assays such as quantitative real-time PCR (qtRT-PCR.) This technique allows investigators to study early stages of development that were previously difficult to access.

INTRODUCTION:

The overall goal of this technique is to obtain zebra finch (*Taeniopygia guttata*) embryos from the earliest stages of embryogenesis for use in a wide range of developmental studies. Zebra finch have become the predominant songbird model organism and have been used extensively in a variety of fields, including toxicology[1,2], behavior[3], memory and learning[4,5,6], comparative neuroanatomy[7,8], and language development[9,10]. As the only songbird with a sequenced genome, the zebra finch allows molecular and genetic study of the Passeriformes order, which represents over 50% of known bird species[11,12,13].

Despite the use of adult and juvenile zebra finch in a diverse array of fields, few studies have been performed on zebra finch embryos, particularly during early stages of development. This can be attributed to the small size of their eggs and embryos, and their newer status as a model organism[14,15,16] for studies in which the chicken (*Gallus gallus domesticus*) was previously used as a predominant model system[17,18,19,20,21]. However, as non-vocal learners, chickens are not an appropriate model system for studying the genetic basis of vocal learning, development of vocal learning, heritability, behavior, and the cortical-basal ganglia circuitry involved in motor learning[10].

It is important to note that zebra finch embryos are much more delicate and more easily damaged than chick embryos during dissection and molecular procedures. In particular, greater care is required when performing permeabilization steps on zebra finch embryos. Strong detergents and enzymes that would not harm a chick embryo can damage zebra finch embryos. In terms of general care, it is necessary to put zebra finch eggs in small cups before placement in an incubator to prevent them from breaking when rolling during incubation.

Zebra finch are amenable to behavioral studies, easily and prolifically breed year-round in captivity, and are vocal learners. These characteristics allow the use of zebra finch to address the need for a model organism that integrates development, genetics, and behavioral aspects of language. The dissections methods detailed below, combined with a recently developed staging guide specific to zebra finch[22], , make the zebra finch an increasingly useful standardized developmental model organism. However, obtaining embryos at early stages can

be daunting. This protocol allows investigators to easily obtain early stage embryos. Studies investigating the early development and the molecular developmental basis of complex behaviors in zebra finch, or the toxicological effects on development in other small, passerine birds will find this dissection methodology useful.

PROTOCOL:

Ethics Statement: The methods were conducted with domesticated zebra finches from the breeding colony at the College of William and Mary. All procedures followed RSPCA guidelines[23] and were approved by the College of William and Mary's OLAW (Office of Laboratory Animal Welfare) Animal Welfare Assurance (#A3713-01) and had Institutional Animal Care and Use Committee (IACUC) approval (#2013-06-02-8721-dacris).

1. Egg Collection and Incubation

1.1) Establish zebra finch pairs on a 14:10 light: dark cycle. Supply food, water, hay, and a nest box *ad libitum*. Note: Routine care and breeding for zebra finches has been well described[23].

1.2) Prepare a standard chick incubator with tilting shelves. Note: Maintain the artificial incubator at 37.5 (+/- 1) °C with 80-95% humidity by adding water to the bottom of the incubator on a daily basis. Allow the incubator to equilibrate for two days before using.

1.2.1.) Select small feed cups (5 X 7.6 cm) at least 2.5 cm deep to hold eggs in the incubator. Line the bottom and lower edges of the cup with two layers of paper towel so that the cup is padded, while still allowing the eggs to easily roll. Place these cups on the tilting shelves of the incubator. Note: Too much padding can inhibit rolling, which will prevent successful incubation due to embryonic adhesion to the shell interior.

1.3) Collect the eggs at two hours following the onset of the light cycle. Note: This minimizes discrepancies in stage development for a given incubation time due to parental incubation.

1.3.1.) Pick up eggs gently with thumb and forefinger at the tip and base along the length of the egg. Use a dull, soft 4B graphite pencil to label the date laid and time collected from the nest. Note: The softer 4B pencil reduces the risk of the pencil breaking the fragile eggshell.

1.3.2.) Place 1 - 5 labeled eggs in each cup in the incubator so that eggs can freely roll to prevent embryonic adhesion to the interior of the shell. Note: Placing more than 5 eggs in a cup may prevent rolling of the individual eggs and reduce viability of embryos.

2. **Removal of embryo from egg**

2.1) To prepare for dissection, assemble the following materials: a clean scalpel, fine tipped forceps, and two extra fine tipped forceps. Place 10 x 10 cm weigh paper on the base of the

dissecting scope to provide a clean, non-absorbent surface for dissection. Note: The weigh paper is crucial because it allows easy manipulation of the yolk and is the best surface for cutting the delicate membranes with a scalpel.

2.2) Prepare an aliquot of phosphate buffer saline (1X PBS) in a 50 ml polypropylene tube, and acquire at least three transfer pipettes and a small waste bucket. If fixing the embryos, prepare an aliquot of 4% paraformaldehyde (PFA). **Caution**: PFA vapors are toxic, and all steps involving PFA should be done inside a fume hood to minimize exposure. If flash freezing the embryos, obtain liquid nitrogen and keep it near the dissecting scope. Sterility is not an issue for this dissection protocol, but ensure all materials are clean.

2.1) Remove the egg from the incubator at the time point needed for the desired stage as described in the zebra finch staging guide[22]. Note: Remove eggs after 36 hours of incubation to dissect stage 6 embryos (Fig. 4, A)[22] and remove eggs after 56 hours of incubation to dissect stage 12 embryos (Fig. 3, A)[22].

2.2) Using a fiber optic illuminator lamp, candle the egg by holding it along its vertical axis and shine light through the egg to illuminate the interior. Place the tip of the light behind the egg and locate the yolk and the developing embryo.

2.2.1.) Position the scalpel on the side opposite the yolk and cut along the egg from tip to base with faint pressure.

2.3) Remove the contents of the egg by carefully applying pressure on the tip and base of the egg with the thumb and the forefinger, or by gently prying apart the eggshell along the cut with forceps. Open the egg directly above the weigh paper so that the yolk gently rolls out.

2.4) Examine the yolk for the white zone of junction, which is visible as a faint white ring encircling embryonic development and orient the yolk using extra fine tipped forceps so that the embryo is located in the center. Note: If a faint white circle on the surface of the yolk is not observed, take the fine forceps and gently roll the yolk over until the embryo is on top of the yolk. Note: For stages 1-9[22], the embryonic disk is less than 6 mm in diameter and is visible as a faint, slightly opaque disk on the surface of the yolk - see Figure 1 for reference. For stages 10 and older[22], blood pools allow improved visibility of the embryo, but take care to avoid puncturing the yolk sac, as this makes locating the embryo difficult.

3. Separation of embryo from extra-embryonic tissue

3.1) Puncture the edges of the yolk to relieve pressure (Figure 1, B), and make single cuts across the length of the yolk diameter alongside the embryonic disk. Repeat making these diagonal lines until the section of the yolk containing the embryo has been successfully separated (Figure 1, B). Note: This step allows the yolk mass to remain intact, but the reduced pressure on the surface of the yolk allows for more precision when cutting around the embryo, preventing

damage to embryonic structures.

3.2) Remove the edges of the yolk with a transfer pipette. Note: Remove as much yolk as possible, but leave a small amount so that the embryo does not adhere to the dry surface of the weigh paper and tear.

3.3) Wash the embryonic disk by dispensing 1X PBS at a 45° angle towards the bottom of the embryo. Place the tip of the transfer pipette next to—not above—the embryonic disk. If additional yolk needs to be removed, separate any yolk with the scalpel on the weigh paper before transferring the embryo to the petri dish. Note: This method removes the embryo from the surface of the weigh paper.

3.4) Transfer the embryo using a transfer pipette along with a minimal volume of 1X PBS to a small, plastic petri dish. Wash the embryo by adding more 1X PBS into the petri dish and dripping 1X PBS near, but not directly onto, the embryo. Swirl the petri dish to wash and remove residual yolk, tilt the petri dish, and remove waste 1X PBS with the transfer pipette. Note: When the 1X PBS is removed, the embryo typically does not adhere to the bottom of the petri dish because the plastic surface is slippery with residual 1X PBS. However, more 1X PBS can be added if the embryo sticks to the dish.

Note: If fixing the embryo, follow steps 3.5 and 3.6. If flash freezing the embryo, skip to step 3.8 immediately.

3.5) If fixing the embryo for *in situ* hybridization, immediately add 4% PFA to the petri dish to submerge the embryo. Drip 4% PFA directly onto the top of the embryo in order to flatten it; this prevents the embryo from curling. Fix the embryo in 4% PFA at 4 °C for 12 hours.

3.5.1.) After fixation, dehydrate embryos in graded methanol (MeOH) solutions and store at -20 °C in 100% MeOH.

3.6) If the embryo is between stages 1 – 8[22], remove the vitelline membrane adhered to the embryo. Note: This step can be done during or after fixation. Embryos younger than stage 8[22] are not easily visualized because they adhere to the vitelline membrane, obscuring key structures as seen in Figure 2, A.

3.6.1.) Grip the edge of the membrane that extends beyond the zone of junction with extra fine forceps. Carefully flip the embryo over multiple times to wash away residual yolk granules and to loosen the adherence of the embryonic disk to the vitelline membrane. Note: Do not touch the center of the embryo, as this will damage embryonic structures.

3.6.2.) If a gap does not appear between the zone of junction and the vitelline membrane, gently scratch the zone of junction with the extra fine tipped forceps to loosen it from the vitelline membrane. Grip the vitelline membrane with the extra fine tipped forceps and gently

pull it away from the embryo. If necessary, gently pull the embryo away from the vitelline membrane by gripping the peripheral edge of the embryonic disk at the zone of junction.

3.6.3.) Discard the vitelline membrane in biohazard 1 waste (biosafety level 1) after removal.

3.7) When conducting an ISH assay on young embryonic stages, follow standard whole mount ISH protocols for chick embryos[24, 25], but consider the following suggestions.

3.7.1.) To reduce damage to the embryos during the ISH procedure, use a single 5 ml glass vial with a screw cap for each embryo. Only fill vials with 2-3 ml of solution, ensuring that the embryos are fully submerged. Nutate vials vertically by placing 5 ml vials into a styrofoam rack (or any rack that will keep the vials secure) that is secured to a nutator. Note: This precaution prevents the embryo from being torn, which can occur when it comes into contact with the lid of the vial during horizontal nutation.

3.7.2.) For embryos stage 0-6[22], treat with 5 µg/ml proteinase K in 1X PTw for 5 minutes at room temperature. Treat embryos staged 7-12[22] with 10 µg/ml proteinase K in 1X PTw for 10 minutes at 37 °C to reduce background staining.

3.7.3) To produce a sufficient color reaction to detect low levels of gene expression in stages 1-10[22], incubate with 10 µg/ml probe concentration for 12 hours. For older stages, incubate with 1 µg/ml probe concentration at 60 °C .

3.8) If flash-freezing the embryo, quickly add 2-3 ml 1X PBS to the petri dish and tilt the petri dish so that the embryo slides along the bottom of the dish, leaving yolk granules behind. Remove liquid and repeat 2-3 times to remove all yolk. Using fine tipped forceps, transfer embryo to a pre-labeled microcentrifuge tube and flash freeze in liquid nitrogen before storing at -80 °C.

4. EdU cell proliferation assay. Incorporation and detection of EdU in zebra finch embryos.

4.1) Candle the egg using a fiber optic illuminator lamp to locate the embryo or yolk.

4.2) Mark the side of the egg opposite to the location of the embryo or yolk within the egg to ensure that the embryo is not damaged during the microinjection process.

4.3) Line a 60 mm plastic dish with modeling clay and mold it in the shape of a bowl to hold the egg in the desired orientation. The marked spot should be oriented to allow insertion of the microinjection needle using the micromanipulator. Note: The clay will stabilize the egg during microinjection and throughout the incubation in step 4.11.

4.4) Pull two glass capillary microinjection needles. Blunt one needle to poke a hole into the egg at the marked spot. Prepare the second microinjection needle for injection by backloading

it with mineral oil and inserting it into the microinjector.

4.5) Set the volume of solution released per injection to 59.8 nl on the microinjector.

4.6) Load the microinjection needle with the 10 mM stock EdU solution.

4.7) Create a hole in the shell on the marked spot with the blunted glass capillary, taking care to not shatter the shell or drop pieces of the shell into the egg cavity. Orient the egg so that the hole is at a 45^{o} angle, allowing the microinjection needle to be inserted directly through the cavity to the yolk or embryo.

4.8) Using the micromanipulator, insert the loaded needle approximately 1.0 cm into the cavity.

4.9) Inject the desired amount of the EdU solution directly onto the developing embryo or yolk.
Note: A maximum of 478 nl of EdU can be injected without resulting in embryonic death.

4.10) Immediately wrap the egg and the clay holder with multiple layers of plastic wrap to cover the egg and the dish to prevent desiccation of the embryo during incubation. Tape the edges of the plastic wrap to the bottom of the dish to prevent the plastic from unwrapping during incubation. Note: The plastic wrap should only be removed immediately before dissection.

4.11) Allow the newly proliferating cells to incorporate the EdU by incubating the egg at 37 °C until the desired stage is reached. After injection, do not return egg to tilting shelves in incubator. Place the plastic-wrapped clay lined dish on stable surface inside the incubator.

4.12) Remove the plastic wrap and dissect the embryo following the aforementioned protocol.
Fix the embryo in 4% PFA and incubate 12 hours at 4 °C as described above. After fixation, wash with 100% ethanol (EtOH) for 5 minutes and store in fresh 100% EtOH at – 20 °C until further analysis.

5. EdU “click” reaction protocol

Note: The following steps are all performed in glass vials.

5.1) Rehydrate embryos with successive 5 minute washes of the following:
100% EtOH, 75% EtOH and 25% sterile deionized distilled (sdd) water, 50% EtOH and 50% sdd water, 25% EtOH and 75% 1X PTw, 100% 1X Ptw

5.2) Wash three times in 100 % 1X PTw for 10 minutes each.

5.3) Dilute the reaction buffer additive (Kit Component F) by combining 1 part of the 10X stock buffer solution (10 µl) to 9 parts sdd water (90µl).

5.4) Prepare the reaction mix in a separate tube by mixing the following: 875 µl 1X PBS, 20 µl $CuSO_4$, 5 µl azide, 100 µl diluted reaction buffer additive. Note: Volume of the reaction mix can be scaled down. The reaction will work if the embryo is completely covered in the reaction mixture. Add the diluted reaction buffer additive (step 5.3) immediately before use. All subsequent steps are performed in the dark. Cover the embryos with foil to protect from the light.

5.5) Cover the vials with aluminum foil, and incubate them vertically at room temperature for two hours by placing them in a styrofoam rack attached to a nutator.

5.6) Wash the embryos 3 times in fresh 1X PBS for 10 minutes each.

5.7) Incubate the embryos in fresh 4% PFA overnight at 4 °C.

5.8) Wash 3 times in 1X PBS for 10 minutes each and store in fresh 1X PBS at 4 °C. Cover the embryos with foil until further analysis.

REPRESENTATIVE RESULTS:

The steps diagrammed in Figure 1 indicate the appearance of the embryo while attached to the vitelline membrane (A) and demonstrate the proper method to separate the embryo from the yolk (B). The embryo can be identified by the zone of junction, which is much lighter than the vitelline membrane. The embryo itself is often difficult to distinguish until the yolk is cut away. Once the embryo is dissected from the egg, it can be fixed or flash frozen for future use. If an *in situ* hybridization is planned for the dissected embryo, it is necessary to remove the vitelline membrane that is adhered to the embryo via the zone of junction. Figure 2 illustrates the improved visibility of the embryo once this membrane is removed (C), and the proper way to peel the vitelline membrane (A, B). After dissection and fixation, whole mount *in situ* hybridization was performed as seen in Figure 3 (A, A', B, B') and Figure 4 (A, A', B, B') and Figure 5 (A,B,C) to detect differences in *orthodenticle homeobox 2* (*Otx2*) expression in embryos developmentally exposed to low doses of methylmercury. Figure 5 (A,B,C) shows sense probe results, demonstrating lack of background. In Figure 4, despite being dissected from the egg at the same time point, the embryo developmentally exposed to methylmercury (MeHg) progressed to stage 5^{22} (B, B'), while the control embryo developed to stage 6^{22} (A, A'). The group of embryos dissected and shown in Figure 4 were collected from the nest and taken from the incubator at the same times. Although some natural variation is present in development, based on previous dissection data, it is unlikely that temperature fluctuations in the incubator would cause only the 2.4 ppm methylmercury embryos to be developmentally delayed. The differences in stages indicate changes in cell proliferation in embryos developmentally exposed to methylmercury.

Before dissection, EdU was injected into a day 2 egg and allowed to incubate overnight. After dissection and fixation of the stage 16^{22} embryo, EdU was visualized using "click" chemistry,

allowing detection of proliferating cells as seen in Figure 6 (A, B, C). It is important to carefully monitor time points when placing eggs in the incubator and during dissections, as exposure to methylmercury or performing the EdU assay may disrupt developmental progression. The earliest injection was performed on day 0, which was the day of collection as specified in step 1.3. This embryo was dissected approximately 38 hours later (stage 7[22]). The survival rate was found to be approximately 90% (same rate as control embryos) as long as the injection amount was under 478 nl.

This dissection methodology also allows for high quality RNA extraction. After dissecting stage 16[22] embryos, an RNA extraction was performed according to manufacturer's protocol with no optimization required, as seen in Figure 7. The removal of the vitelline membrane was unnecessary for RNA extraction and later qRT-PCR applications.

Figure Legends:

Note: All embryo figures are oriented so that the anterior and posterior regions are at the top and bottom of the images, respectively.

Figure 1: Procedure for locating and dissecting zebra finch embryos, stages 1-10[22]. Locate embryo by gently rolling the yolk until the faint white disk is apparent (A). Once the embryo is located at the center of the yolk, the yolk is dissected in a stepwise fashion (B) where the first cut relieves pressure of the vitelline membrane (1) and subsequent cuts (2) border the zone of junction (zj) which is adhered to the vitelline membrane. Scale bars represent 1 mm.

Figure 2: Removal of vitelline membrane and visibility of embryonic structures.
Following removal of the embryo from the yolk, place embryo in a petri dish containing 4% PFA. If an *in situ* hybridization needs to be performed, visibility of the embryonic structures is essential and can be achieved by removing the vitelline membrane (A). Grip the vitelline membrane with extra fine tipped forceps and gently peel it away from the embryo by handling the embryo directly at the outermost edge, if necessary (B). Vitelline membrane removal increases clarity of embryonic structures, and allows embryos to be imaged or processed with *in situ* hybridization (C). Scale bars represent 1 mm.

Figure 3: Whole mount *in situ* hybridization performed on zebra finch embryos developmentally exposed to methylmercury. Expression patterns of *orthodenticle homeobox 2* (*Otx2*) were characterized in embryos exposed to 0.0 ppm methylmercury (A, A') and 2.4 ppm methylmercury (B, B') via parental diet. The dorsal (A) and ventral (A') expression of *Otx2* is visible throughout the midbrain and optic vesicles during stage 12[22]. The treatment group embryos were dissected at the same time point, but were developmentally delayed as seen in the dorsal (B) and ventral (B') view of head structures, which are characteristic of stage 11[22]. Abbreviations: mb, midbrain; op, optic vesicle. Scale bars represent 1 mm.

Figure 4: Whole mount *in situ* hybridization performed on zebra finch embryos developmentally exposed to methylmercury. Expression patterns of *orthodenticle homeobox 2* (*Otx2*) were characterized in stage 6[22] embryos exposed to 0.0 ppm methylmercury (A, A') and stage 5[22] embryos exposed to 2.4 ppm methylmercury (B, B') via parental diet. Abbreviations: am, anterior margin of mesoderm; no, notochord, notochord mesoderm; po, proamnion, anterior blastopore; ps, primitive streak[22]. Scale bars represent 1 mm.

Figure 5: Whole mount *in situ* hybridization performed on zebra finch embryos using sense probe. (A) Stage 5[22] embryo. (B) Early stage 6[22] embryo. (C) Stage 11[22] embryo. Scale bars represent 1mm.

Figure 6: EdU incorporation and detection in zebra finch embryos. EdU "click" chemistry was used to detect proliferating cells in a stage 16[22] embryo (A, B, C). EdU is incorporated into the DNA in the place of thymidine[26,27] and is detected using click chemistry[27]. Proliferation is clearly visible in the lateral edges of the somites, and the tailbud. Panel A shows proliferation occurring exclusively in the posterior of the embryo and also shows individual proliferative cells. Panel B shows the proliferative locations in the whole embryo. Panel C shows the anterior region, and shows the highly proliferative telencephalon (te) in greater detail. Abbreviations: af, amniotic fold; flb, forelimb bud; hlb, hindlimb bud; le, lens vesicle; ms, mesencephalon; mt, metencephalon; opc, optic cup; pa, pharyngeal arch; sm, somite mesoderm; tb, tailbud; te, telencephalon. Scale bars represent 1mm.

Figure 7: Quality of RNA extracted from dissected zebra finch embryos. Control (0.0 ppm) and 1.2 ppm methylmercury embryos were dissected and flash frozen as described in Step 3.8. Each lane shows RNA extracted from two homogenized embryos from each treatment group.

DISCUSSION:

Recent development of an embryological staging guide[22] and genome annotation make the zebra finch a desirable model organism for developmental studies. However, the small size and fragility of the zebra finch embryos, which range from 3 to 7 mm in stages 1 – 10[22], can make dissections difficult[11,14]. Locating and cleanly removing embryos from the surface of the yolk can be challenging. This protocol provides sufficient detail to perform the procedure with ease. This protocol demonstrates the critical steps that are not typically known, but are necessary to ensure a successful dissection. For example, it is essential to leave a small layer of yolk between the embryo and sheet of weigh paper to preclude sticking.

Both the identification and removal of the embryo can be difficult. To troubleshoot identifying the embryo on the surface of the yolk, shine light directly above the yolk after it has been removed from the egg, and look at the yolk at a 45° angle to find the embryo. Once the embryo is located, cut the yolk on weigh paper taking care to not tear the embryo.

If further applications include imaging for anatomical differences, *in situ* hybridization, or cell proliferation assays, it is important to remove the vitelline membrane in stage 1 – 8[22] for better

visualization of structures. If experiencing difficulties when removing the yolk or the vitelline membrane in early stages, fix the embryo in 4 % PFA before washing it in 1X PBS to reduce embryonic fragility. By first removing the vitelline membrane during dissection as described, structures are clearly visible and intact in zebra finch embryos after performing an *in situ* hybridization.

A limitation of EdU is that administration of dosage volumes over 478 nl leads to embryonic fatality. However, the vast dosage range allows varying levels of proliferative cell tagging.

The "click" reaction used in this kit is the Copper(I)-Catalyzed-Alkyne-Azide-Cycloaddition (Cu(I)AAC). In this specific reaction, an alkyne-containing thymidine analogue molecule (EdU) is incorporated by actively dividing cells. The alkyne group in the EdU protrudes from the helical structure of the DNA, and is detected by exposure to an azide molecule conjugated to a green, fluorescent molecule which binds to the free alkyne group. The green fluorescence shows the newly proliferating cells in the embryo. The bio-orthogonality of the azide and the alkyne groups prevents non-specific staining because these reactive species are not naturally present in organisms. Also, because the DNA does not need to be denatured in order for the reaction to occur, further DNA-dependent analysis can be easily performed[27].

An inherent limitation of this method is the small size and fragility of zebra finch embryos. Removing the vitelline membrane of embryos stages 1 – 15[22] can result in damaging embryonic structures if not performed with caution. However, this protocol simplifies the dissection method, allowing investigators to use early embryonic stages to examine structural anomalies and gene expression that have not been previously studied in depth. This protocol opens the way for a plethora of cellular-molecular assays that will allow investigators to determine the developmental origins of adult phenotypes. For example, it will be possible to examine gene expression implicated in vocal learning under various environmental conditions or following pharmacological treatments at the earliest stages of development[28,29,30,31]. Although not demonstrated in this paper, this method potentially allows for other procedures such as radioactive *in situ* hybridization on zebra finch tissue sections and electroporation/*in ovo* surgery[32,33,34]. Given that the zebra finch has been established as an important model organism within a vast body of literature, such studies provide untapped opportunities to link developmental mechanisms with adult physiology and behavior, in particular the development of language[7,9].

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The authors have nothing to disclose.

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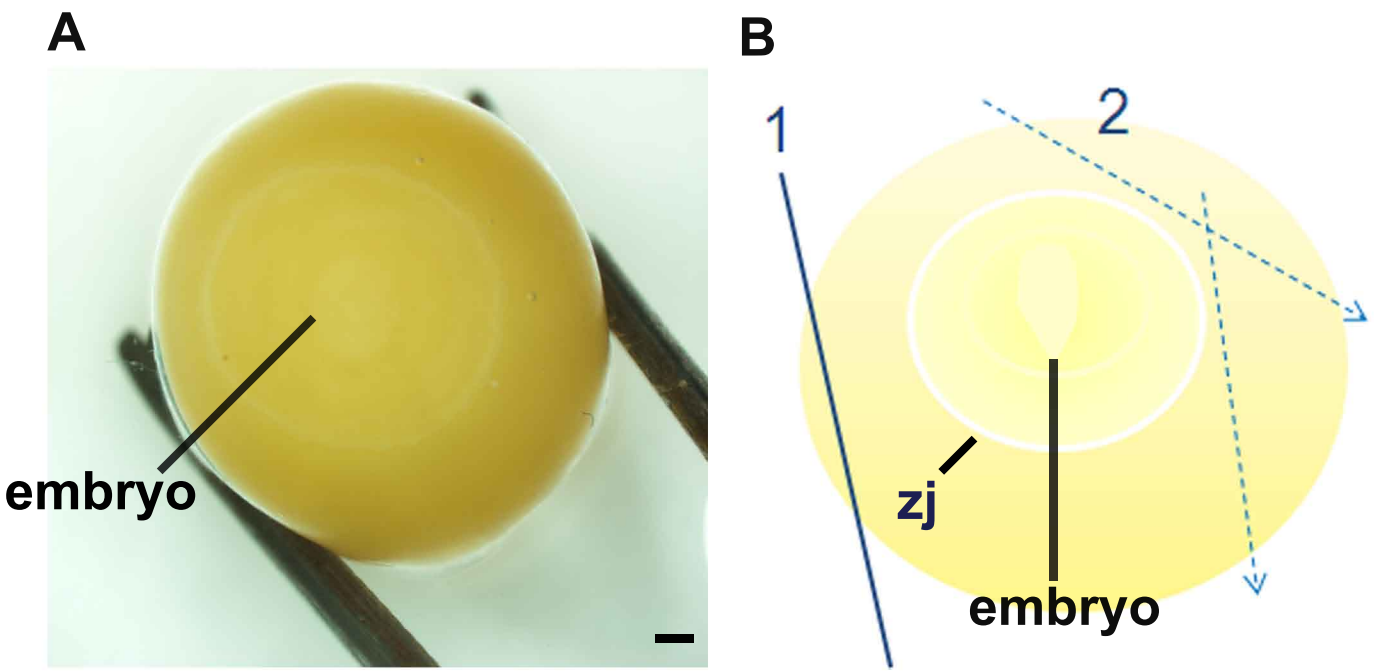

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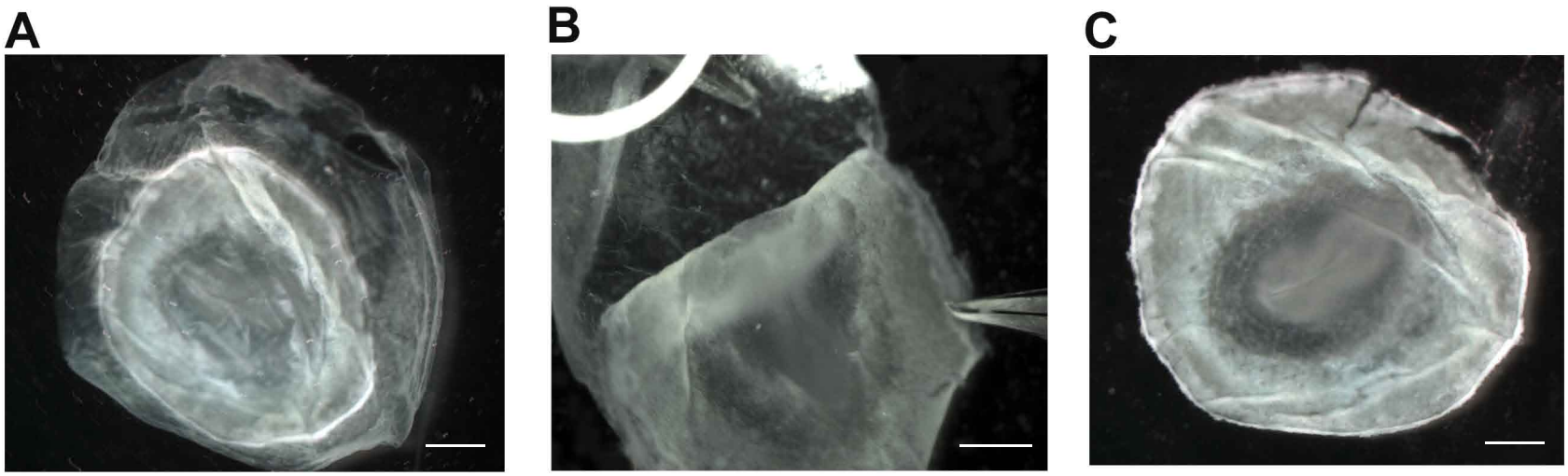

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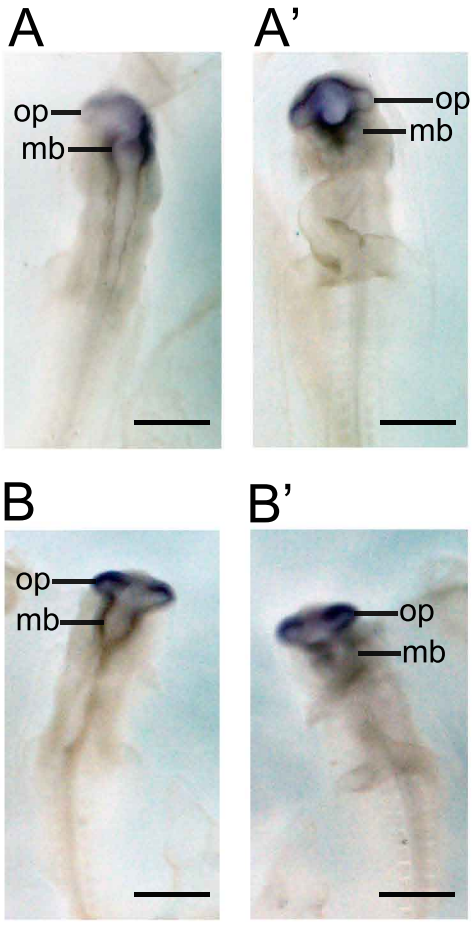

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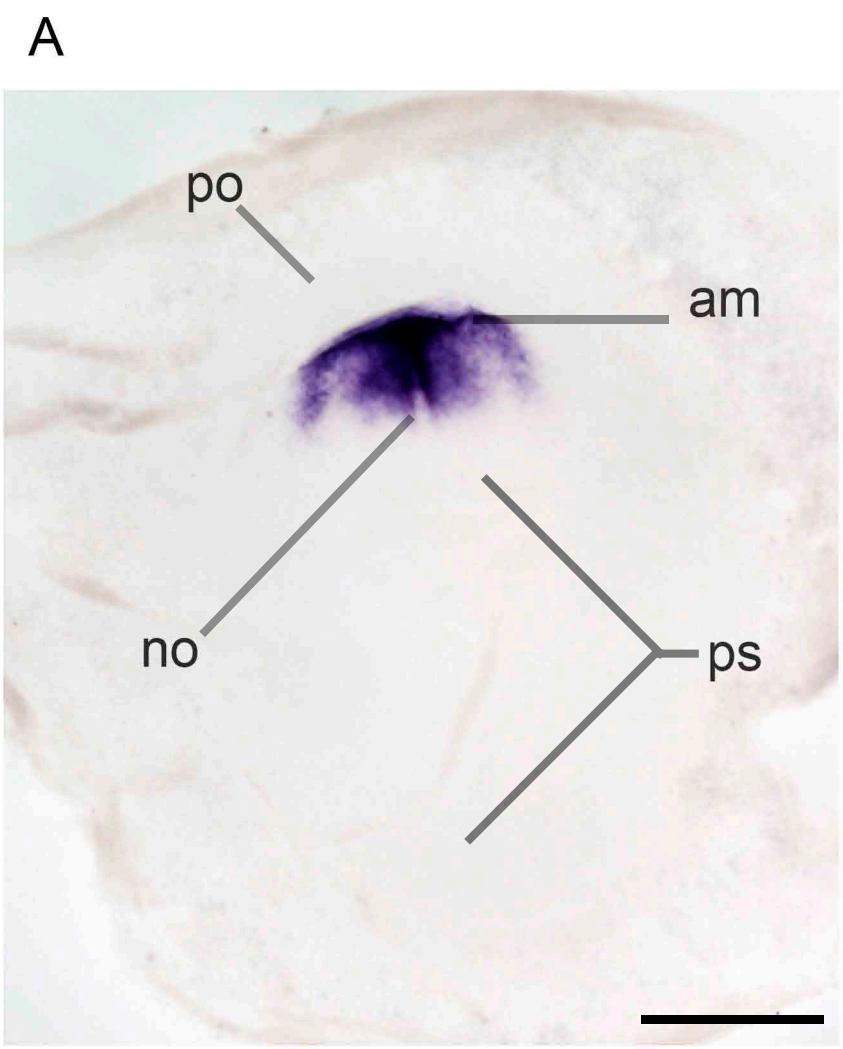

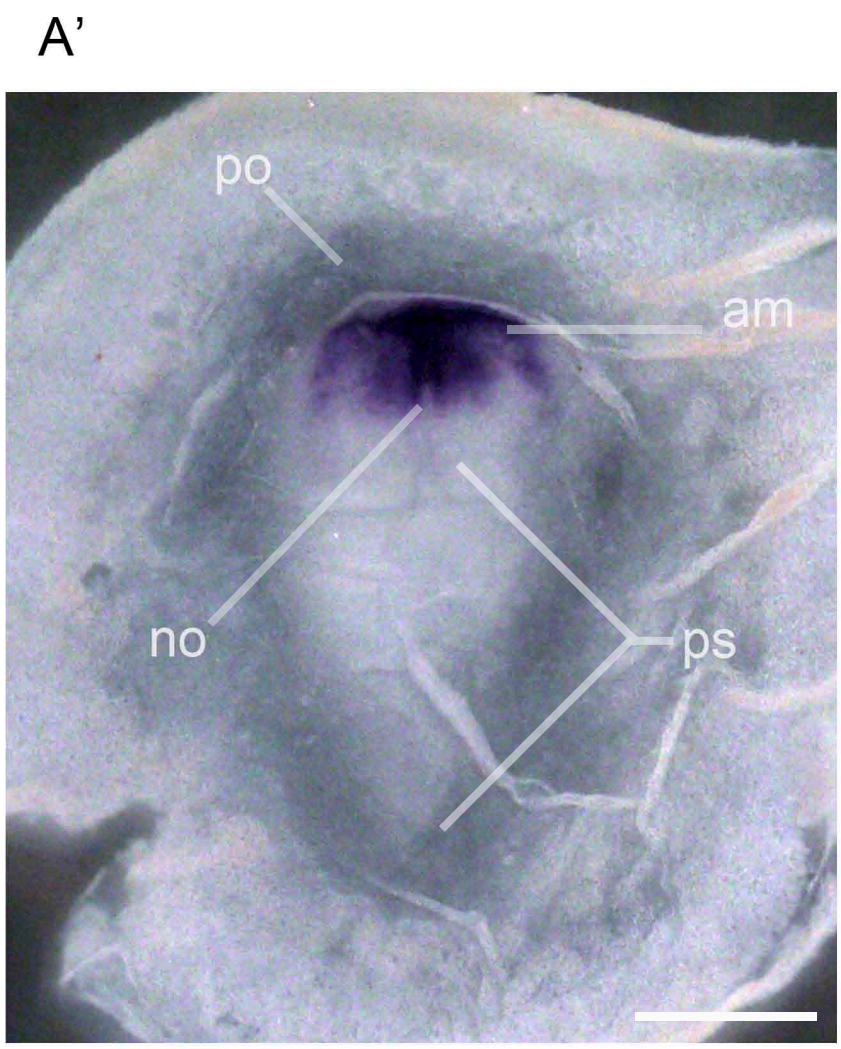

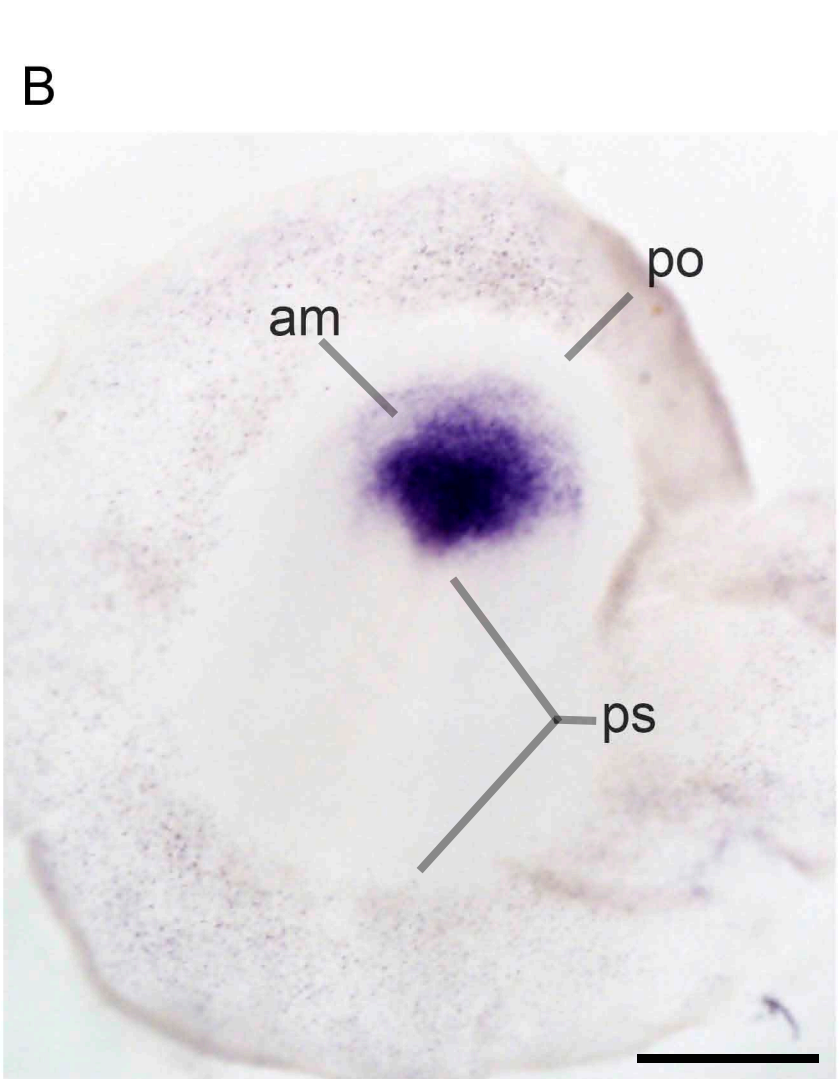

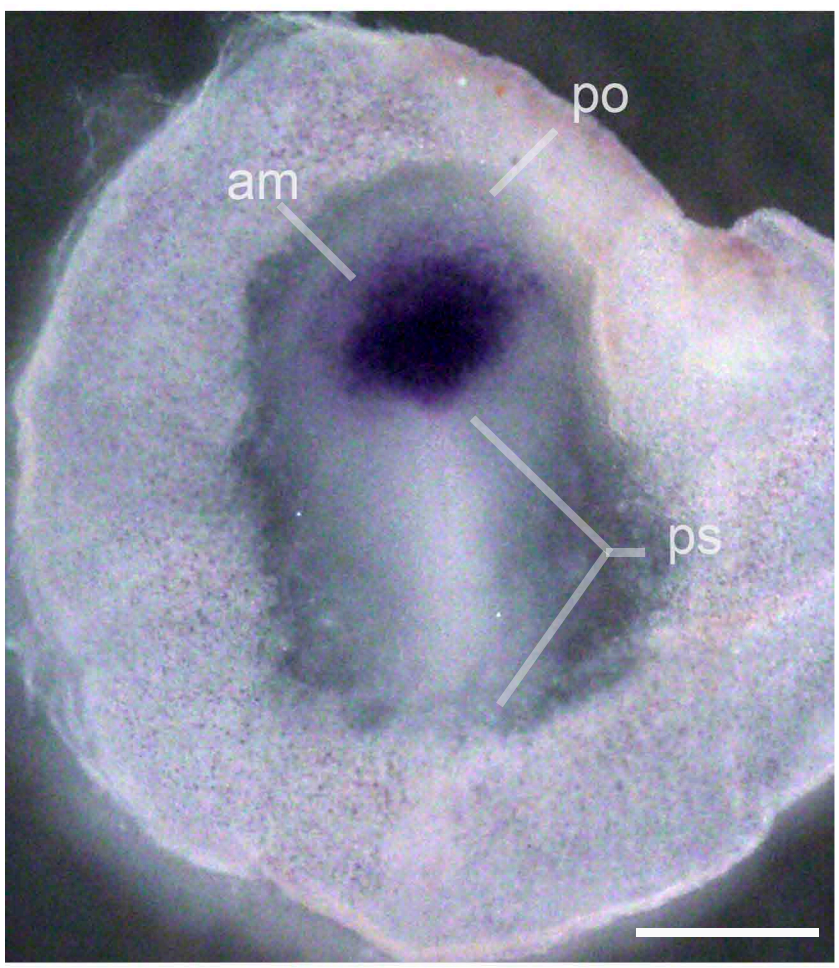

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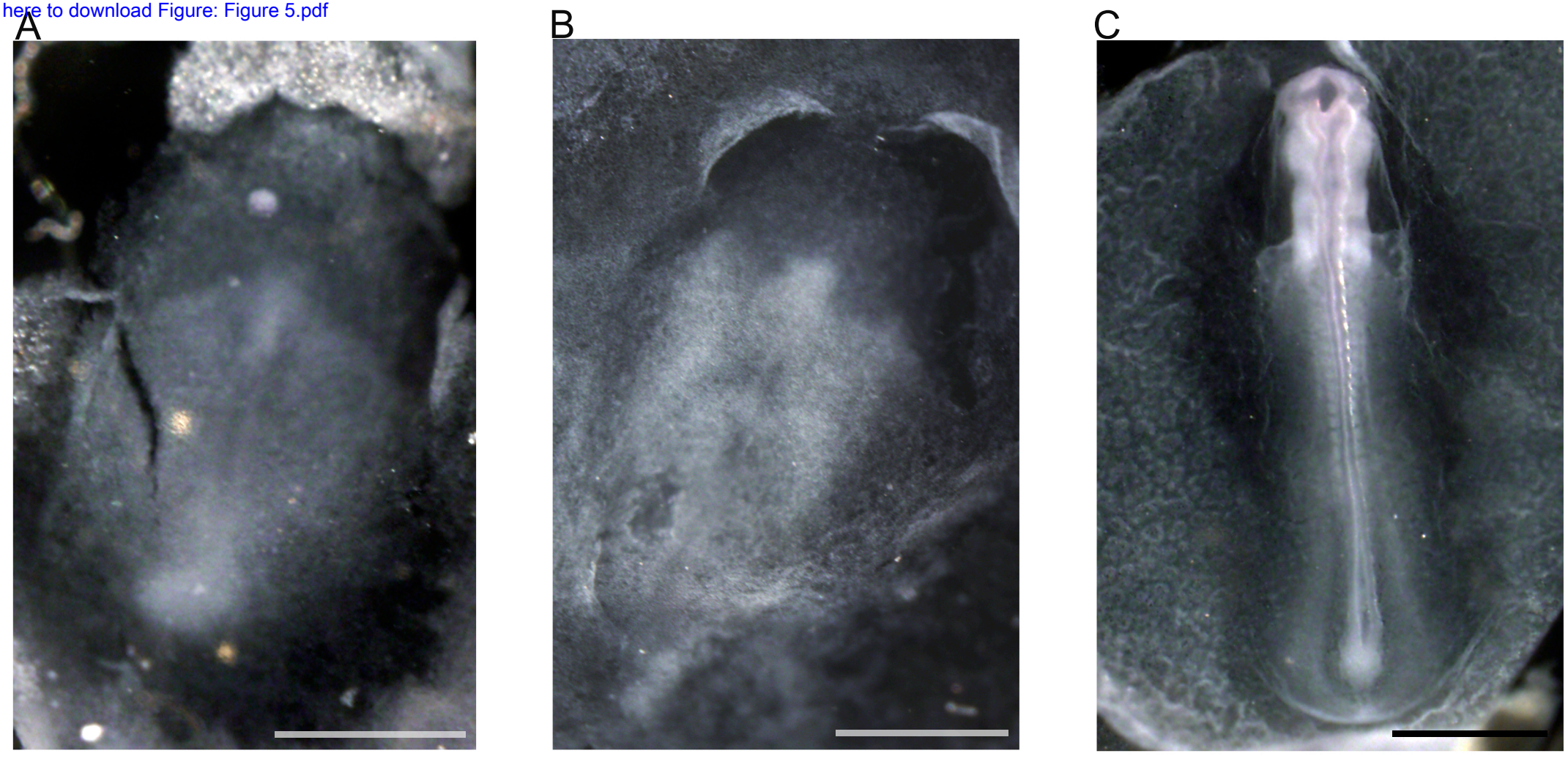

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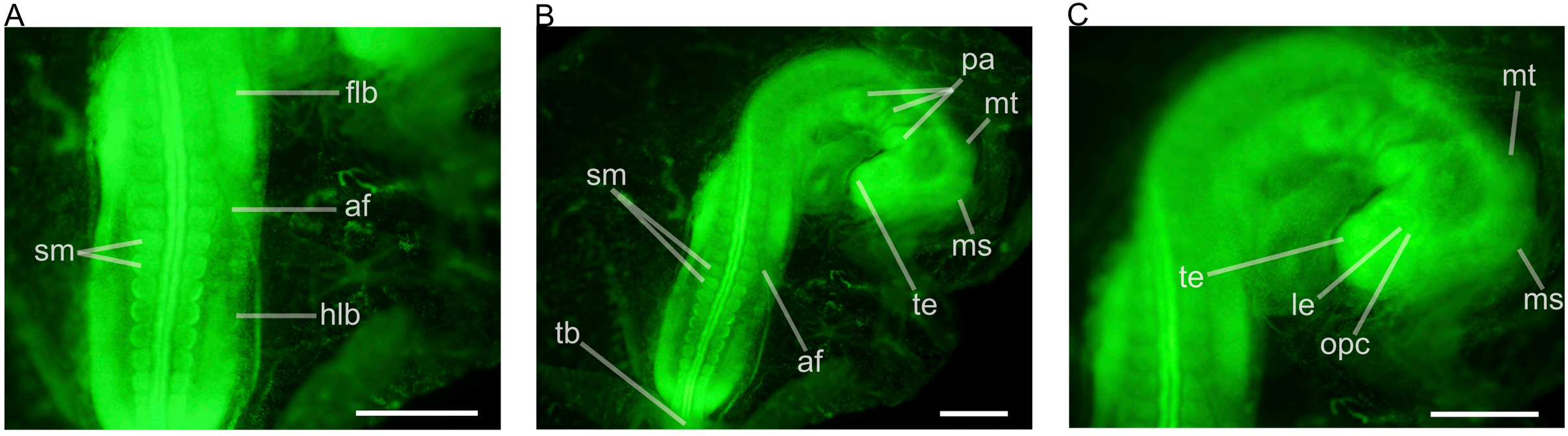

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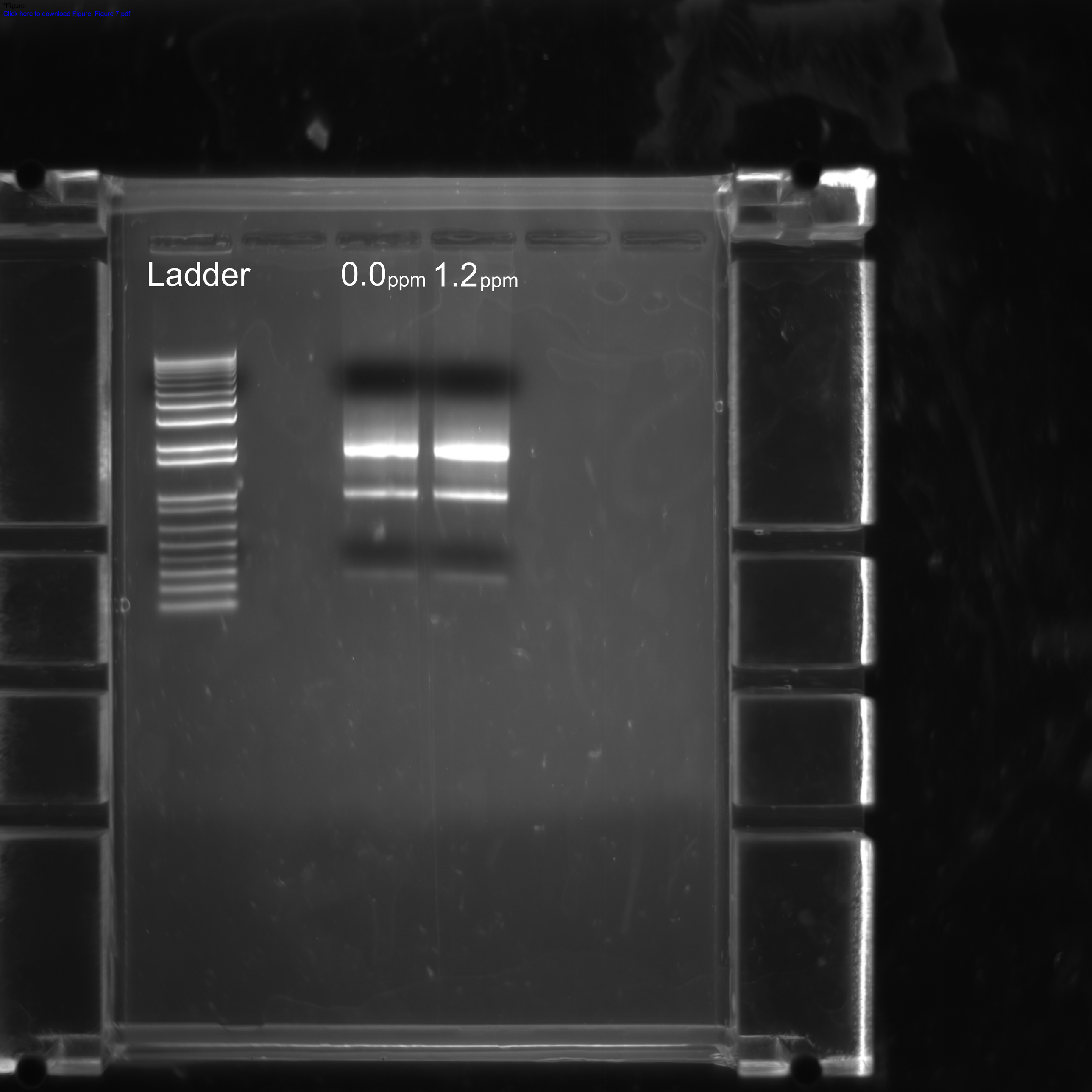

*Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Chicken egg incubator			We use a Picture Window H
Click-iT EdU Alexa Fluor 488 Imagin	Invitrogen	C10337	Detection of cell proliferati
Dissection microscope			We use Olympus SZ61
7'' Drummond capillary for Nanojec	Drummond	3-00-203-G/X	
Drummond Nanoject II microinject	Drummond		
Dumont Tweezers #55	World Prec	14099	
Ethanol (EtOH)			
50mL Falcon tube (polypropylene)	Fisher Scier	06-443-18	
FastPrep Lysis Matrix H tubes	MP biomed	6917-100	Used for RNA extraction, us
Fiber optic illuminator lamps (High	Dolan-Jenn	Fiber-Lite MI-150	
Glass vials with screw cap (DEPC tre	Fisher Scier	03-338A	
Microcentrifuge eppe tube (1.5 mL	Fisher Scier	05-408-129	
Mineral oil	Sigma-Aldri	M-8410	
Modeling Clay			Any brand
Narshige PB-7 needle puller	Narshige		
Omni Bead Ruptor Homogenizer	OMNI	19-010	Used for RNA extraction
4% Paraformaldehyde (4% PFA): 20 mL 8% PFA (32g paraformalde			Fixation of embryos. Cautio
Phosphate buffered saline (1xPBS): 200mL 10X PBS, 1800 mL Barnstead H_2O, adjust pH to 7.4			
Phosphate buffered saline (10xPBS): 800mL Barnstead, 2.013g KCL, 80.063g NaCl, 2.722g KH_2			
Phosphate buffered saline with 0.1% Tween-20 (1xPTw): 1800mL Barnstead H_2O, 200mL 10X			
35mL Plastic petri dish	Fisher Scier	08-757-100A	
plastic wrap			Any brand
Prepease RNA Spin Kit	PrepEase, A	78766 1 KT	Used for RNA extraction, us
Reaction Mix: 875µL 1xPBS, 100mM	Invitrogen	C10337	Detection of cell proliferati
sdd H_2O			
Seed cup			We use it as a container wh
Stainless steel scalpel			
Standard nest box			We use Abba Plastic Finch N
4mL, Teflon Lined Cap, Glass Vials	Fisher Scier	02-912-352	
Transfer pipettes (polyethylene)	Fisher Scier	13-711-7M	
4x4 weigh paper	Fisher Scier	09-898-12B	

Nova-Bator Incubator, Circulated Air Model
on.

sed to homogenize embryos

on, is harmful and do not inhale.

PO_4 monobasic, 14.196g Na_2HPO_4 dibasic, 200mL Barnstead H_2O, adjust pH to 6.5
PBS, adjust pH to 7.4, 2mL Tween-20

sed to homogenize embryos
on.

nen incubating eggs

Nestboxes

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Article Title: Dissection and Downstream Analysis of Zebra Finch Embryos at Early Stages of Development

Signature: Margaret O Saha Date: 8-30-2013

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Dear Editor,

Below (in bold) are our responses to the Reviewers' comments. We have made all of the suggested edits. Thank you for this opportunity to submit to JoVE,

Jessica Murray, Monika Stanciaukas, Tejas Aralere, and Margaret Saha

Reviewer #1:
Manuscript Summary:
This nicely written manuscript from Murray et al. documents how to manipulate the zebra finch embryo in order to collect embryos for later experimentation. The zebra finch is one of the best model organisms for a variety of different areas of research, but early embryo studies (especially ones molecular in nature) have been limited due to the establishment of critical techniques for embryo isolation. In particular, for vocal learning the zebra finch often serves as a better model than the chick due to the fact that chicks are non-vocal learners. As such, the ability to isolate embryos for further experimentation is critical for the zebra finch to become a model organism that permits early embryo assays. In this manuscript, the authors document how to dissect out and remove embryos for use in in situ hybridization or RNA extraction experiments, as well as how to label whole embryos for cell proliferation assays. This manuscript is well suited to the general readership of JoVE and should be acceptable for publication with the following minor revisions:

1. In Figure 1, a label on the figure indicating the location of the embryo proper would be useful.
We have added the requested label to the figure showing the location of the embryo proper.

2. In 3.4, will the embryo stick to the bottom of the petri dish if one removes all of the 1X PBS? Please clarify this.

We have now clarified this in the text. We state that it usually does not stick to the bottom of the dish because the plastic surface is slippery with the residual 1X PBS, however more 1X can be added if it does happen to stick.

3. It would be useful to include the in situ hybridization suggestions in the Discussion section right after 3.6.4, as well as the information in lines 252-253 with regards to post-fixation procedures (if these are applicable for in situ hybridization experiments).

We have now included the ISH suggestions in the section after 3.6.4 as requested. It is now steps 3.7 through 3.7.3.

4. In 4.10, for how long should the egg remain after injection of the EdU? Just long enough to cover it with plastic wrap? Please clarify this.

We have now clarified this in the text as follows: The clay is used to hold the egg in the correct orientation throughout the microinjection and the incubation process and the entire dish along with the egg is wrapped plastic wrap immediately after microinjection; it is unwrapped after the incubation, immediately before dissection. The purpose of the plastic wrap is to prevent desiccation of the embryo during incubation.

5. In line 301, the authors suggest that the difference in development for zebra finch embryos in Figure 4 could be due to incubation with methylmercury. While I agree that this is probably the case, another option could be that some embryos just develop more slowly than others, so that at any given point in development you could get a stage 5 embryo when the embryo should be stage 6. If the time difference between stages 5 and 6 in zebra finch is 6 hours, how likely is it that one would see a stage 5 embryo when it should really be stage 6? I think this should at least be mentioned in the text.

The set of embryos dissected and shown in Figure 4 were collected from the nest and pulled from the incubator at the same times. Although some natural variation is present in development, based on previous dissection data, it is unlikely that temperature fluctuations in the incubator would cause only the 2.4 ppm embryos to be developmentally delayed. However,

we do have data showing that methylmercury does delay development.

6. In Figure 3, it would be useful to include labels for the midbrain and optic vesicles. Also, is it possible to include a sense probe image for both Figures 3 and 4 to indicate the level of background staining, if any?

We have now included labels for the midbrain and optic vesicles. - Midbrain and optic vesicles are easy enough to label. We have also included a sense probe image. This is now Figure 5.

7. In Figure 6, please clarify what is in each lane of the gel. Is it RNA from 2 embryos (per lane)?

We have now clarified the contents of each lane. This is now Figure 7.

8. As an optional figure, it may be useful to see what the embryo/yolk looks like as it sticks to the weigh paper sheet (line 366).

This picture would look very messy and be relatively uninformative. This concern will, however, be addressed in the video.

Editorial comment:
[Please keep JoVE's protocol guidelines and length requirements in mind while addressing reviewer comments (use short steps, imperative tense, proper spacing, etc).]

We have done our best to address every one of the reviewers' comments while adhering to JoVE's guidelines.

Reviewer #2:
Manuscript Summary:
The authors described how to dissect the avian embryos from small size eggs and to process the harvested tissue. The authors also show that the techniques that used on chicken embryos can apply on zebra finch embryos.

Major Concerns:
N/A

Minor Concerns:
1. The embryo sizes between chicken and zebra finch are different in the same developmental stage, but similar in very early embryonic stages. Could the authors emphasize more the differences between dissecting (or treating) chicken embryo and zebra finch embryo, beside the size, in the introduction?

We have now emphasized the following differences in the introduction: Zebra finch embryos are smaller and more delicate; structures are more easily damaged during the dissection. Zebra finch eggs also need to be placed in smaller cups before being placed in the incubator racks. We also included differences in permeabilization steps between finch and chick.

2. In Step 4: BrdU assay, which embryonic stage do the authors perform the assay in? And what is the survival rate of this application?

We have now clarified that the assay can be completed as early as Stage 7 and that survival rate is 90% (the same rate as control embryos) as long as less than 478 nl volume is injected.

3. Step 4 is interesting and will be easier to understand by video. Could the authors add it in the video?

Yes, we have now added this into the video.

4. The term, yolk membrane or vitelline membrane, is more commonly used in biology, compare to yolk film.

We have now used the term vitelline membrane.

5. Other methods could apply on zebra finch embryos, such as, radioactive ISH in embryo sections (Chen et al., 2012, JoVE and Chen et al., 2013, Journal of comparative neurology) or electroporation/ in ovo surgery (Chen et al., 2012, PLoS One). Please discuss those other possible applications and include some references in the discussion section.

We have now added these methods as well as the appropriate references.

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
As the manuscript nicely describes, this technique will be very helpful for those wishing to use a small bird for embryological assays. While I agree that the zebra finch is an important system that is widely used, which is an excellent rationale for this work, I assume that the approach will be helpful in studies of other small bird species. I am not certain that the claim that studying early stages will add much to the understanding of language acquisition, though, because this is a fairly late event in telencephalic development. But for toxicology and environmental studies, it is a great model.

Major Concerns:
none

Minor Concerns:
Point 2.4: What instrument is used to "orient the yolk"?

We used closed extra fine tip forceps; this is now clarified in the text.

Point 3.4: What instrument is used to transfer the embryo to a petri dish?

We used a transfer pipet to transfer the embryo in a minimal volume of 1X PBS; this is now clarified in the text.

Point 5.3: I do not know what the "reaction buffer" is; this step is too vague for me because it assumes the reader knows the assay.

This is listed in the "Materials" spreadsheet, but this is a component of the Click-iT EdU kit from Invitrogen. A reference to the specific kit component was inserted in this step in the manuscript for further clarification.

Figure 5: I think the "click chemistry" method should be described, at least in principle, in a little more detail. I assume the green cells are the ones proliferating? This should be made explicit. Why are three panels shown? They are not individually described in the figure legend.

We have now described click chemistry in more detail. We have also described the green cells as the proliferating cells and clarified that the three panels are different regions and magnifications. This is now Figure 6.

Additional Comments to Authors:
N/A

Reviewer #4:

This is an interesting technique paper that will be of much interest to researchers who study avian development, including brain development, both in songbirds and in poultry.

The introduction would be strengthened by highlighting how easy it is to get zebra finches to breed in captivity. The birds are opportunistic breeders and can bred year round (rather prolifically) making developmental studies very feasible.
We have now added a statement highlighting the ease of breeding zebra finches in captivity.

It would be useful to know how the authors staged the embryos prior to the 2013 Murray et al., publication. I'm fairly certain this work begun before that paper was published (just earlier this year) so how did the authors determine the appropriate time points for dissection? Can any of the extra-embryonic tissue be used in sexing PCR? If so, that should be addressed.
This work arose from the Murray et al. paper (these are the same research groups) because as we were doing the work for the staging paper, it became clear that there was no published methodology for dissecting zebra finch embryos. A PCR test for sexing is available and can be used in conjunction with the dissections (Soderstrom et al., 2007).

Also, more information should be provided in point 3.1 (line 160) regarding puncturing the edges of the yolk to research relieving the yolk pressure. What happens to the integrity of the yolk mass?
We have now clarified this. Specifically, this procedure allows the yolk mass to remain intact, but the reduced pressure on the surface of the yolk allows more precision when cutting around delicate embryonic structures.

Overall, this is an interesting and useful manuscript. The two revisions have improved the overall quality of the writing and descriptions of the protocol.