DEVELOPMENTAL DYNAMICS 220:284–289 (2001)

BRIEF COMMUNICATION

Improved Method for Chick Whole-Embryo Culture Using a Filter Paper Carrier

SUSAN C. CHAPMAN[1]* JÉRÔME COLLIGNON,[1,2] GARY C. SCHOENWOLF,[3] AND ANDREW LUMSDEN[1]
[1]*MRC Centre for Developmental Neurobiology, King's College London, Guy's Hospital, London, United Kingdom*
[2]*Departement de Biologie du Développement, Institut Jacques Monod, Paris, France*
[3]*Department of Neurobiology and Anatomy, University of Utah School of Medicine, Salt Lake City, Utah, USA*

ABSTRACT **We describe a simple method of chick whole-embryo culture, which uses a filter paper carrier to hold the early blastoderm and vitelline membranes under tension while the embryo grows on a substratum of agar-albumen. This is a quick and efficient means of setting up cultures of chick embryos beginning at pre-primitive streak stages to stage 10 (stages X–XIV, Eyal-Giladi and Kochav [1976] Dev Biol 49:321–337; stages 1–10, Hamburger and Hamilton [1951] J Morphol 88:49–92). This is an improvement on the original method of New, which used a glass ring and watch glass (New [1955] Exp Morphol 3:320–331). Our modification of New's method, which we call EC (Early Chick, pronounced EASY) culture, facilitates several manipulations in early chick embryos, including microsurgery, grafting, bead implantation, microinjection, and electroporation. Using the EC method, embryos at stage 8 and older can be readily cultured either dorsal-side up (in contrast to New's method) or ventral-side up, as desired; embryos younger than stage 8 can be culture only ventral-side up (as with New's method). We also discuss some alternative methods for setting up these cultures.**

Key words: avian development; in vitro; gastrulation; neurulation

INTRODUCTION

To culture intact gastrulating and neurulating chick embryos, we have developed a method, called EC (Early Chick, pronounced EASY) culture, that is quicker and easier to perform than the method developed by New (New, 1955; reviewed in New, 1966) and that eliminates the glass rings and watch glasses required in his original procedure. Additionally, development of the extraembryonic vasculature occurs significantly better in EC culture. Our method uses a piece of filter paper, with a central aperture, as a frame to hold the blastoderm and vitelline membranes under tension. The EC method provides an efficient and simple means of setting up a large number of whole chick embryos for culture at one sitting. In essence, our method derives from Sorokin's technique, which has been used for many years as a way of harvesting whole-mount embryos, either for fixation or as an easy way to isolate embryonic material for grafting (Menkes et al., 1961; Flamme, 1987). We have extended this technique to whole-embryo culture, as described below.

Several procedures have been developed to culture chick embryos after explanting them from the yolk (Spratt, 1947; New, 1955; DeHaan, 1963; Flamme, 1987; Kucera and Burnand, 1987; Flamme et al., 1991; Connolly et al., 1995; for reviews see Hamburger, 1960; New, 1966; DeHaan, 1967; Stern, 1993; Stern and Holland, 1993; Selleck, 1996; Darnell and Schoenwolf, 2000; see also Cockroft, 1997, for a review of culture methods in various vertebrates). New's method, in which embryos are cultured on their vitelline membranes, is thus far unsurpassed for the culture of early stages (e.g., unincubated through neurulation; stages X–XIV, Eyal-Giladi and Kochav, 1976; stages 1–10, Hamburger and Hamilton, 1951).

New's method made it possible to perform microsurgical manipulations on whole chick embryos in vitro (New, 1955, and reviewed by New, 1966; DeHaan, 1967; Selleck, 1996; the last shows an excellent picture series of the procedure), thereby providing much easier access to very early stages as compared to in ovo approaches. When manipulated in ovo, young embryos often fail to develop further without severe morphological defects (especially in the neural tube and cardiovascular system; e.g., Fisher and Schoenwolf, 1983), due at least in part to dehydration and/or changes in mechanical tension after windowing of the egg shell (Fineman et al., 1986). Moreover, manipu-

Grant sponsor: NIH; Grant numbers: NS18112, DC 04185; Grant sponsor: MRC.
S.C. Chapman and J. Collignon contributed equally to this work.
*Correspondence to: Susan C. Chapman, MRC Center for Developmental Neurobiology, King's College London, 4th Floor New Hunts House, Guy's Hospital, London SE1 1UL United Kingdom. E-mail: susan.chapman@kcl.ac.uk
Received 11 October 2000; Accepted 7 November 2000

lations can be done only from the dorsal side on embryos exposed in ovo, and unless a contrast agent such as ink is injected sub-blastodermally, it is difficult to see and to stage young embryos.

New's method utilized glass rings to hold the vitelline membranes under tension, with the embryos mounted ventral-side up on a watch glass containing a pool of albumen. Although improving access to early embryos immeasurably, New's method still has severe limitations. First, setting up embryos for culture is an exacting, laborious, and time-consuming process. Second, obtaining the correct amount of tension is empirical, as is the adjustment of the amount of fluid contained within the well formed by the ring. Third, stretching the vitelline membranes to achieve the correct amount of tension often results in mechanical damage to the embryo, and the blastoderm frequently becomes detached from the vitelline membranes, preventing further development. Finally, it can be difficult to obtain appropriate glass rings and watch glasses necessary to perform the culture. Variations of New's classic protocol can be found in DeHaan (1967), Selleck (1996), and Darnell and Schoenwolf (2000). In one of the most useful variations, embryos are cultured in a 35-mm Petri dish containing a semi-solid agar-albumen substrate, instead of in a watch glass containing liquid albumen (Darnell and Schoenwolf, 2000).

Here we describe EC culture and delineate its major benefits as compared with New culture. Prior to stage 8, embryos can be cultured only ventral-side up as in New culture, but after stage 8, embryos can be cultured either side up, as desired. The range of manipulations that can be performed include microsurgery, microinjection, grafting, bead implantation, and electroporation. The embryo can be viewed directly in EC culture using transillumination or epi-illumination, and its development can be followed using time-lapse video microscopy.

EXPERIMENTAL PROCEDURES AND RESULTS

EC culture eliminates the need for a glass ring and substitutes standard (Whatman no. 1–5) laboratory filter paper, providing a surface for the vitelline membrane to absorb onto and thereby attach. It is simple, effective, and requires no specialized equipment. Four overlapping holes (each about 7.5 mm in diameter and collectively forming a four-leaf clover shape) are cut into filter-paper carriers using a standard hand-held, single-hole paper punch. The embryos are cultured ventral-side up, at least until they reach stage 8. Embryos from pre-primitive streak to stage 10 have been cultured successfully using this method. In general, the older the embryo at the time the culture is initiated, the more successful the culture. Up to stage 3a/b, about 50% of the cultures develop successfully, whereas for embryos from stage 3c onward, the success rate increases to at least 80%. At early stages, the yolk is often firmly attached to the blastoderm. This necessitates cleaning with saline washes, as yolk interferes with further development of the embryo if it is left in place. For later stages, yolk is more easily removed and the embryo is more durable.

A major benefit of the EC method is that large numbers of embryos can be set up for culture in a short period of time. Depending on the investigator, in the time that it takes to make one dozen New cultures, 2–4 dozen EC cultures can be made. This economy of time and effort is especially beneficial if egg batches vary in quality, as it allows for the rapid screening of several dozen eggs to pick the best ones. Unfertilized eggs and those with defects such as blistering of the blastoderm, detachment of portions of blastoderm from the vitelline membranes, or embryonic abnormalities, are easily recognized and embryos can be quickly discarded.

Before beginning EC culture, prepare a batch of agar-albumen culture dishes as described below.

Preparation of Agar-Albumen Culture Dishes (Darnell and Schoenwolf, 2000)

Consumables required to make approximately 80 culture dishes:

1. 35 mm Petri dishes (Falcon, 3001).
2. 50 ml Falcon tubes (Falcon, 2098).
3. 10 ml pipettes
4. 120 ml of thin albumen, collected from 2 dozen unincubated eggs.
5. 120 ml of simple saline, autoclaved (7.19g NaCl/ 1 l distilled water).
6. 0.72 g Bacto-Agar (Difco).
7. Penicillin/Streptomycin (Sigma, P0906).

Steps to follow:

1. Heat a water bath to 49°C.
2. Add the saline to a sterile 500 ml flask and bring it to boiling, using a hot plate/stirrer. Add the agar and stir until it is dissolved.
3. While the agar is dissolving, collect the thin albumen in a sterile Falcon tube (50 ml) or similar container. Place the tubes into the water bath at 49°C.
4. Once the agar is dissolved, put the flask into the water bath. Allow the liquid to equilibrate at 49°C.
5. On a flat surface, lay out 80 35-mm sterile Petri dishes with their lids removed.
6. Add the albumen to the flask containing the dissolved agar, and mix by swirling for 30–60 sec. Also add the penicillin/streptomycin to this mixture, 5 U/ml.
7. Using a sterile 10 ml pipette and pipette-aid (e.g., Drummond, Bibby Jet) or similar device, aliquot 2.5 ml of the mixture per Petri dish. Do this reasonably quickly, without introducing bubbles into the dishes. If more than 2.5 ml agar-albumen mixture is pipetted, the substrate will be too thick, subsequently degrading the imaging of embryos with transillumination.
8. Once the aliquoting is complete, replace the lids of the Petri dishes and leave the dishes for several hours or overnight at room temperature to dry.

Place dishes upright in an airtight container at 4°C and use them as required. The dishes can be stored at 4°C for 1–2 weeks, provided that sterile conditions are maintained.

Preparation of EC Cultures

1. After incubating eggs for the desired length of time, allow each egg to cool for 15-30 min before cleaning the shell with 70% ethanol and allowing it to dry, thereby providing some measure of sterility. Crack the egg cleanly so that the contents are deposited directly into a 10-cm glass Petri dish (Fig. 1A). To do this, first crack the shell by holding the egg with its long axis oriented horizontally and tap its lower surface against a solid work surface. Then, place the egg in the glass Petri dish with its cracked surface downward, push the egg firmly against the bottom of the dish crushing the shell, and rock the egg back and forth while pulling the two ends of the shell (blunt and pointed) away from one another, releasing the egg's contents onto the bottom of the dish. The blastoderm should be positioned uppermost and roughly centered on the yolk, and the vitelline membranes should be intact. Discard any eggs containing embryos that are not perfect.
2. Remove the thick albumen covering the blastoderm by using a piece of folded tissue paper (e.g., a Kimwipe). Do this by placing the paper onto the albumen at the edge of the blastoderm and gently drawing it away from the center (Fig. 1B). This should gradually lift the albumen away from the blastoderm. The albumen interferes with the ability of the vitelline membranes to attach to the filter paper. Thus, its removal is crucial. Failure to perform this step will result in loss of tension on the blastoderm, and the embryo will either develop poorly or be lost. In general, the older the embryo at the time of collection, the less the thick albumen covers the blastoderm.
3. Once an area of vitelline membranes is cleared, a piece of filter paper with a central aperture is placed gently onto the vitelline membranes, such that the embryo is framed as if a picture (Fig. 1C). The paper will immediately absorb liquid from the surface of the vitelline membranes, drawing the membranes tightly onto the paper. We have tried several filter papers including Whatman no. 1, 2, 3, 4, and 5, and all work equally well. Provided that the paper can be sterilized and it attaches to the vitelline membranes, it can be used. To prepare the filter paper, cut 1.5–2.0 cm squares, and using a hole punch, cut out a four-leaf clover shape in the center of each square.
4. Using scissors, cut through the vitelline membranes around the filter paper (Fig. 1D), taking care to cut completely around its perimeter (Fig. 1E). With forceps, gently pull the filter paper away from the yolk in an oblique direction (i.e., not vertically upward away from the yolk). It is best to pull in the direction of the initial flow of yolk through the cut vitelline membranes and/or in the direction of the craniocaudal axis of the embryo (Fig. 1F). Doing so, helps to prevent the blastoderm from separating from the vitelline membranes, and in pre- and early gastrula stage embryos, separation of the tissue layers from one another.
5. Holding the filter paper with forceps, gently remove any attached yolk using blunt forceps. Do this by stroking the vitelline membranes in a centrifugal direction (Fig. 1G). Any remnants of albumen should also be pulled away and discarded at this stage. This cleaning step is an important one. Because a shallow well forms over the cultured embryo, any remaining debris will inevitably flow toward the center of gravity and into the well, obscuring the embryo.
6. Two alternative methods can be used to remove the yolk from the filter paper.
   A. In the first alternative, the filter paper and attached embryo and vitelline membranes is placed into a Petri dish containing saline plus 5 U/ml Penicillin/Streptomycin (Sigma, P0906). To avoid detaching the blastoderm from the vitelline membranes or separating the germ layers from one another, do this by immersing the filter paper into the saline obliquely and in the direction of the craniocaudal axis of the embryo (Fig. 1H). Try to remove as much of the remaining yolk as possible using gentle swirling. After washing, remove the filter paper from the saline and dab an edge on a piece of tissue paper to absorb excess liquid. Then place the filter paper onto an agar-albumen culture dish, generally with the blastoderm ventral-side up (Fig. 1I). Be careful to avoid trapping air bubbles between the filter paper and the semi-solid medium.
   B. In the second alternative, the filter paper is placed directly onto the agar-albumen substrate. With a little practice, this method may be more satisfactory than the immersion method just described. The use of a semi-solid base allows for "washing" in situ. The blastoderm should be positioned ventral-side up, with the vitelline membranes acting as a barrier between the embryo and semi-solid medium. Wash off excess yolk, using a saline jet from a Pasteur pipette (Fig. 1J). Be careful not to detach the blastoderm from the vitelline membranes or separate the germ layers from one another. Tilt the dish slightly and suck off the yolky liquid. Try not to disturb the agar-albumen substrate in the culture dish.
7. When washing is complete, replace the lid of the Petri dish and incubate several dishes together in a covered 150-mm Petri dish, with moistened tissue paper lining the base. Embryos in EC culture can develop essentially normally for up to 36 h, with some surviving less reliably up to 72 h. Excellent development of the extraembryonic (vitelline) vasculature frequently occurs (see Fig. 1L).

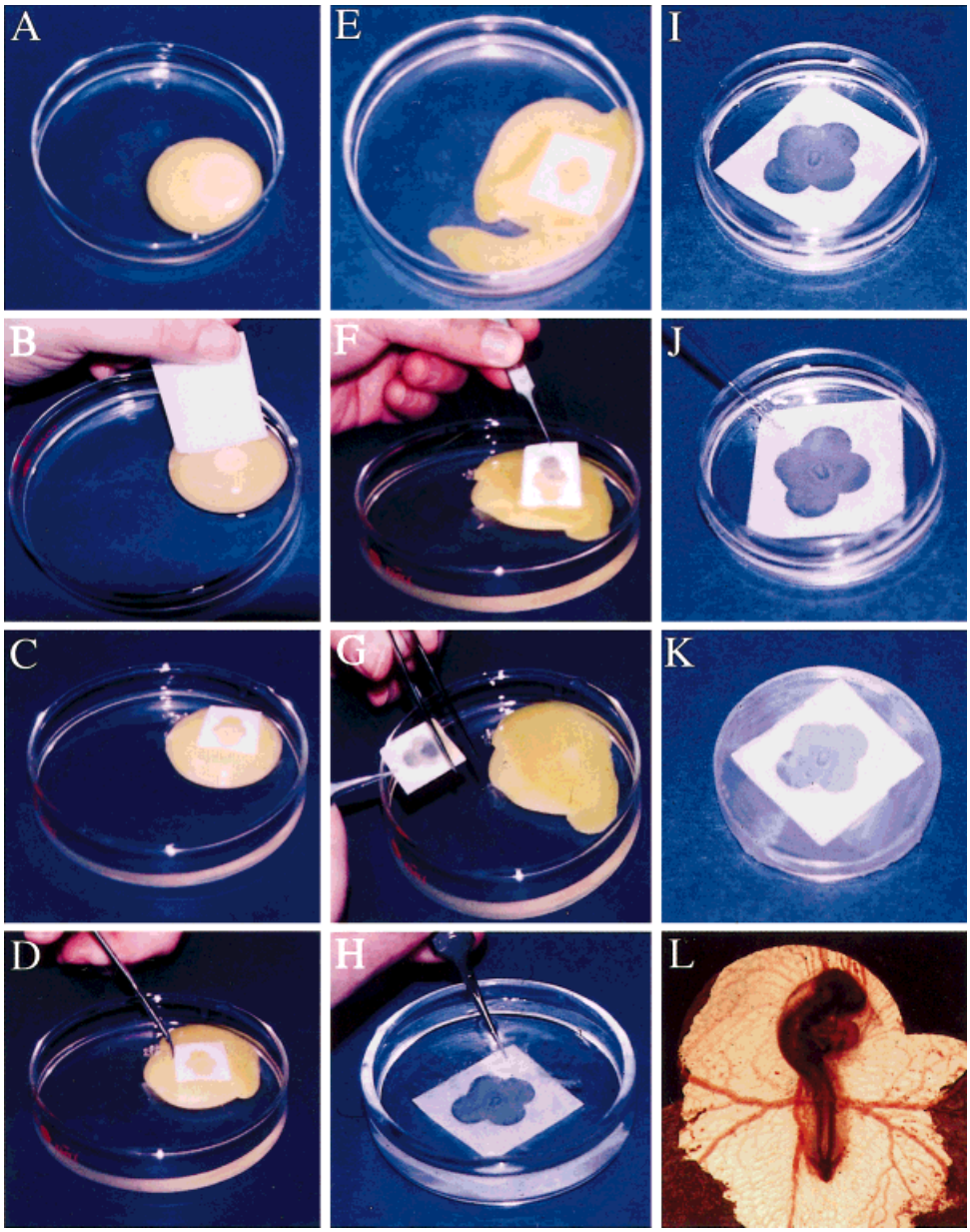

Fig. 1. Sequence of steps in preparing embryos for EC culture. **A:** Eggs should be incubated on their side until the desired stage is reached. After cooling for 15–30 min, break each egg into a glass Petri dish. **B:** Using a piece of tissue paper (e.g., Kimwipe) neatly folded, place the tissue paper on the thick albumen and pull the thick albumen away from the blastoderm in a centrifugal direction. **C:** Center the filter paper over the blastoderm. If the craniocaudal axis is apparent, align the filter paper with respect to this axis as desired. **D:** Cut the vitelline membranes around the filter paper, ensuring a complete cut. **E:** The filter paper will remain attached to the vitelline membranes after cutting if the albumen has been properly removed. **F:** Pull the filter paper away from the yolk in an oblique direction; that is, usually in the direction of yolk flow and/or the craniocaudal axis. **G:** Using blunt forceps, wipe excess yolk off the filter paper in a centrifugal direction. **H:** For removing as much of the remaining excess yolk as possible, use a small glass Petri dish, filled with saline and 5 U/ml Penicillin/Streptomycin, and swirl the EC culture to wash away excess yolk. **I:** Place the filter and contained blastoderm onto the substrate and cover the dish with a lid. Incubate dishes in a larger Petri dish containing moistened tissue paper lining the bottom of the dish. **J:** As an alternative method of washing away yolk, place the filter paper directly onto an agar-albumen culture dish. Using a Pasteur pipette, wash the yolk off with a jet of saline. After washing, remove excess fluid while tilting the dish. **K:** For an alternative culture substrate, construct a Parafilm covered Petri-dish lid, containing a central hole. Fill the lid up to the level of the hole with thin albumen. The culture is placed on top of the Petri dish, over the central hole, ventral-side up. **L:** An embryo after 44 hr of EC culture (beginning at stage 3/4). Note the excellent vascular development. The rostral portion of the brain shows signs of retarded development by this stage, while the rest of the embryo appears normal. However, the rate of development is essentially that as occurring in ovo (i.e., according to Hamburger and Hamilton, 1951, stage 4 embryos are at 18–19 hr of incubation and stage 17 embryos are at 52–64 hr of incubation; thus, embryos progress in ovo from stage 4 to stage 17 in about 39.5 hr; in EC culture, the embryo shown progressed through these stages in 44 hr).

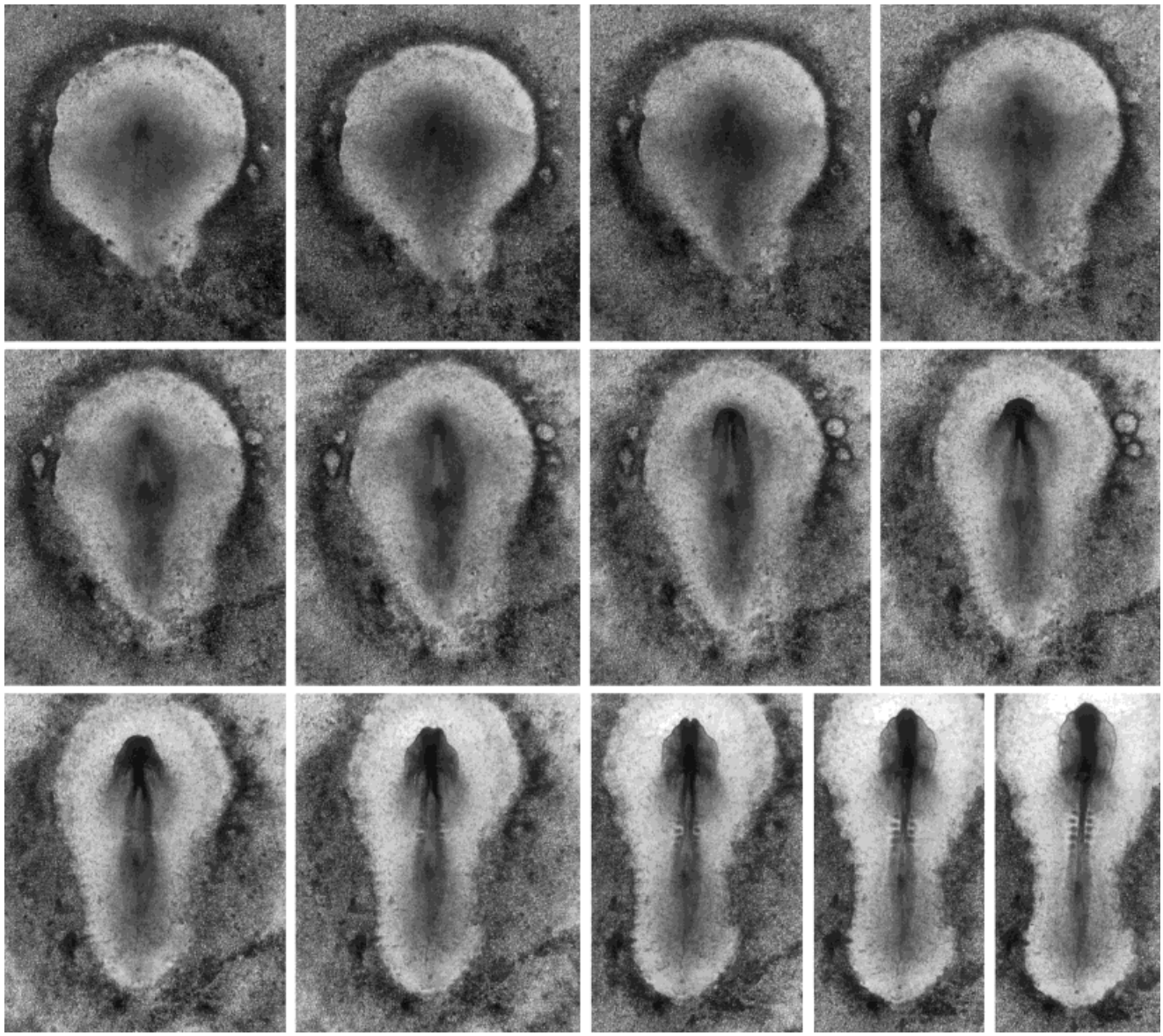

Fig. 2. Thirteen pictures in temporal sequence (from top left to bottom right) from a QuickTime movie showing the development of the embryo over a 12-hr period in EC culture. Frames were selected at 1-hr intervals, beginning at time 0 (Hamburger and Hamilton, 1951, stage 4) and end at 12 hr of incubation (Hamburger and Hamilton, 1951, stage 9-) in EC culture. Gastrulation and neurulation occur normally and at approximately the same rate as they do in ovo (i.e., according to Hamburger and Hamilton, 1951, stage 4 embryos are at 18–19 hr of incubation and stage 9 embryos are at 29–33 hr of incubation; thus, embryos progress in ovo from stage 4 to stage 9 in about 11.5 hr; in EC culture, the embryo shown progressed through these stages in 12 hr).

Alternative Methods for EC Culture

Culturing embryos dorsal-side up:

Follow the seven-step procedure described above for EC culture, and incubate the embryos until they reach stage 8 or older. Then, peel the culture off the substratum (or add saline to float it off) and replace it dorsal-side up on the substratum in the same dish (if saline is used to float the culture off, remove excess saline as described in step 6B). Alternatively, if the embryo is at stage 8 or older at the time it is removed from the egg, initiate the culture with the embryo oriented dorsal-side up. In either case, the vitelline membranes will now be uppermost, and the embryo will be sandwiched between the vitelline membranes and the agar-albumen substrate. Complete all washing prior to placing the embryo onto the substrate, as the yolk and debris cannot easily be removed when the embryo is sandwiched on the culture dish.

Unfortunately, embryos younger than stage 8 fail to develop in EC culture when oriented dorsal-side up. We have tried several modifications, such as using a more liquid medium and supporting the filter paper on a solid base, but none of these has proved successful. The

most likely explanation for the failure of young embryos to develop dorsal-side up is that the pressure on the early embryo is too great when it is sandwiched between the vitelline membranes and substrate.

Use of a liquid albumen culture medium:

Instead of using a semi-solid medium, some investigators prefer to use thin albumen alone as a substrate. To do this, follow steps 1–5 above for EC culture. Then,

1. Follow step 6A above for EC culture, removing the yolk by immersion.
2. Use the lid of a plastic 35-mm Petri dish (Falcon, 3001) turned onto its back and covered with Parafilm into which a 1-cm square hole is cut to make the culture dish. With a Pasteur pipette, fill the culture dish through the hole in the Parafilm with thin albumen, using care to avoid bubbles. Remove the embryo culture from the saline used in step 6A above, and dab one edge of the filter paper onto a piece of tissue paper to remove excess liquid. Place the filter gently down onto the albumen-filled culture dish, with the blastoderm centered on the hole in the Parafilm (Fig. 1K). The vitelline membranes should be in contact with the albumen and the blastoderm should be ventral-side up. Adjust the amount of albumen so that it "glues" the filter paper to the Parafilm but does not overflow onto the uppermost side containing the blastoderm.

SUMMARY OF THE BENEFITS OF EC CULTURE

There are several benefits of EC culture as compared with classical New culture: (1) It does not require either glass rings or watch glasses; (2) Standard laboratory filter paper is inexpensive and readily available, and it can be sterilized by autoclaving; (3) Tension on the blastoderm is maintained without the need to adjust the vitelline membranes on a glass ring. The correct tension is attained by placing the filter paper directly onto the intact yolk before cutting through the vitelline membranes; (4) Cultures can be made very quickly; (5) Identical conditions are maintained for each embryo; (6) Liquid does not pool on the embryo; (7) Embryos can be easily removed and replaced on the substratum and, therefore, can be cultured first ventral-side up and then dorsal-side up (after they reach stage 8); (8) Embryos typically develop at about the same rate as in ovo, and development of the extraembryonic vasculature is extensive (Fig. 1L); (9) Embryos can be routinely cultured until they reach stages 15–17, although expansion of the rostral brain region is retarded in such embryos (Fig. 1L) and sections reveal that some degeneration of the neural tube occurs (data not shown; degeneration of the neural tube also occurs in postneurula stages of embryos cultured by the method of New, 1955).

TIME-LAPSE VIDEOMICROSCOPY OF AN EC CULTURE

See http://www.interscience.wiley.com/jpages1058-8388/suppmat for a QuickTime movie of an embryo developing in EC culture. Selected frames of this movie are shown in Figure 2.

ACKNOWLEDGMENTS

We thank Jean-Francois Colas, Victoria Gallego Diaz, and Raj Ladher for critically reading the manuscript. We also thank Valerie Dupé and Jean-Francois Colas for technical assistance. Supported in part by NIH NS18112 and DC 04185 (G.C.S.), the MRC (A.L.) and a Wellcome Prize Studentship, Company of Biologists, Ltd., Travel Award and a BSDB Travel Award (S.C.C.).

REFERENCES

Cockroft DL. 1997. A comparative and historical review of culture methods for vertebrates. Int J Dev Biol 41:127–137.

Connolly D, McNaughton LA, Krumlauf R, Cooke J. 1995. Improved in vitro development of the chick embryo using roller-tube culture. Trends Genetics 11:259–60.

Darnell D, Schoenwolf GC. 2000. Culture of avian embryos. In: Tuan RS, Lo CW, editors. Methods in molecular biology, vol. 135. Developmental biology protocols. Clifton, NJ: Humana Press, Vol. 1. p 31–37.

DeHaan RL. 1963. Organization of the cardiogenic plate in the early chick embryo. Acta Embryol Morphol Exp 6:26–37.

DeHaan RL. 1967. Avian embryo culture. In: Wilt FH, Wessels NK, editors. Methods in developmental biology. New York: Thomas Y. Crowell, p 401–412.

Eyal-Giladi H, Kochav S. 1976. From cleavage to primitive streak formation: A complementary normal table and a new look at the first stages of the development of the chick. I. General morphology. Dev Biol 49:321–337

Fineman RM, Schoenwolf GC, Huff M, Davis PL. 1986. Animal model: Causes of windowing-induced dysmorphogenesis (neural tube defects and early amnion deficit spectrum) in chicken embryos. Am J Med Gen 25:489–505.

Fisher M, Schoenwolf GC. 1983. The use of early chick embryos in experimental embryology and teratology: Improvements in standard procedures. Teratology 27:65–72.

Flamme I. 1987. Prolonged and simplified in vitro culture of explanted embryos. Anat Embryol 176:45–52.

Flamme I, Albach K, Muller S, Christ B, Jacob HJ. 1991. Two-phase in vitro culture of explanted chick embryos. Anat Rec 229:427–433.

Hamburger V. 1960. A manual of experimental embryology. Chicago: University of Chicago Press, p 1–221.

Hamburger V, Hamilton HL. 1951. A series of normal stages in the development of the chick embryo. J Morphol 88:49–92.

Kucera P, Burnand M-B. 1987. Routine teratogenicity test that uses chick embryo in vitro. Teratogen Carcinog Mutagen 7:427–447.

Menkes B, Miclea C, Elias St, Deleanu M. 1961. Researches on the formation of axial organs. I. Studies on the differentiation of the somites. Acad Repub Pop Rom, Baza Cercet Stiint, Timisoara, Stud Cercet Stiint Med 8:7–34.

New DAT. 1955 A new technique for the cultivation of the chick embryo in vitro. J Embryol Exp Morphol 3:320–331.

New DAT. 1966. The culture of vertebrate embryos. London: Logos Press, p 1–245.

Selleck MAJ. 1996. Culture and microsurgical manipulation of the early avian embryo. In: Bronner-Fraser M, editor. Methods in cell biology, vol 51: Methods in avian embryology. New York: Academic Press, Inc., p 1–21.

Spratt NT. 1947. A simple method for explanting and cultivating early chick embryos in vitro. Science 106:452.

Stern CD. 1993. Avian embryos. In: Stern CD, Holland PWH, editors. Essential developmental biology: a practical approach. Oxford: IRL Press at Oxford University Press, p 45–54.

Stern CD, Holland PWH. 1993. Essential developmental biology: a practical approach. Oxford: IRL Press at Oxford University Press, p 1–333.