Short Technical Reports

Culturing of Avian Embryos for Time-Lapse Imaging

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ABSTRACT

Monitoring morphogenetic processes, at high resolution over time, has been a long-standing goal of many developmental cell biologists. It is critical to image cells in their natural environment whenever possible; however, imaging many warm-blooded vertebrates, especially mammals, is problematic. At early stages of development, birds are ideal for imaging, since the avian body plan is very similar to that of mammals. We have devised a culturing technique that allows for the acquisition of high-resolution differential interference contrast and epifluorescence images of developing avian embryos in a 4-D (3-D + time) system. The resulting information, from intact embryos, is derived from an area encompassing several millimeters, at micrometer resolution for up to 30 h.

INTRODUCTION

Vast morphogenetic change during a brief time interval is the hallmark of embryogenesis—cells replicate, migrate, change shape, differentiate, and form tissues that can further move, fold, or separate to become a 3-D structure or organ. These dynamic processes occur on a minute-by-minute time scale, each requiring the appropriate environment in which to proceed. For example, gastrulation, neurulation, notochord extension, somitogenesis, foregut formation, and vasculogenesis are all occurring contemporaneously, but in spatially different regions within a vertebrate embryo. Typically, cells observed in culture do not behave as they would in situ; therefore, it is critical to examine cells within their natural environment whenever possible. A long-standing goal of developmental cell biologists is to monitor morphogenetic processes in a dynamic manner at high resolution over a wide length scale.

Imaging of a live tissue explant or embryo, in three dimensions, can be accomplished using noninvasive optical sectioning microscopy; images are acquired stepwise, at multiple distinct focal planes within a specimen, using a focusing motor. With the creation of new image-processing algorithms, it is now practical to examine embryos in four dimensions (X, Y, Z, time) at the level of the cell (1,3,4,10,13,15).

Mammalian embryos are difficult to image, while other vertebrates (zebrafish, frogs, and avians) have properties that make them excellent subjects for imaging. For example, the entire vasculature of the developing zebrafish up to seven days post-fertilization has been documented using fluorescence microangiography (8). Similarly, events during *Xenopus laevis* gastrulation have been recorded using time-lapse microscopy (5). Avian embryos make fine specimens for studying early development, as they are easily accessible and their developmental stage is readily determined. In addition, because they are planar and thin, they exhibit excellent optical properties. Finally, avians are warm-blooded animals with four-chambered hearts and body plans virtually identical to those of mammals at early stages of embryogenesis. As a result, many long-term in vitro and *in ovo* avian culturing techniques were developed compatible with microscopic study (2,6,9–12).

In considering long-term microscopic imaging, two practical issues must be addressed: sustaining specimen health and maintaining a clear optical path. For embryo cultures to remain healthy and develop normally, they require the following: (*i*) a humidified chamber at the proper temperature; (*ii*) a sterile, nutrient-rich, pH-stabilized medium; (*iii*) proper O_2/CO_2 balance; and (*iv*) for avians cultured *ex ovo*, a mechanical support that not only allows for natural deformations during

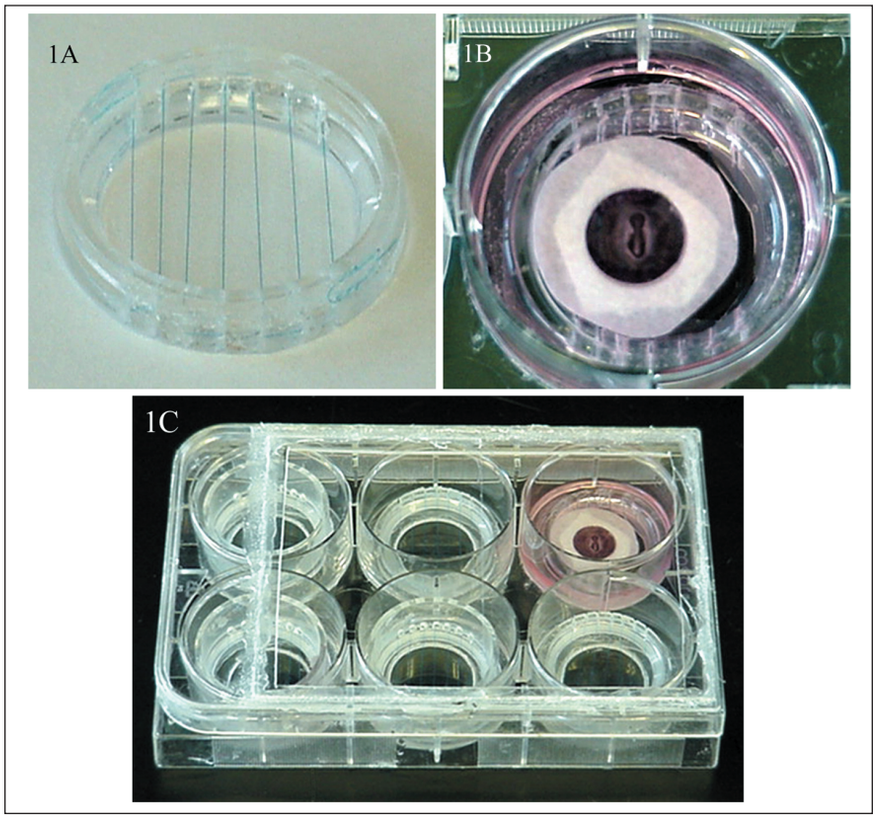

Figure 1. Avian culture system. (A) A picture of the culture insert, created from a Millicell cell culture insert and surgical suture. The sutures are spaced approximately 2 mm apart. (B) An embryo is shown on the suture bed within the culture chamber. Note that the sutures are sufficiently wide so that the embryo rests between two strands that do not interfere with the light path. (C) The culture chamber. With the configuration shown, four embryos can be imaged within one experimental session.

embryo growth but also allows for proper tension fields to be generated within the embryo. The latter condition requires the embryonic vitelline membrane to remain intact. Documenting developmental events using both differential interference contrast (DIC) and epifluorescence microscopy brings its own challenges. The use of DIC optics precludes the use of plastic surfaces in the optical path, as it randomizes plane-polarized light. Additionally, all surfaces between the objectives and the embryos must be kept free of condensation for optimal image clarity.

We describe here a culture system devised to meet all requirements for culturing avian embryos in a 4-D microscopic system. This configuration is capable of imaging avian embryos with high-resolution DIC and epifluorescence optics (4) for up to 30 h, encompassing any morphogenetic event from Hamburger and Hamilton (HH) stages 5–15 (7). A description of our culture system and a demonstration of the resulting DIC optical quality, as well as the documentation of the normal development of a quail embryo, are described below. This culture system can be adapted for use with other microscope configurations and may be used for other transparent embryos.

MATERIALS AND METHODS

Culture Insert

To optimize the quality of DIC optics, a culture insert was created by removing the filter material of a Millicell® cell culture insert (Millipore, Bedford, MA, USA) and replacing it with an array of polypropylene surgical suture fibers (7-0 SURGIPRO™; USSC, Norwalk, CT, USA), creating a supportive bed. The cell culture insert was notched using a Dremel® tool, and the suture material was woven through the notches (Figure 1A). The distance between the parallel-running sutures is approximately 2 mm. This is ample distance to allow an early avian embryo to be observed in its entirety without interference from the sutures, yet close enough to provide support for the embryo on its vitelline membrane (Figure 1B).

Culture Chamber

A closed culture chamber is created from a Costar® polystyrene 6-well plate (Corning, Corning, NY, USA). To permit DIC optics, the plastic in the light path is replaced with glass. A 19-mm hole is created in the base of each well by pushing a heated cork borer through the plastic. The rough edges are then filed smooth, and a 24 × 30 mm glass #1 coverslip is sealed over the hole using Marine Goop® (Eclectic Products, Pineville, LA, USA). The plastic in the lid is replaced by cutting a 70 × 90 mm rectangle within the lid near one end and sealing two 75 × 50 × 1 mm glass slides over the opening with adhesive around the outside edges, but not the abutting inside edges (Figure 1C). This allows viewing in four of the six chambers. Alternatively, if all six wells are to be used for image acquisition, holes may be bored through the plastic lid and sealed with coverslips as described for the base. With this alteration, it is important that the holes within the chamber and lid are concentric so that plastic at the periphery of the hole does not interfere with the light path.

Microscope Incubator

To culture embryos for dynamic imaging, a microscope-attached incubator was fabricated from cardboard (4 mm thickness) to enclose the stand and associated optics of a Leica DMR upright microscope (Leica Microsystems, Wetzlar, Germany) (Figure 2A). A small portable room heater and standard ductwork are used to direct air (38.5°C) into the incubator. Notably, the airflow is distributed across the cover of the six-well culture chamber using a manifold with multiple outlets. Further, an aluminum plate below the culture chamber is heated to 37°C (Figure 2B). The combination of the two heating systems maintains culture media within the chamber at 37.5°C, as calibrated with liquid crystal thermometer foils (Edmund Industrial Optics, Barrington, NJ, USA). Additionally, the vertical temperature difference (1°C) between the culture medium and the upper glass of the culture chamber lid prevents condensation on surfaces in the optical path. The temperature is regulated using Love 1600 controller units (Dwyer Instruments, Michigan, IN, USA) with thermocouple sensors (PT6; Physitemp Instruments, Clifton, NJ, USA).

Embryo Preparation

Quail embryos (*Coturnix coturnix japonica*; Smith Farms, Bucyrus, KS, USA) from HH stages 5–10 are removed from eggs and mounted on paper rings to allow their *ex ovo* manipulation (6). Briefly, eggs incubated for the appropriate times are carefully cut open, keeping the vitelline membranes on the dorsal aspect of the embryos intact. The egg, with embryo and vitelline membrane present, and most of the albumin are placed into a sterile plastic petri dish. The albumin is removed using a transfer pipet to expose the vitelline membrane. Paper rings pre-

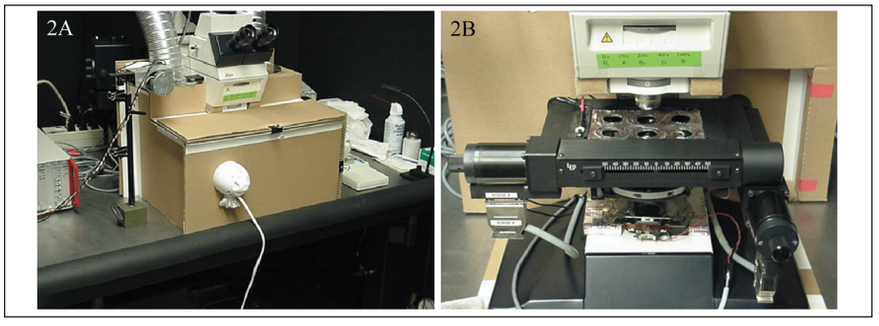

Figure 2. Microscope incubator. (A) A picture of the microscope incubator, encompassing the stand and associated optics of a Leica DMR upright microscope. Heated air (38.5°C) is piped into the incubator and directed across the surface of the culture chamber. Note the metal ductwork entering the top of the incubator. (B) The microscope stage and optics. The heated aluminum plate, shown on the stage, and the warmed air from above establish a vertical temperature gradient that prevents condensation on surfaces in the optical path.

pared from Whatman® 52 filter paper (Whatman International Ltd., Maidstone, UK), cut with an inner diameter of 11 mm and an outer diameter of 22 mm, are placed onto the vitelline membrane with the embryo directly centered within the ring. After 3–5 min, the paper ring adheres to the vitelline membrane. Using fine scissors, the paper ring/membrane assembly is cut along its perimeter and lifted off the yolk in the direction of the initial cut. It is best if the initial cut is made at the embryo's cranial or caudal end and the embryonic plate is pulled free of the yolk at a low degree of angle (2). Specimens are then gently immersed in embryo PBS (137 mM NaCl, 2.69 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.68 mM $CaCl_2$, 0.49 mM $MgCl_2$) and allowed to soak for 3–5 min. To remove loosely adherent yolk particles, the ring is gently agitated up and down and side-to-side. It is essential to remove as much yolk as possible to avoid obscuring the embryo during imaging. At this time, embryos are placed ventral side up on a solid agarose bed (5% agarose in embryo PBS, cast in 35-mm petri dishes) for microinjection. If no injection is required, then embryos can be placed directly into the culture chamber.

Embryo Culture

Embryos, on ring assemblies, are placed ventral side up on the suture-mesh scaffolds within the multi-well culture chamber containing 3–4 mL CO_2-independent Leibovitz-L15 medium supplemented with 2 mM L-glutamine, 10% chicken serum, and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). The culture medium should support the embryo from beneath but not flood the surface of the embryo. A second paper ring is gently placed on top of the embryo, forming a sandwich (bottom ring, vitelline membrane, embryo, top ring). The bottom paper ring and the vitelline membrane are thus in contact with the sutures (see Figure 1B). Any unused wells are filled with sterile deionized water to maintain high humidity during incubation. The culture chamber lid is sealed to the multi-well chamber using a vinyl electrical tape (3M Scotch 130C tape; 3M EPD, Austin, TX, USA). The culture chamber is then positioned on a multi-well microscope stage insert (Ludl Electronic Products, Hawthorne, NY, USA) within the attached incubator.

Microscopic Viewing and Image Collection

4-D analytical microscopy is accomplished on a Leica DMR upright microscope with attached motorized BioPrecision stage (Ludl Electronic Products) and a Photometrics Quantix cooled CCD camera (Roper Scientific, Tucson, AZ, USA). A 10× (0.25 N.A.) objective with a working distance of 20 mm is used for imaging. A more complete description of image acquisition, instrumentation, and software used is described in Reference 4.

RESULTS AND DISCUSSION

To demonstrate DIC optical quality and progression of normal development in this culture system, normal quail embryos were recorded in time-lapse. A number of physical features can be monitored to indicate normal development; addition of somites, extension of the notochord, cardiogenesis, and ingression of the anterior intestinal portal, to name a few. Figure 3 shows an embryo before, during and after a 12-h incubation within the culture system, demonstrating normal morphogenesis. Each image is created by alignment of 30 individual frames taken at 10× magnification to create a montage. The representative embryo added eight somites over a 12-h incubation, consistent with published rates of somite addition (1 somite pair/90 min) (14). The primitive streak and Hensen's node engaged in typical regression behavior, during which time extension of the notochord and normal neural tube formation occurred. The heart began beating shortly after HH stage 10 and proceeded to loop properly while the anterior intestinal portal moved posteriorly with proper ingression of the splanchnopleura. The blurring of the heart is caused by two factors. First, the plane of focus

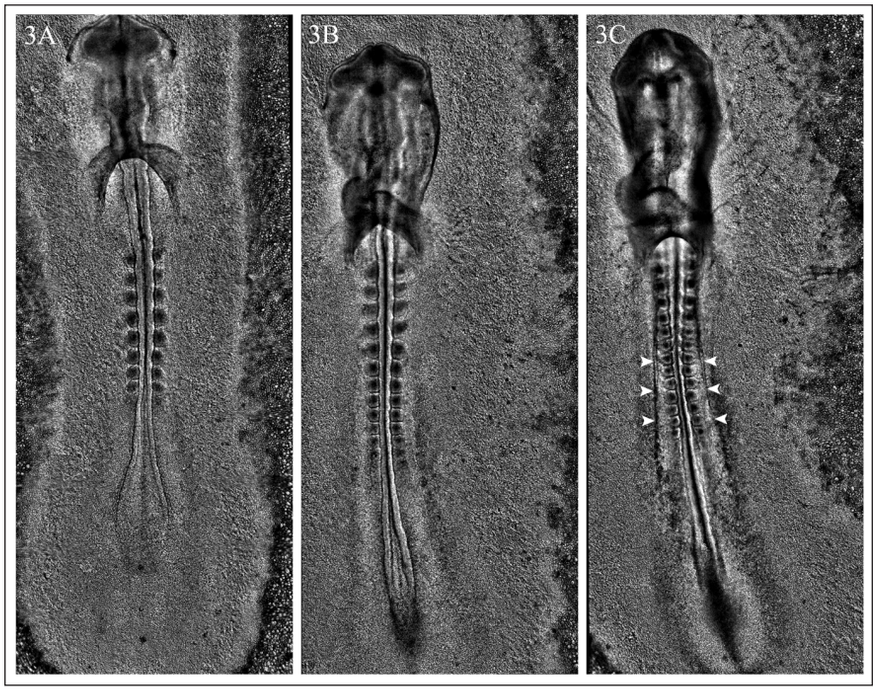

Figure 3. Normal quail development. The normal development of a quail embryo over a 12-h period is depicted. (A) An HH stage 9+ embryo at the initial time of observation. (B) The same embryo after 6 h incubation (HH stage 11). (C) The embryo after 12 h incubation (HH stage 12/13). The arrowheads point to the developing dorsal aortae. The montage images in each panel are created from 30 overlapping collapsed 10× fields. All images are from the ventral aspect of the embryo. A movie of the development of this embryo can be found at http://www.BioTechniques.com/Movie/Feb03/Little.mov.

was positioned at the level of the somites, not the heart. Second, the heartbeat causes a blurring of the image. The dorsal aortae can be seen running parallel and ventrolateral to the somites (Figure 3C). Although not apparent in this micrograph, proper extension of the pronephros occurred as well.

Using our system, embryos have been recorded for up to 30 h (60 h total incubation, HH stage 15) during which time head-turning and cranial and cervical flexures were observed. Additionally, the omphalo-mesenteric (vitelline) vessels formed, and in some but not all embryos, circulating blood was observed. We cannot extend imaging much beyond 30 h (60 h total incubation) using this culture method for several reasons: (*i*) there is a limited amount of yolk present for nutrients; (*ii*) the embryos, because of head-turning and flexures, do eventually pull free from their ring supports; and (*iii*) the cranial tissue becomes too thick for quality imaging.

The flexibility of the software allows a variety of image acquisition scenarios. The smoothest-appearing movies are created from merged images taken every 10–15 min (48–72 frames over a 12-h period). Longer cycling times are an option, but this will disrupt the apparent motion of the movies. If one changes a time-lapse parameter, then another can be changed to compensate; however, there is an upper limit to specimen number and time-lapse frequency. Ideally, in one session, three embryos, each with a 3×4 image grid with 10 z-sections in both DIC and epifluorescence modes, can be recorded to create relatively smooth motion. If more embryos are to be recorded, then the grid size or number of z-sections must be reduced to keep the cycling time appropriate. Thus, the operator can trade specimen number for fewer images or vice versa. However, decreasing the number of z-sections will decrease the quality of the resulting images. Obviously, the best movies will be created by imaging a few embryos with a small grid in only one imaging mode (DIC or epifluorescence). This will keep the cycling times short and maintain high-quality optics. An example of 4-D image acquisition can be found in the form of a QuickTime™ movie at http://www.BioTechniques.com/Movie/Feb03/Little.mov. This movie shows the developmental progression of the embryo depicted in Figure 3 with the following parameters: one embryo, 3×10 grid, 10 z-sections, one imaging mode, and a 10-min cycling time.

We show only DIC images here; however, the instrumentation works equally well with epifluorescence optics (4). If one is only interested in epifluorescence optics, then plastic and semitransparent membrane filters can be employed, but for proper DIC optics, glass optical surfaces are required. In addition to the optical quality, there are other benefits to this culture system as well: (*i*) use of paper rings allows easy manipulation of embryos; (*ii*) proper tension is maintained for normal embryo development; (*iii*) embryos can be cultured ventral-side up as late as HH stages 14 and 15, allowing for easier observation of ventrally-located events without interference from the vitelline membrane; and (*iv*) embryos develop at approximately the same rate as *in ovo*.

Our goal is to investigate temporal and spatial changes during the early stages of embryogenesis. Accordingly, we developed a culture system that allows us to monitor and record events (4) in the embryo with minimal interference to ongoing biological processes. At the same time, we can obtain information on the whole embryo, extended over an area of several square millimeters, with micrometer resolution.

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DNA Shuffling Method for Generating Estrogen Receptor α and β Chimeras in Yeast

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ABSTRACT

To facilitate our study of the molecular basis for the estrogen receptor (ER) subtype selectivity of novel ligands, we used DNA shuffling to construct chimeric ERs having ligand binding domains derived from both ERα and ERβ. The efficiency of chimera generation was low with traditional DNA shuffling protocols. Furthermore, ER ligand binding domain sequences lack convenient restriction sites for introducing chimeric ligand binding domain sequences into expression vectors. To overcome these problems, we developed a modified strategy whereby chimeric sequences were exclusively amplified from among the reassembled products from DNA shuffling using a special pair of PCR primers whose 3′ ends specifically match the α and β sequences, respectively, and whose 5′ ends match sequences outside the ERβ ligand binding domain. When chimeric ligand binding domain DNA sequences, amplified with these primers, were co-transformed into a yeast strain with a linearized expression vector for ERβ, an active expression vector was produced by homologous recombination. Twenty-two different crossover sites were found; most occurred when there was a stretch of eight or more identical base pairs in both sequences, and many were concentrated in the regions important for studying ligand binding and transactivation. This method should prove to be useful for generating chimeric gene products from parent templates that share relatively low sequence identity.

INTRODUCTION

Chimeric gene products can be produced from two homologous parent genes using restriction enzyme digestion and fragment ligation (15,19). However, the number and position of corresponding restriction sites are often very limited, making this strategy less general. Restriction sites can be engineered into the genes to facilitate gene fragment swapping, but this quickly becomes tedious when numerous chimeras are needed. Also, it is not always possible to introduce a restriction site at a desired position without changing the coded amino acids. Blunt-end ligation of PCR-generated fragments can be used to generate chimeras at any position, but this becomes cumbersome for more than one crossover and requires the rational design of crossover positions.

DNA shuffling involves in vitro or in vivo recombination methods for generating chimeric genes that rely on short stretches of identical DNA sequence in the homologous parent genes. DNA shuffling for chimeragenesis in gene families (13,17,18) has been widely used in directed evolution, examples of which include changing enzyme substrate specificity (24), improving enzyme thermostability (3), distinguishing functional and nonfunctional mutations (25), and probing protein structure-function relationships (4). The original Stemmer method for DNA shuffling uses DNase I fragmentation and gene reassembly (17); however, a variety of modified methods has been developed to improve the efficiency of in vitro recombination: staggered extension (5,26), shuffling with restriction enzyme-cleaved DNA fragments (6), shuffling using ssDNA (7,23), random chimeragenesis on transient templates (RACHITT) (1), the homology independent ITCHY method (12), and the