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New Methods for Chicken Embryo Manipulations

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ABSTRACT The capacity to image a growing embryo while simultaneously studying the developmental function of specific molecules provides invaluable information on embryogenesis. However, until recently, this approach was accomplished with difficulty both because of the advanced technology needed and because an easy method of minimizing damage to the embryo was unavailable. Here, we present a novel way of adapting the well-known EC culture of whole chick embryos to time-lapse imaging and to functional molecular studies using blocking agents. The novelty of our method stems from the ability to apply blocking agents ex ovo as well as in ovo. We were able to study the function of a set of molecules by culturing developing embryos ex ovo in tissue culture media containing these molecules or by injecting them underneath the live embryo in ovo. The in ovo preparation is particularly valuable, because it extends the period of time during which the developmental function of the molecule can be studied and it provides an easy, reproducible method for screening a batch of molecules. These new techniques will prove very helpful in visualizing and understanding the role of specific molecules during embryonic morphogenesis, including blood vessel formation. *Microsc. Res. Tech. 73:58–66, 2010.*

INTRODUCTION

The chicken embryo has long been used as a developmental vertebrate model for its adaptability to manipulation in experimental studies. The chick embryo develops outside the mother in a self-sufficient egg, is easy to manipulate, and is amenable to transplantation, explantation, and micro-dissection techniques. It is also amenable to overexpression and knock-down studies through techniques including viral infection, injection of cells, and electroporation of morpholinos and expression vectors. In addition, the chick embryo's similarity to human embryos at early stages has validated its use in morphogenetic studies (Hamburger and Hamilton, 1951; Patten and Corliss, 1976).

The study of the developmental processes often demands disruption of the embryo. Several methods have been used to observe chicken embryonic development with minimal disruption, starting with the classical windowing of the egg (Barfurth, 1902; Waddington and Cohen, 1936). A popular, alternative in vitro approach is the New technique. New (1955) devised an elegant, now classical, method of explanting and culturing the chicken embryo in vitro. He accomplished this feat by submerging the contents of the egg in saline and removing the embryo from the yolk saving as large a portion of vitelline membrane as possible. The embryo was then transferred ventral side up on a watch glass and cultured until Hamburger and Hamilton stage 18 (HH18). This method has been subsequently modified due to its intrinsic difficulties, which include: the high level of skill required to master the technique, the paucity of teachers who can train others in this technique, the extensive time required to complete the process for a single embryo, the difficulty in finding glass rings, and the adaptations needed to apply this method to a petri dish for time lapse imaging. Thus, Chapman and colleagues (2001) and Rupp and colleagues (2003) modified the New method to make it a two to four times faster process. Because their modified technique requires a low-skill level, no glass rings, is easily adaptable to imaging on a microscope, other "chicken labs" have devised their own modifications to suit their particular needs (Chapman et al., 2001; Dugan et al., 1991; New, 1955; Rupp et al., 2003; Stern and Bachvarova, 1997).

However, in recent times, the need for time-lapse imaging and the use of screening molecules has highlighted the drawbacks of the New and the EC culture techniques. These drawbacks include (a) reduced visibility for embryo observation in the case of windowing, (b) difficulty to view morphogenesis of one of the surfaces of the embryo in the case of windowing, and (c) inadequacy for molecular studies best addressed with blocking agents in the case of semisolid media.

Genetic manipulation has been a standard in genetic model organisms like *Drosophila*, *C. elegans*, and mice. However, although the use of these organisms has a clear advantage, they carry the limitation that a single or multiple genetic changes can have wider implications within the developmental program of their embryos. Manipulations like conditional knockouts in mammalian embryos or RNAi treatment of worms

Additional supporting information may be found in the online version of this article.

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by-pass certain critical developmental periods or have wide-ranging effects on multiple organ systems. Altogether, it has been difficult to assess the specific functions of certain proteins in real time using these genetic techniques in these model systems.

In this work, we provide (1) an adaptation of the well-known culture EC method of Chapman and colleagues to time-lapse imaging (Chapman et al., 2001; Streit, 2008) and (2) a new technique that allows functional experimentation using blocking agents. Thus, within the intact embryo, we can specifically observe both the behavior of cells and tissues and the function of specific secreted factors. These powerful techniques can provide an easy, direct, real-time method of determining the developmental function of secreted molecular factors in the whole embryo.

MATERIALS AND METHODS

Our first method is similar to those described in Chapman et al. 2001 with the following changes. Briefly, we used either thin egg white collected using sterile techniques (a less-used technique than the semisolid medium, though also presented in Chapman and colleagues (Chapman et al., 2001) or tissue culture medium. In addition, we used large rings cut in nitrocellulose or PVDF membranes. Last, for convenience, we incubate embryos in a 5% CO_2 incubator instead of an egg incubator.

Ex Ovo Culture

Embryos are transferred, ventral side up, to 10-cm or 6-cm Petri dishes containing about 3 mL or 1–1.5 mL of sterile thin egg white or culture media, respectively (Dulbecco minimal essential medium, DMEM). Both egg white and culture media are clear liquids and therefore allow flexibility in imaging the developing embryo. Chicken eggs have two kinds of egg white where one is thick and sticky and the other is more fluid with a liquid consistency. The latter thin egg white is cheap, and fast and easy to collect. We used conditioned standard culture media, [DMEM (OMEGA Scientific) with 10% fetal bovine serum (FBS, OMEGA Scientific)], that was either collected from cultures of soluble Slit2 or Slit2-Receptor constitutively expressing cells or was media that included a specific concentration of the molecule of interest. Serum was specifically included, because it increased embryo quality and survival. Embryos were cultured in a total of 3 mL of conditioned medium.

Unlike the two published techniques of Chapman or Rupp, who collected embryos on rings cut from Whatman filter paper, we collected embryos on rings cut from nitrocellulose or PVDF membranes to avoid air bubble formation commonly seen using Whatman paper rings on the embryonic membrane (Chapman et al., 2001) or embryo detachment from the whatman filter papers. In our hands, air bubbles often formed along the thick inner edge of the Whatman filter paper ring, on the embryonic membranes. This was especially true when the embryos were cultured in thin egg white. Air bubbles were especially problematic during time-lapse imaging, because they would often obscure the view or damage the embryo. The thinness of rings made of nitrocellulose or PVDF membrane allows little space between the embryonic membrane and the bottom of the dish, and therefore air bubbles rarely formed underneath the embryo. Nitrocellulose membrane must be handled with Millipore forceps.

To promote the development of a normal a blood vessel network, we cut rings as wide as possible (~3–4.5 cm) of a 1-cm diameter. The nitrocellulose or PVDF ring holding the embryo was placed at the bottom of the Petri dish, with a thin layer of the appropriate conditioned media covering both the bottom of the dish and the lower side of the embryo.

For the embryonic membranes to remain attached to the nitrocellulose or PVDF, it is important to push excess thick egg white away from the surface of the embryo and surrounding membranes, as is required for the New culture system. This can be accomplished by using the blunt edge of a pair of forceps (VWR International, cat. No. 25729-503) held almost horizontally. The ring of nitrocellulose or PVDF membrane is placed in direct contact with the cleaned embryonic membranes (Figs. 1B and 1C). We found that overrinsing the embryo is detrimental to its survival; the key is to balance expediency in cleaning the membranes and removal of most, but not necessarily all, of the egg yolk. Last, the embryos are incubated at 37°C in a CO_2 incubator (5%), where temperature and humidity are stable and controlled. Embryos are incubated up to 48 h until the blood vessels reach the edge of the nitrocellulose or PVDF ring (usually stage HH19-21), at which point the embryo dies (Flamme, 1987).

We used two imaging modalities. For low-magnification video-microscopy on a stereoscope, we minimize drying of the embryo by placing the Petri dish containing the embryo in a larger glass dish filled with water. The inside surface of the glass cover of the larger glass dish is thinly smeared with egg white to avoid water condensation. To maintain the appropriate incubation temperature, we place the dish onto a slide or coffee mug warmer set to 36–38°C. We used a Nikon Coolpix 4300 camera, attached to the microscope with a Scopetronix adapter (www.scopetronix.com) and use the Krinnicam software (krinnicam.cjb.net) to capture time-lapse images at 10-min intervals. For fluorescent imaging at higher magnification or to visualize layers deep within in the embryo, we used confocal microscopy. We set the embryo in one of the chambers of a six-well culture dish (Figs. 1E–1G). To use an inverted microscope, 3-cm holes are drilled in the bottom of one or two wells and covered with 45 × 50-1 cover-slips (Fisher Scientific, catalog # 12-545-H) secured with silicon grease. Empty wells are filled with sterile water to provide enough humidity in the chamber and prevent dehydration of the embryo. The cover of the imaging chamber is wiped with thin egg white to prevent water condensation along the light path. To maintain incubation temperature, a cardboard chamber covered with reflectrix insulation is built around the microscope stage, and the chamber is heated with a multipurpose heater. The temperature inside the chamber is monitored with the probe of a temperature controller (Fisher scientific 11-463-47A) taped down onto the microscope stage, right next to the six-well plate containing the embryo. The temperature is set to 38°C. ImageJ is used to analyze the movies and the Stackreg Plugin is used to register the movies when necessary.

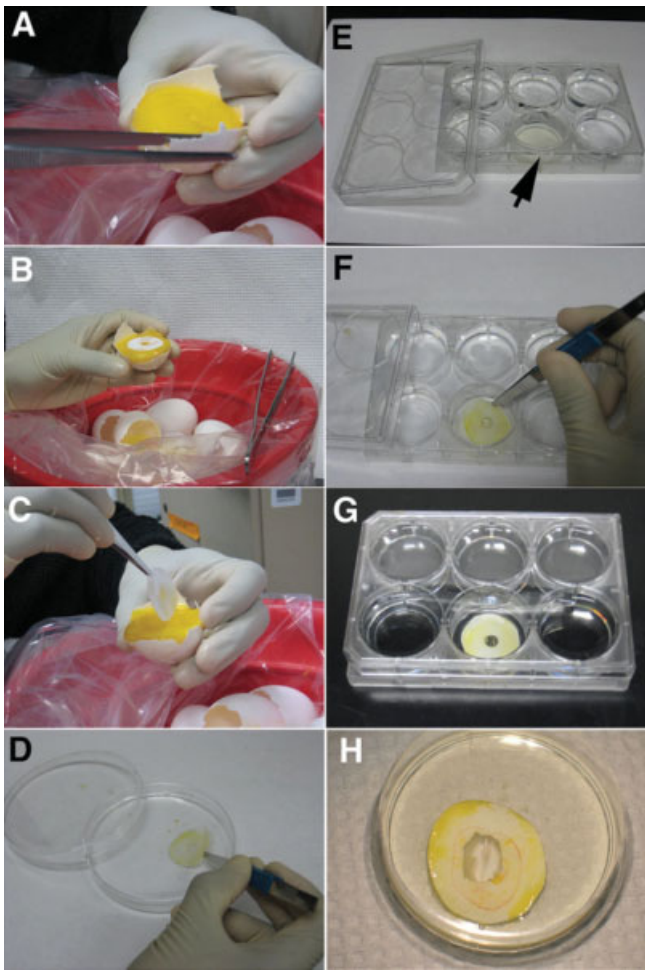

Fig. 1. Collecting and culturing embryos for imaging or blocking agent studies. **A:** The egg is opened with forceps to expose the embryo. Alternatively, the egg can be cracked into a Petri dish. **B:** The membranes surrounding the embryo are cleaned of thick egg white with the large forceps held horizontally and nitrocellulose or PVDF ring is placed around the embryo with a Millipore forceps. Alternatively, the thick egg white can be removed with Kimwipes. **C:** The embryo is released from the yolk sac by cutting around the ring with scissors and the ring is picked up with Millipore forceps. **D:** The embryo is quickly and gently rinsed in Ringers. **E:** A six-well tissue culture plate is prepared in advance. A 3-cm hole made in one of the wells is covered with a cover slip and filled with ~2 mL of thin egg white (arrow). The other wells are filled with distilled water. **F:** The embryo is transferred into the well containing thin egg white. **G:** The inside of the cover of the tissue culture plate is wiped with thin egg white and placed on the six-well plate. The six-well plate dish is then placed on the stage of a microscope, around which we assemble a cutout cardboard box covered with insulating reflectrix. We install a heater plugged into a temperature controller set to 37–38°C whose probe is taped on the microscope stage right next to the six-well plate. **H:** For embryos that will be kept in the tissue culture incubator or used for blocking agent studies, wider holes can be cut in the nitrocellulose or PVDF membrane with a razor blade to allow more flexibility when collecting the embryo from the egg.

In Ovo Manipulation

Eggs incubated to stages HH9-15 were windowed and injected subblastodermically with 0.3 mL of media conditioned for 3–5 days with HEK293 cells expressing the soluble form of Slit2 receptor (RoboN) or control cells (De Bellard et al., 2003; Wang et al., 2003) using an insulin (1 mL) syringe fitted with 25G 1-1/2 needle. We did not ink the embryos underneath. The eggs were sealed with transparent scotch tape (Office Depot packaging tape, Cat. No.363792) and incubated for 24–48 h. Embryos were then removed from the egg and fixed overnight at 4°C in 4% paraformaldehyde.

Whole Mount Immunofluorescence

After overnight incubation in blocking buffer [phosphate buffered serum (PBS) with 1% Triton-X100, 10% FBS], embryos were incubated with 1:100 HNK-1 supernatant in PBS overnight at 4°C. HNK-1 antibody was obtained from cell-culture supernatant from the Developmental Studies Hybridoma Bank, University of Iowa. The next day, embryos were extensively washed with PBS and incubated with an anti-mouse IgM-specific Alexa 488 conjugated antibody (Invitrogen, Molecular Probes). The following day the embryos were washed extensively and photographed with either an A-1 AxioImager or Z-scanned with a 410 LSM confocal microscope and projected into a single file with LSM 5 Image Browser by Zeiss.

To determine whether this method was truly efficient for testing molecules that may affect development, we focused on our established technique of neural crest migration (De Bellard and Bronner-Fraser, 2005). After rinsing the embryos in PBS to remove the paraformaldehyde, embryos were processed for whole mount antibody staining with HNK-1, a marker for migrating neural crest cells. To determine the scope of the effects from our embryo manipulation with the ex ovo and in ovo techniques, after wholemount staining with HNK-1 antibody the migratory pattern of neural crest cells was assessed as follows. In the instance of tail neural crest quantification, we looked at the last five somites for presence or absence of migrating neural crest cells that delaminated from the dorsal neural tube. It is known that neural crest will be present in the fourth and fifth from the last somites. Thus, presence of neural crest cells in the second and third somites is an indication that neural crest cells delaminated earlier than usual. In the case of cranial ganglia, we measured the area covered by neural crest cells and compared it with control. Finally, for the experiments using Paclitaxel, we performed in situ hybridization (De Bellard et al., 2007) with a probe for chicken Seraf (Wakamatsu et al., 2004) to determine if Schwann cell development was perturbed by this anticancer drug.

Embryo Electroporation

Chicken embryos at stage HH8-9 were electroporated as described elsewhere (De Bellard et al., 2007). In brief, we removed 3 mL of egg white with a needle from the horizontally incubated egg and cut an oval shaped window in the shell of the egg to expose the embryo. A 3–5 mg/mL solution of pCIG-GFP plasmid DNA (Megason and McMahon, 2002) was injected under the vitelline membrane into the neural tube or onto the ectoderm of the embryo. A picospritzer (General Valve) was used to deliver compressed air in pulses. L-shaped platinum wires were placed on either side of the neural tube. Two electrical square wave

pulses of duration 50 ms and of 25 mV were applied with a pulse generator. twenty microliters of Ringer's solution were deposited onto the embryo to maintain moisture and prevent drying. The eggs were resealed with tape and reincubated. When needed, embryos were removed from the egg, as detailed earlier, and prepared for time-lapse imaging.

RESULTS

Motivation and Embryonic Cultures

We attained two objectives in this study. First, we devised a flexible ex ovo method that combines the ability to image the live, whole embryo with the application of blocking agents. Second, we devised a simple method for studying the function of secreted factors through the application of blocking agents.

These aims required transparent culture conditions (for imaging) that allowed the embryo to be in direct contact with the culture medium (for blocking agents). For imaging without functional molecular studies, we cultured the embryos in thin egg white (New, 1955). For blocking-agent study, we used cell-culture media, because the egg white is probably inadequate (or less specific for that purpose than defined media) and because the numerous proteins (especially avidin) and factors contained in egg white can mask the effect of a blocking agent. We used tissue culture media with serum, which is also transparent enough for imaging. Culture media with serum is widely used in cell culture and live embryo experiments despite having many unknown factors (Hadjantonakis and Papaioannou, 2004; Passamaneck et al., 2006). For the embryo to grow in liquid media for extended periods with normal vascularization, we provided extra space for the expansion of the embryonic vascular plexus by cutting wide rings (Kucera and Burnand, 1988).

Collected embryos that were not used for imaging were placed in a tissue culture incubator for the duration of the experiment (Fig. 1). Embryos stage HH9 and older usually could be cultured and development would proceed as when inside the egg for 1 day. Interestingly, we found higher death and abnormalities after 2 days until stage HH19-21 in our culture system (this is when embryos are 37–44 somites, the eyes show signs of pigmentation and the extra-embryonic blood vessels are quite developed). For embryos cultured for 24 h, the results were 69% normal, 25% grossly abnormal (small head, missing eye), and 6% were dead (N = 36). For embryos cultured for 48 h, 50% showed normal development, and the rest were dead or showing signs of abnormal morphology (N = 26).

We were able to collect embryos as old as stage HH17 using nitrocellulose rings and thin egg white and cultured them with reliable success (N = 20). However, collection and cultures of younger the embryos (HH4-8) for this extended periods of time were very unreliable using our method, although survival with good morphology was achieved for 24 h but almost rarely to stage HH19-21. We attribute this to the fact that, when collected on such large rings, embryonic membranes often detach from the nitrocellulose membrane under the weight of attached egg yolk. Furthermore, the tension in the embryonic membranes is most often easily disturbed when these small embryos start growing to larger sizes. Therefore, for imaging embryos younger than stage HH9, we recommend the use of Chapman's technique; small Whatman paper rings coupled with thin egg white and imaging for shorter length of time (12–18 h) (Chapman et al., 2001; Rupp et al., 2003). For experiments with blocking agents, embryos stage HH9 and older attached to PVDF or nitrocellulose membranes are bathed in culture media containing the blocking agent. On average, 55% of embryos developed normally for 24 h (N = 86 embryos; used four different blocking agents) with ~70% of the surviving experimental embryos showing a phenotype (Table 1). Whether for imaging or blocking agent, embryos can only be cultured until the extra-embryonic vasculature reaches the edge of the ring. This occurs around stage HH19-21, even earlier sometimes.

TABLE 1. Summary of survival rates and phenotypes observed with both techniques

Ex ovo	Alive	Dead	%
Control	25	20	55.6
Experimental	76	58	56.7
Total	101	78	
Ex ovo	**Normal**	**Phenotype**	**%**
Control	16	9	36
Experimental	23	53	69.7
Total	39	62	101
In ovo	**Alive**	**Dead**	**%**
Control	38	21	64.4
Experimental	48	41	55.8
Total	86	62	
In ovo	**Normal**	**Phenotype**	**%**
Control	30	8	21.1
Experimental	6	42	87.5
Total	36	50	86

Bright Field Low Magnification and Fluorescent 4-D Confocal Microscopy

Using a Zeiss Stemi11, we imaged a developing chicken embryo in vitro for 26 h (Fig. 2 and Supporting Information Fig. 1). The movie in the Supporting Information shows that a stage HH12 chicken embryo (imaged until stage HH18) developed similarly in vitro (ex ovo) as it would in ovo. We were able to culture other embryos in vitro for 24–48 h in a tissue incubator (data not shown), and embryos were within one stage of their counterparts left in their eggs. We found that 30% of cultured embryos while showing normal development of their heart, nervous system, and general morphology had shorter trunk regions, although the number of somites was appropriate. Of interest is that development of vasculature on such large rings closely resembles the development of vasculature in the egg. In comparison, the vasculature of embryos collected and cultured on smaller rings is aberrant, showing very thin vessels in almost all cases. Notice in the Supporting Information movie the growth of blood vessels (arrows in Fig. 2).

With this method, we can visualize either ventral or dorsal embryonic morphogenesis at high magnification and is easily adapted to the imaging of a variety of moving cells. After fluorescently labeling, the embryos

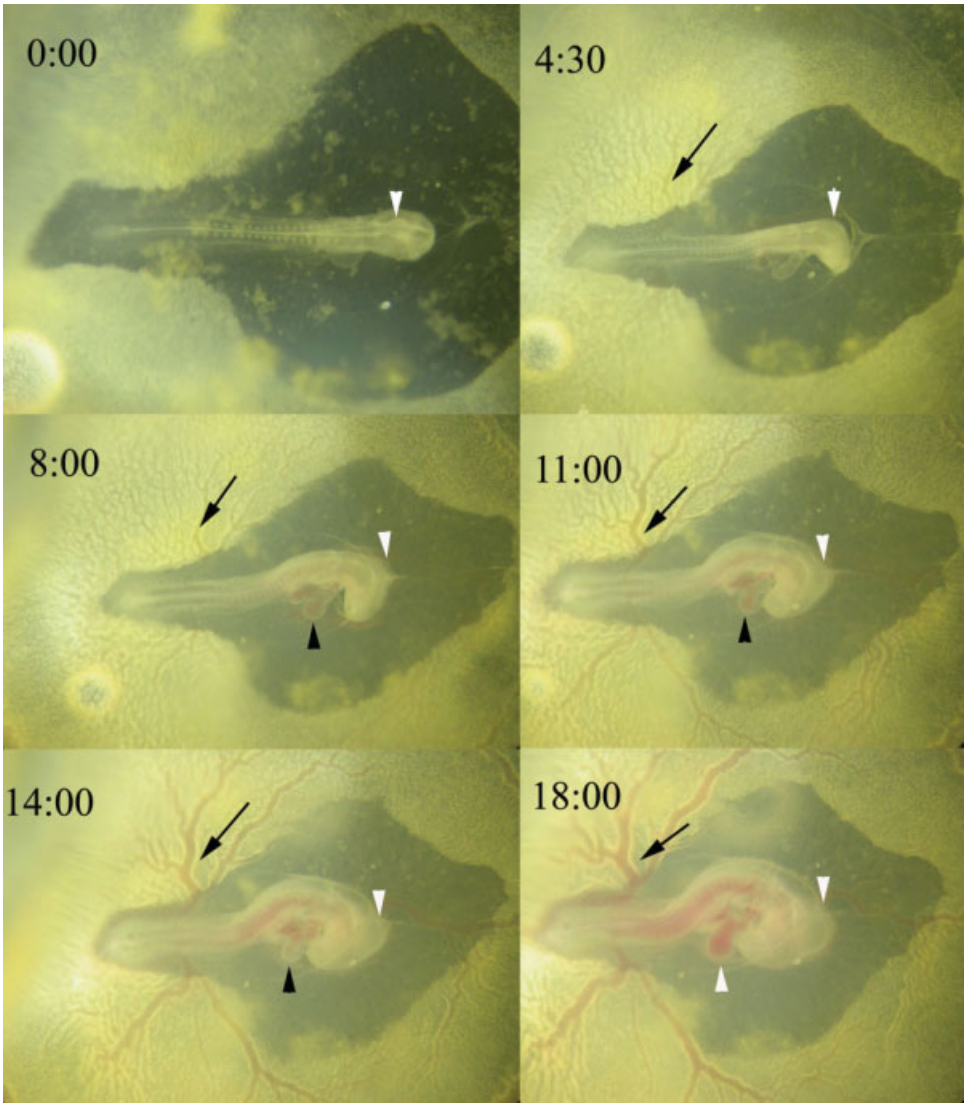

Fig. 2. Bright field time-lapse imaging is compatible with extended culture and shows the overall morphogenesis of the embryo. A stage HH12 chicken embryo was dissected and glued to a ring, placed ventral side down on 3 mL of clear egg white and covered with a glass top to prevent desiccation during the filming (see "Methods" section). It was imaged for 18 h until it reached stage HH18. Frames of embryo developing were taken every 90 s for 26 h. Notice how the embryo after 4.5 h has turned its head (white arrowheads), and the midbrain starts to develop and grow properly. The embryo's heart also grows and loops as expected (black arrowhead). Finally, the blood islands give rise to a wide vessel-network in our explanted embryos (black arrows). For the full video of the embryo development, see Supporting Information Figure 1S.

in ovo at stage HH10 by electroporation of pCIG-GFP construct injected into the lumen of the neural tube, the embryos were reincubated for 24 h until explantation ("Methods"). Low-magnification fluorescent imaging showed the migration of fluorescently labeled neural crest cells en route to the branchial arches using a confocal microscope (Supporting Information Fig. 2). We also were able to visualize ectodermal and vitelline cells and Z-stacking in time showed the movement of neural crest cells in deeper layers of the branchial arches. The overall movement of the embryo, resulting from a vigorous heartbeat and strong blood circulation, is a testimony of the health of the embryo even as it undergoes time-lapse imaging in culture.

Ex Ovo Blocking Agent Studies

We cultured stage HH11-12 chicken embryos for 24 h in direct contact-or submerged-in media conditioned with a blocking protein for the Slit2 ligand (De Bellard et al., 2003). Table 1 shows the total number of embryos that were alive with this technique and with neural crest phenotype. As shown in Figure 3, the migration of the neural crest was significantly disrupted in embryos cultured with a soluble form of the receptor for the chemorepellant Slit2 (RoboN) compared with embryos growing in the presence of control conditioned media. Notice how the trigeminal ganglia condensation is abnormal (Fig. 3E) or disrupted (Fig. 3F) when RoboN was present compared with con-

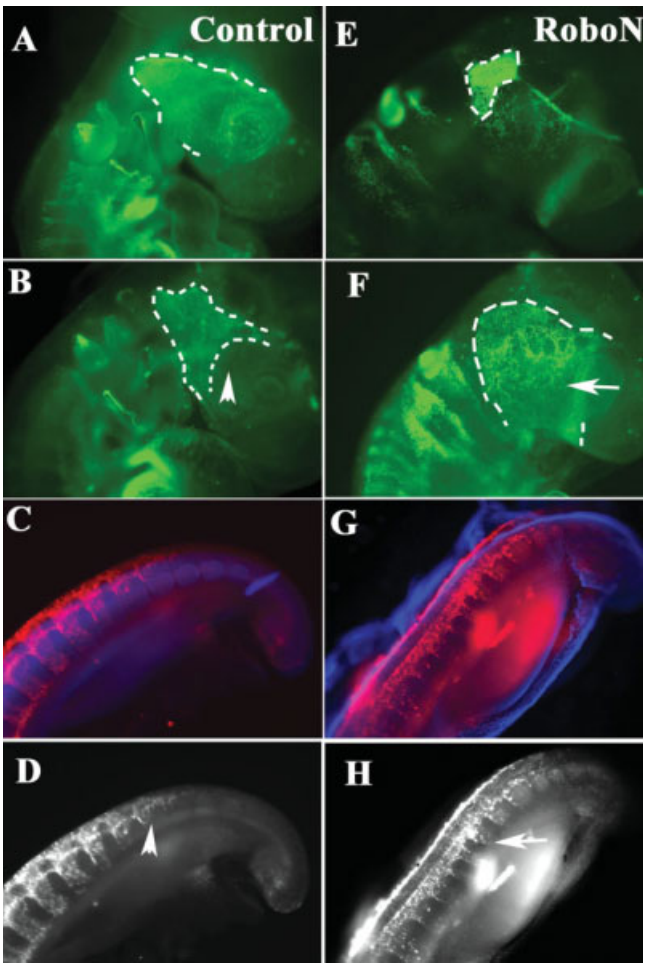

Fig. 3. Neural crest disruption with the "ex ovo" and "in ovo" techniques. EX OVO: Chicken embryos HH13 were dissected and bound to a nitrocellulose membrane ring, placed ventral side down in media conditioned by control HEK293 cells **(A** and **B)** or by cells permanently expressing a soluble form of Slit2 receptor (RoboN) **(E** and **F)** for 24 h. RoboN inactivates the endogenous Slit present in the embryo. Embryos were fixed and stained for HNK-1 to visualize the migrating neural crest with a secondary green fluorescent antibody. Notice how the trigeminal area (dotted lines) is either significantly reduced or enlarged due to cell dispersion after 24 h in the experimental embryos (**E** and **F**, respectively) while in controls, the trigeminal neural crest is starting to condense (**A** and **B**). IN OVO: Chicken embryos HH13 were injected sub-blastodermically with control media (**C** and **D**) or with RoboN (**G** and **H**) for 24 h. Embryos were fixed and stained for HNK-1 to visualize migrating neural crest cells with a secondary red fluorescent antibody and DAPI for visualizing the nuclei. Notice how the neural crest is present in the last somites in the RoboN exposed embryos compared with control treated embryo that shows neural crest starting at the fourth somite and up (Arrowhead in D and arrow in H).

trol-treated embryos. Measurements of the area covered by the neural crest showed that there was an increase of 25% ($N = 12$) in experimental compared to control treated embryos ($P < 0.05$ in a Student t-test). In addition, exposure to RoboN causes an increase in HNK-1 positive neural crest cells delaminating for the neural tube in the embryonic trunk and tail (data not shown). When looking at the trunk and tail end for signs of neural crest transition into a mesenchymal, migratory population, we observed that in the presence of RoboN there were more HNK-1 positive neural crest present in lateral sides of the neural tube after their delamination from the neural tube, compared with control-treated embryos (data not shown). These findings corroborate previous experiments showing that Slit2 is an important molecule in guiding migration of neural crest cells (De Bellard et al., 2003; Shiau et al., 2008) and further demonstrates that our method can be used to determine, relatively quickly, whether a given molecule has a function in cell guidance or growth.

TABLE II. Summary of Survival rates for the in ovo techniques by embryo stage

In ovo HH stages	Survival	Survival %
7–9	22/38	58
9–10	18/35	51
11–12	35/61	54
14–15	11/14	79
Total	$N = 148$	Ave = 60

In Ovo Blocking Agent Studies

A second method for conducting functional studies was tested in our search for new efficient, less-invasive techniques. This method takes advantage of the fact that the chicken embryo is quite resilient to manipulations while inside the egg. Thus, we cultured stage HH9-15 chicken embryos for 24–48 h inside the egg with the addition of 0.3–0.5 mL of (a) control conditioned media or RoboN (Fig. 3) or (b) 10 μM of Paclitaxel (Supporting Information Fig. 3).

We observed that in this experimental approach with the same Slit2-blocking protein, as in the previous ex ovo method, there was a significant change in the migration of the neural crest in these embryos at the level of the developing tail (Figs. 3C, 3D, and 3G–3H) and in the size of the condensing ganglia (data not shown). It is noticeable how neural crest cells labeled with HNK-1 antibody are present all the way to the third somite (counting the newest/last somite as the "first" somite), while control-treated embryos showed migrating neural crest cells mostly starting from the fifth somite on. Importantly, as in the previous ex ovo technique, the size of the ganglia was affected. RoboN-treated embryos had larger sympathetic ganglia as well disrupted trigeminal condensation (data not shown). The survival rate with this technique was between 51% for embryos stages HH8-9 and 60% for embryos at stages HH11-12, but when embryos were older (HH14-15) the survival rate increased to 79%. The percentage of experimental embryos showing a phenotype was 60% of live embryos, with all of these embryos showing earlier delamination at the level of the tail (as pictured in Figs. 3G and 3H) and 50% of the embryos showing the cranial ganglia phenotype (as pictured in Figs. 3A, 3B, 3E, and 3F).

These experiments were repeated at least six times with similar consistent results. The survival was much higher (51–79%) than with the ex ovo (50%) and, more

importantly, the embryos showed a similar phenotype as when exposed to the same blocking agent ex ovo (87%). These results show that the in ovo technique is reliable, simultaneously providing a less-invasive method than ex ovo culture, and an easier way to functionally manipulate the behavior of neural crest cells. As for the ex ovo technique, the survival of embryos increased with developmental age, older embryos had better survival than younger ones (Table 2).

DISCUSSION

In this work, we outline modifications to methods used in imaging the chicken embryo for a prolonged period of time ex ovo, and we present a new way of manipulating the embryonic environment as a way to look at the function of specific molecules during early development. The combination of these two methods will provide an easy and powerful way of understanding the role of secreted factors in cell and tissue behavior within the forming embryo.

The first method shown here is similar to the EC method published by Chapman and colleagues, one key difference consisting of the size and material used for holding the embryo to allow improved blood vessel growth. The fragility and growth of the vascular plexus (sinus terminalis) that surrounds the chick embryo had been an obstacle to extended culture in liquid medium (Patten, 1971) but solved by several groups (Chapman et al., 2001; Schoenwolf and Sheard, 1989; Stern and Bachvarova, 1997). However, the pattern of the vascular plexus is abnormal and the vessels that form are thin on smaller Whatman filter paper rings used for these techniques. In our method, we were able to maintain the integrity of the growing circulatory system by using larger NC or PVDF membranes rings. This change provided sufficient adhesion to the vitelline membrane for the extended period of time required for imaging and vascular development. Once the blood vessels reach the edge of the rings, however, extra growth is impossible and, if left in culture, the embryo dies (Flamme, 1987). A second key aspect of our modified ex ovo culture technique is the observation that chicken embryos can be cultured outside their natural surroundings of egg white. We successfully cultured embryos in the presence of tissue culture media that contained molecules/proteins important during development. The ex ovo technique described here thus provides the advantage of (a) studying the embryo for extended periods of time, especially during imaging, (b) studying the formation of the vascular tissue for an extended period of time in the vitelline membrane, and (c) studying the effect of molecules on chicken development.

Several earlier methods have made the embryo accessible for observation and allowed for live imaging of morphogenesis using light microscopy (Bortier and Vakaet, 1992; Jaskoll et al., 1991), epifluorescent microscopy (Schoenwolf and Sheard, 1989), or confocal microscopy (Kulesa and Fraser, 2002). Time-lapse imaging of embryos through a window in the egg can be achieved but is difficult, and visibility is reduced unless subpopulations of cells are fluorescently labeled (Kulesa and Fraser, 2002). Prior methods have allowed culture of the embryo for extended periods of time, and these include windowing the egg and exposing the embryo even until hatching (Giamario et al., 2003). However, the method presented here increases the range of organs, specifically blood vessel formation that can be viewed by time-lapse while allowing functional manipulations with blocking agents.

The second novel approach for the study of embryo development dealt with the manipulation of development ex ovo and in ovo. Currently, one of the preferred approaches is by electroporating either morpholino antisense oligonucleotides or plasmids that will make shRNA in chicken embryo. However, although being powerful techniques, they have the inconvenience that their effect is mostly limited to the electroporated cells only. The pattern of migratory neural crest disruption we observed with both ex ovo and in ovo techniques is extremely similar to the results obtained by electroporating morpholino for Slit receptor in trigeminal neural crest (Shiau et al., 2008). This similarity validates our method. In addition, we repeated this method with other molecules obtaining different neural crest phenotypes, which further confirm these new techniques as useful to determining if certain molecules may play a role in development. The use of blocking agents for functional studies present two distinct advantages over morpholino or dominant negative DNA electroporation; it is easier and cheaper. As a result, treating larger number of embryos with larger number of molecules is no longer a daunting task and makes statistical analysis easier.

Although they both allow functional studies with blocking agents, the ex ovo and in ovo technique each presents advantages and disadvantages. The ex ovo method is easily compatible with time-lapse imaging by light or confocal microscopy (Supporting Information Fig. 2), because the embryo develops in an environment that is optically clear, which does not scatter light. However, the shortcomings of the ex ovo method consist of shorter and lower embryo survival. Indeed, embryos can be cultured up to 2 days, and no more. In addition, the death rate is relatively high ($\sim$50%). In contrast, the in ovo technique is great for good survival ($\sim$65–80% depending on stage) and offers the possibility of extended culture periods. However, that method is not the most conducive for live imaging. First, setting up for time-lapse imaging in ovo is not trivial (Ezin and Fraser, 2008). Second, because the egg yolk is highly light-scattering, the embryo must be fluorescently labeled (by electroporation or DiI labeling for example) and only those labeled cells can be viewed by time-lapse. In addition, because of the yolky background, the larger embryonic context cannot be imaged simultaneously in bright field, as is routine ex ovo. In brief, it is important to choose the method that suits one's purposes best.

Studying the role of molecules in the context of the whole embryo, instead of pieces of explanted tissues, leads to a better understanding of the role of these molecules during development. That the embryo can grow in tissue culture media is important, because the numerous unknown factors in thin egg white might otherwise quench the effect of biological-blocking agents. However, substituting thin egg white with fetal calf serum in the tissue culture media does not

completely circumvent the underlying issue of unknown factors present in the assay. There are a large number of unknown factors present in fetal calf serum that could likewise interfere with substances added to the culture media. In addition, because the embryo can be cultured for a relatively long period of time, later effects of blocking antibodies or biochemical drug can be assessed with comparative ease. This is very important for those using the chicken embryo as their developmental model organism; there are no genetic screening techniques for chicken as there are for mouse, zebrafish, nematodes, and others. This has been a disadvantage when using the chicken as a model organism. However, with our technique, we can now "screen molecules" just as mutants are screened for the above organisms to determine those that are involved in development. Indeed, a somewhat similar technique of injecting retinoic acid has been successfully used with chondrichthyans (Dahn et al., 2007). In addition, we have the flexibility of timing the application of the blocking molecules and can therefore circumvent stages during which the absence or presence of the molecule is lethal to the developing embryo. One difficulty that comes from this technique is the possibility that some drugs/molecules could be blocked by interacting with those abundant proteins present in the yolk and egg white. Currently, we have no evidence for or against this possibility, but we observed reproducible results in our Slit experiments comparable to ex ovo culture. In brief, this method is somewhat similar to and provides the same type of results as treating nematodes with RNAi or adding chemicals in the water of developing zebrafish (Boyd et al., 2007; Cvejic et al., 2008; Poteryaev and Spang, 2008; Qadota et al., 2007; Redd et al., 2006). Typically RNAi shows phenotypes in 25% of worms and only 50% in the best case scenario (Shao et al., 2007). Our results consistently showed phenotypes in 50% of embryos, albeit with a lower survival rate. In the two well-known techniques using worms and zebrafish, the amount and rate of RNA or chemicals that penetrate the embryo cannot be accurately determined. Furthermore, researchers cannot determine whether the observed altered phenotype is a direct or indirect consequence of the RNA or chemical within the cells of the embryo. Thus, the same shortcomings that apply to those two techniques also relate to our application of blocking agents.

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