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Early chick embryos *in vitro*

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ABSTRACT In 1955, Denis New described a technique for the *in vitro* culture of early avian embryos that has formed the basis for nearly all of the experimental embryological studies performed on these species since that day. Many modifications to this technique have also been described in these four decades for specific experimental purposes. Here, we review the effects of some parameters that appear to be important for different aspects of the growth of embryos in this type of culture, and conduct a small experimental comparison between different modifications of the technique as described by various authors. We conclude that the original technique still compares favorably with its alternatives.

KEY WORDS: *New culture, chick embryo, avian embryo, techniques, primitive streak*

Introduction

McWhorter and Whipple (1912) are reported to be the first to have succeeded in culturing early (primitive streak stage) chick embryos *in vitro*. However, most of the important studies in the 1930's-1950's that contributed so much to avian experimental embryology followed the pioneering efforts of Waddington (1932). He adapted the plasma clot method used at the Strangeways laboratory for organ and tissue culture to support the growth of intact embryos. These cultures were difficult to set up, required sterile conditions, and did not allow the embryos to expand normally as they do in the egg. In addition, the peripheral regions of the embryo frequently became vacuolated and the embryonic axis was often stunted. In 1947, Spratt modified Waddington's technique by replacing the plasma clot with a mixture of agar and either egg albumen (Spratt, 1947a,b) or egg-yolk dialysate (Spratt and Haas, 1960a). Although this variation allowed cultures to be set up more easily, development of the embryo was not substantially improved over what Waddington had obtained. Nevertheless, this technique allowed Spratt and his followers to establish some important facts about morphogenetic movements (e.g. Spratt and Haas, 1960a) and the regulative capacity (e.g. Spratt and Haas, 1960b) of the early chick embryo.

A major advance was made when Denis New (1955) published a method for the culture of avian embryos that differed radically in its approach from those in current use. He aimed to emulate the relationship of the early blastoderm to its normal substrate (the vitelline membrane), on which it expands in the egg (New, 1959; see also New, 1966) and to use pure egg albumen as the culture medium. The strong bacteriostatic properties of the albumen made it possible to dispense with strict sterile techniques, and it turned out that the vitelline membrane allowed expansion of the extraembryonic regions and elongation of the embryonic axis at the same rate as in the egg. With this novel method, embryos could be maintained *in vitro*, in an optically clear system and accessible to manipulation, from pre-primitive streak stages to about stage 16 (Hamburger and Hamilton, 1951). Briefly, the New (1955) technique consists of explanting the blastoderm still attached to its own vitelline membrane, stretching the latter around a glass ring placed on a watch glass and then making a pool of thin egg albumen underneath the membrane (Fig. 1). The blastoderm is cultured with its ventral side uppermost, exposed to air in a humid atmosphere. Apart from New's own studies with this culture method (e.g. New, 1956, 1959), this technique has formed the basis for nearly all of the experimental embryological studies on the early avian embryo since its publication.

Some variations on the New (1955) technique

Less than a decade after the publication of the New (1955) method, others started to introduce modifications to it. Among them, Nicolet and Gallera (1961, 1963) described the use of a double-ring, allowing the vitelline membrane to be clamped between them to maintain its tension (Fig. 2). They also turned the preparation over, so that the ventral side of the embryo lay over the pool of albumen and its dorsal surface pointed uppermost. Nicolet and Gallera (1963) report that this arrangement not only makes the epiblast surface accessible for manipulation through a small hole in the vitelline membrane, but that the amnion forms normally under these conditions. Nicolet and Gallera's double ring system demands the use of rings of rectangular cross-section, cut from glass tubing (see Figs. 1D and 2), so that the inner and outer rings can be made to fit snugly to tighten the membrane. Jacob (1971) used a different approach, and recommended the use of special steel rings.

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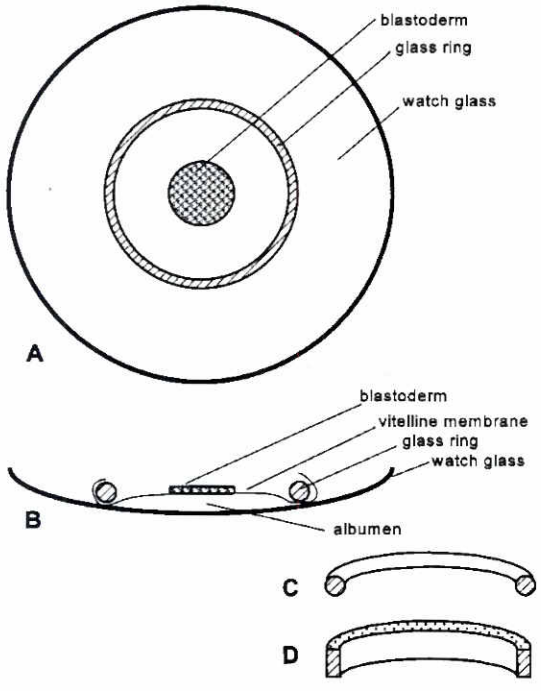

Fig. 1. Culture according to New (1955). (A) *Top view of the culture assembly.* **(B)** *Side view.* **(C)** *Diagram of one half of a ring of circular cross section.* **(D)** *Cross-sectional diagram of a ring of rectangular profile.*

This approach was further modified by Jaffe and Stern (1979) in order to make the epiblast accessible to a vibrating electrode to measure extracellular ionic fluxes. Here, the inverted system of Nicolet and Gallera (1963) was used but the embryo sat on a pool of heavy fluorinated oil (Medifluor) and the vitelline membrane was covered first with a layer of thin egg albumen (as in the embryo) and then with a thin layer of light paraffin oil to prevent evaporation from the open dish while recording (Fig. 3). In short term cultures, these embryos survived quite well, but long term survival was not assessed.

Another modification was introduced by Seidl (1977), who designed an elaborate device consisting of a dish with an integral ring surrounding a central hole, a second ring that clamps over and around the first, and a vertical piston that screws into the central hole (Fig. 4). In this way, the two rings act in the same way as in Nicolet and Gallera's (1963) technique, and the piston can be screwed up or down to adjust the tension of the vitelline membrane. In addition, the piston contains two inner channels that can be connected to syringes to add or withdraw culture medium. However, this device does not appear to allow the blastoderm to be observed during culture.

De Carli (1965), like Seidl, looked for a culture method that was easy enough for an inexperienced worker and one that allowed the solution to which the embryo was exposed to be changed. He designed a device made from a polyethylene vial and push-fit bung (12-14 mm diameter) (Fig. 5). First, a hole is drilled into the center of the bung and then this is glued (top down) to a microscope slide. The rim of the vial is cut, and this used to secure and stretch the vitelline membrane over the bung. According to the author, embryos cultured by this method develop up to Hamburger and Hamilton (1951) stages 15-16.

An important variation was introduced by Kucera and Burnand (1987). They designed a moulded silicon elastomere rubber dish of 51 mm outer diameter containing a raised ring (25 mm in outer diameter) at its centre (Fig. 6). The portion of the base that lies inside the ring is a very thin (0.5 mm) membrane, so as to be gas permeable. The vitelline membrane containing the embryo is transferred, with the embryo uppermost, to the chamber and the space inside the inner ring filled with egg albumen. In this way the membrane is held against the dish simply because the upper surface is dry but no second ring is used to secure it more tightly. Three factors appear to contribute to extend development beyond the period normally possible by the New (1955) technique: the larger volume of albumen culture medium available to the embryo, the unrestricted surface area of membrane over which the peripheral extraembryonic regions can spread, and the gas permeability of the base. With this method, embryos can develop for up to 66 h, to at least stage 17 of Hamburger and Hamilton (1951).

Effects of different parameters on embryo survival and longevity

While the original description of New (1955) and later specific instructions by others (e.g. De Haan, 1969; Stern, 1993) appear simple enough, seven parameters seem to be important: the composition of the saline used for explantation, the shape and size of the glass ring, the shape and size of the culture vessel, the amount of tension applied to the membrane, the amount of fluid above the embryo, the incubation temperature and humidity and the position of the embryo within the ring. The following sections discuss what we have learnt about each.

The saline solution

New (1955) suggested the use of a slightly hypotonic saline, mildly buffered to neutrality with phosphates, Pannett-Compton solution (Pannett and Compton, 1924), for explanting the embryos for culture. However, American authors have tended to use Howard–Ringer's medium, which is an unbuffered salt solution (De Haan,

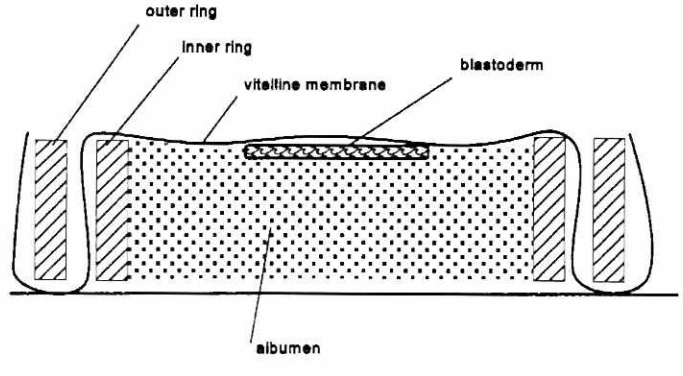

Fig. 2. Double-ring inverted culture according to Nicolet and Gallera (1961). *The assembly is shown in side view. The rings used have the profile shown in Figure 1D. Two rings fitting fairly snugly are used to secure the vitelline membrane so that the embryo can be cultured hanging from it, projecting into the albumen culture medium. Under these conditions, the amnion was reported to form normally by Nicolet and Gallera (1961, 1963).*

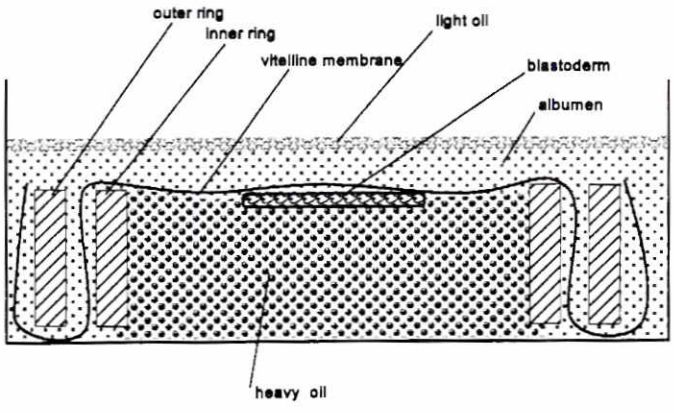

Fig. 3. Double-ring inverted culture according to Jaffe and Stern (1979). *The setup is similar to the one described by Nicolet and Gallera (1961) except that the embryo sits over a pool of heavy fluorinated mineral oil and the whole assembly is covered first with a layer of egg albumen and then with light paraffin oil, allowing the dish to remain open.*

1967). The pH of the explantation medium is probably unimportant because egg albumen is strongly alkaline in stored eggs (as high as 9.5) while the yolk is slightly acid (usually 6.5).

While there is probably no great advantage in the use of either solution, and we have had success even when forced to use plain tap water for explantation, we have found that Pannett-Compton saline tends to lead to more reliable adherence of the embryo to the vitelline membrane and that at early stages (pre-primitive streak) the embryo shows less of a tendency to develop a vacuolated appearance and bulging of the hypoblast layer. However, when the experiment requires that the embryo be submerged in saline for more than an hour or so, we have found that morphology is preserved better if the embryos are collected in Tyrode's solution, which is approximately isotonic and contains glucose (see Stern, 1993). We are not certain whether the more important factor is the salt concentration or the presence of glucose.

The glass ring

New (1955) did not state how the glass rings were made, but his Figure 1 shows a ring with circular cross-section (see Fig. 1B,C in the present paper). Likewise De Haan (1967) suggests that for student classroom use, metal key-rings are adequate. We have found that rings of rectangular cross-section, made by cutting sections of glass tubing (Fig. 1D), can be obtained easily and conveniently. However, the cross-sectional shape of the ring affects several aspects of the culture assembly. Rings with rectangular cross-section, provided they have not been polished, cause the vitelline membrane to adhere firmly to the upper edges of the ring. It is then easy to lift the ring with the vitelline membrane and embryo attached, and then to transfer it to another container for culture. This is of particular advantage if the living embryos will be photographed or followed by time-lapse filming, since a flat dish, preferably one with a thin glass bottom and lid, will have optical advantages over a curved watch glass. However, as will be discussed later, it seems that embryos maintained in a watch glass and with the membrane supported by a smooth glass ring develop slightly better (see below).

New (1955) recommended the use of glass rings of about 33 mm outer diameter and 28 mm inner diameter. These are slightly too large for the eggs that can be obtained here. We now use rings of either 27 or 30 mm outer diameter, 4-5 mm high. To fit the vitelline membrane around the 30 mm rings requires the membrane to be cut below the equator of the yolk. We have found that when the periphery of the *area opaca* reaches the glass ring the development of the embryo slows down considerably (see below), so larger rings are advantageous.

The culture vessel

As mentioned above, while the watch glass used for explanting the embryo is a convenient culture receptacle, as described by New (1955), some applications benefit from the use of other containers. For some time we have used 35 mm plastic Petri dishes. These can be stacked so that they take less space in the incubator and, more importantly, a large hole can be drilled in the base and lid of the dish, and a glass coverslip glued to the inside of both the base and lid to provide an optically clear surface for photography or filming. One problem with the use of 35 mm plastic dishes for filming applications is that their height does not allow a high power objective to be focused on the surface of the embryo unless the working distance of the lens exceeds 3-4 mm. For such high power applications, it is possible to set up the cultures in other types of dishes, such as that of the Cooper type designed for organ culture and again to drill a large hole in both the base and the lid (Stern, 1990) (Fig. 7). Here, the height of the glass ring becomes limiting; we have obtained glass rings of 27 mm diameter but just 1-2 mm high that can fit in the narrow space between the lid and base of Cooper dishes.

One problem when filming embryos in this type of culture, where there is a large air space between the embryo and the lid of the container, is that water condensation often forms on the lid. This is particularly acute when using a heated microscope stage, and not only does it obscure the view of the embryo but the droplets may

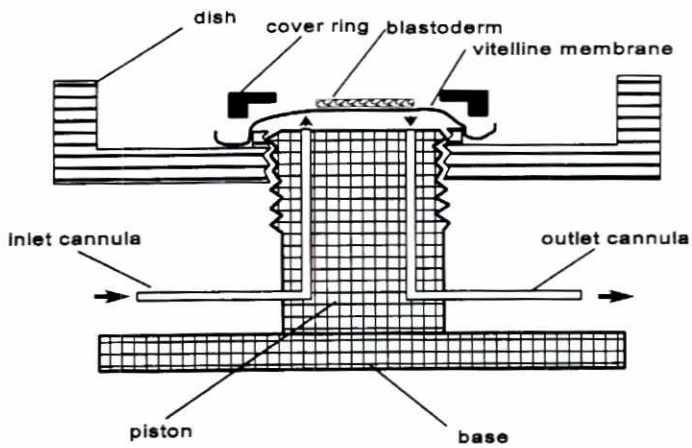

Fig. 4. Culture according to Seidl (1977). *The vitelline membrane is secured by a cover ring, which fits tightly onto a raised ridge in the base dish. This base has a central hole into which a piston can be screwed up and down to adjust the tension of the membrane. The piston also contains canals connecting to inlet and outlet cannulae, allowing the medium under the membrane to be circulated through. However, the embryo cannot be viewed with transmitted light.*

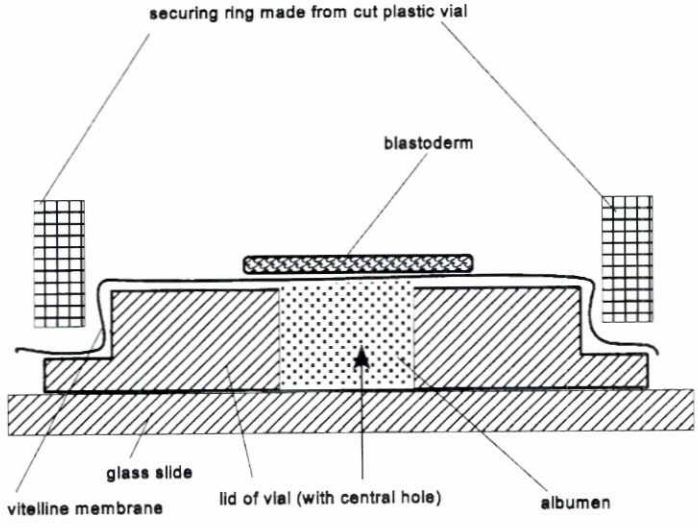

Fig. 5. Culture according to De Carli (1965). *The simple chamber is made by cutting a plastic vial with a push-fit lid. The lid acts as a container for the albumen culture medium (having been glued to a glass slide). A central hole is bored in the centre of the lid, over which the blastoderm will sit, attached to its vitelline membrane. Finally, a ring remaining from the body of the cut plastic vial is used to secure the membrane onto the lid. Although De Carli (1965) proposes the use of 13mm vials, we have found that a more convenient and larger chamber can be made easily from the outer plastic vials in which 35 mm films are usually packaged.*

coalesce and eventually fall on the embryo itself, generally causing substantial damage. There are two ways to avoid this problem. The first consists simply of wetting the inside of the lid with a little thin egg albumen just before placing the culture in the incubator. This both seals the lid preventing evaporation and creates a thin hydrophilic coating that causes condensation to form as a smooth film rather than droplets. The second way to avoid condensation on the lid is to heat it. If filming is done inside a large heated chamber or room that surrounds the microscope stage or the whole microscope, then the lid will be at the same temperature as the base and condensation should not occur.

Bioptechs Inc. (Butler, PA, USA) have recently introduced a device that may help to solve these problems. They adapted the same principle used to prevent condensation on the rear windscreens of cars so that the culture dish itself is heated to the incubation temperature. The lid can be treated in the same way to warm it to just above dew point. The device consists of a temperature controller, and modified 35 mm dishes containing a special coverslip on the base and/or the lid. The coverslips are coated with a conductive material and connected to the temperature controller. No further heating devices are required around the microscope and no condensation occurs if the lids are heated. However, at present the lid of these chambers lies about 3 mm above the surface of the embryo and it is still difficult to obtain films at high magnification except with ultra-long working distance objectives.

The tension of the vitelline membrane

While the vitelline membrane is being wrapped around the glass ring, the tension of the substrate for the blastoderm can be controlled by the amount of albumen culture medium used and by the choice of ring and culture vessel (see above). Smooth glass rings (particularly those of circular cross-section) do not grip the membrane as tightly as those of rectangular cross-section; only the latter allow the membrane to be stretched fully. However, as New (1955) himself remarked, "*Any small wrinkles left in the membrane ... can be ignored as they tend to disappear shortly after the preparation has been returned to the incubator and the membrane then forms a perfectly smooth surface*".

The degree of tension can also be controlled by adjusting the volume of albumen beneath the membrane; lesser volumes allow the membrane to remain more relaxed, while a large amount will cause the membrane to bulge upwards if a flat dish is being used. Even rings of rectangular cross-section make contact less firmly with a curved watch glass than with a flat-bottomed dish, and if a large pool of albumen is placed under the membrane this tends to leak out under the ring, reducing the tension of the membrane.

When the membrane bulges upwards, any fluid accumulated above the embryo during culture tends to run down to the edges, preventing a large volume of fluid from accumulating over the embryo (see below). It also appears that the extraembryonic *area opaca* spreads more quickly over the membrane if this is tense, even though development of the embryo proper does not appear to be affected. On the other hand, any small wounds inflicted on the embryo will expand very quickly and will not heal during subsequent culture when the membrane is tense. This often happens after microsurgical manipulation even if the wound appeared to close early on – if the embryo or membrane is subjected to tension, sooner or later the former will burst, usually at the site of the operation.

The amount of fluid above the embryo

Keeping the upper (ventral) surface of the embryo quite dry appears to improve development (New, 1955, 1956; Stern *et al.*, 1985). This is particularly true when there are wounds involving the epiblast. If fluid remains inside the wound, its edges tend to curl upwards and no healing will take place.

Because of the importance of a low rate of expansion of the blastoderm and of keeping the surface of the embryo dry in the extent of wound healing, we routinely do the following after a microsurgical operation involving the epiblast: (a) any fluid remaining inside the wound is aspirated carefully with a microcapillary pipette; (b) the edges of the "margin of overgrowth" (outer edge of the *area opaca* epiblast; see New, 1959) are carefully nicked with a needle to slow down expansion; (c) the vitelline membrane is carefully checked for holes through which albumen might leak; (d) the membrane is arranged fairly loosely around the glass ring and only a small amount of albumen is used. When this is done, even large wounds heal within a few hours (Psychoyos and Stern, 1996).

The incubation temperature and humidity

Another factor that appears to influence the effectiveness with which wounds heal is the incubation temperature. At lower temperatures (25-32°C) spreading of the margin of overgrowth slows down considerably but the closure of large wounds appears to begin normally. After an operation involving all three germ layers, it is sometimes advantageous therefore to leave the operated embryo at room temperature or at 30°C for a few hours before placing it at the normal incubation temperature of 38±1°C. Incubation in humid air is also important, probably because it prevents drying of the exposed surface of the embryo; cultures in a watch glass are therefore enclosed in a Petri dish containing a supporting ring of cotton wool saturated with water.

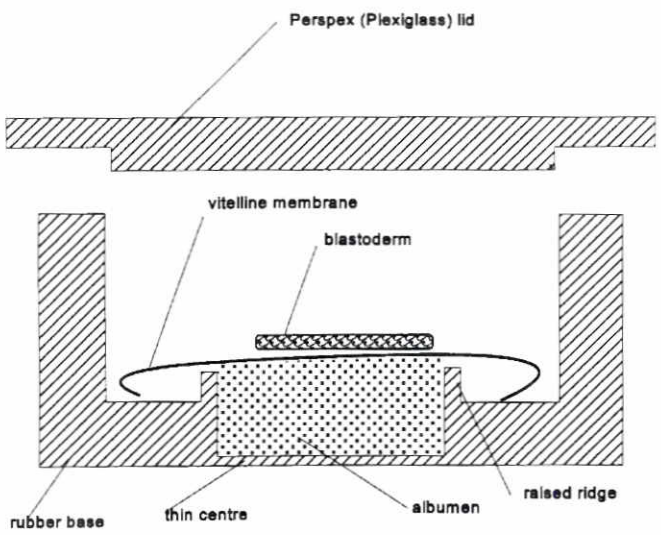

Fig. 6. Kucera and Burnand (1987) chamber. *The base is moulded from silicon elastomere rubber and contains a central raised ridge, surrounding a very thin central region of rubber which is both optically clear and gas permeable. The central chamber enclosed by this ridge is filled with albumen culture medium, and the vitelline membrane arranged over it with the embryo uppermost. The chamber is sealed with a hard transparent plastic (Perspex/Plexiglass) lid that fits snugly, providing a rigid and sturdy skeleton for the otherwise flexible chamber.*

The position of the embryo within the ring

The asymmetric turning of the heart and head that occur at stages 10-11 and 13-14, respectively, only take place predictably when the embryo is well centred in the glass ring (Levin *et al.* 1995). When this is not the case and one edge of the margin of overgrowth meets the ring before the other, and if this occurs before stages 9-10, turning appears to take place in a random direction (Cooke, 1995; Levin *et al.*, 1995).

A new approach

Until very recently, the New (1955) technique and its modifications, listed above, were the only truly successful methods for the culture of early chick embryos *in vitro* up to early organogenesis stages. Last year, however, to improve the exposure of the epiblast to the culture medium, a radically new approach led to the development of the "Cornish pasty" method of Connolly *et al.* (1995). It is probably no coincidence that the principle on which this technique is based is derived from another of Denis New's inventions: his successful method for culturing postimplantation rodent embryos (New *et al.*, 1973).

To set up "Cornish pasty" cultures, chick embryos are explanted from their eggs, freed from their vitelline membranes and folded in half, with the endoderm facing inwards. A small pair of scissors is then used to cut, following the margin between *area opaca* and *area pellucida*, so as to seal the two halves together into a half-moon (Cornish pasty) shape. These embryos are then placed into a small tube in a small amount of culture medium, and the bottles rolled in an incubator as described by New *et al.* (1973) for mammalian embryos. Connolly *et al.* (1995) suggest the use of air buffered tissue culture medium containing fetal calf serum, but we have also obtained equally good results using pure thin egg albumen.

The embryos cultured in this way resemble mammalian embryos in their spherical shape, but in the case of avian embryos, the embryonic body proper develops atop an inflated balloon of extraembryonic membranes resembling a yolk sac. With this technique embryos will also develop up to stages 16-17, as with the New (1955) technique and its modifications. It is possible that even better development may be obtained by including a small amount of yolk together with a larger portion of the *area opaca*, but this has not yet been tried.

As with the New (1955) technique, these embryos do not form an amnion. The main attraction of this technique at present, as explained by its inventors, is that when culture medium is used instead of albumen, the problems of the latter in precipitating phosphorothioate oligonucleotides are avoided. It will also be useful for exposing whole embryos to growth factors at defined concentrations.

Culture of pre–primitive streak stage embryos

Apart from their fragility, pre-primitive streak stage embryos have the problem that they are only weakly attached to the vitelline membrane, if at all. There are several strategies that can be used to culture these according to the methods described in this paper. To culture them on their vitelline membranes, it is sometimes possible, with care, to explant them as done for older embryos. To do this successfully requires the membrane to be peeled very slowly and almost vertically, while using a pair of forceps to push the yolk down, away from the membrane. In our experience, this can be done with success in about 1/3 cases of unincubated eggs containing embryos at stages X-XII (Eyal-Giladi and Kochav, 1976). If the membrane comes off without the embryo attached, it is still possible to free the embryo from the yolk, either by using a spoon to collect the area of the blastoderm and place it in a Petri dish with saline (where it can be dissected carefully under the microscope) or while still in the explantation

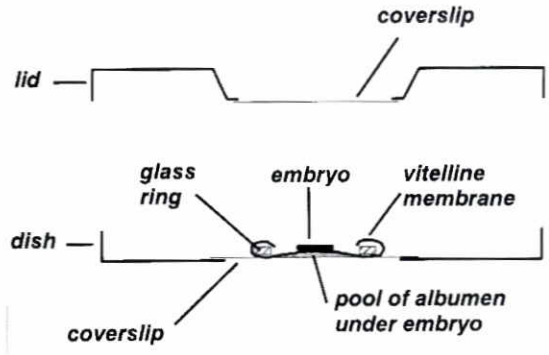

Fig. 7. Filming chamber from Stern (1990). *The culture vessel is a plastic Cooper dish, with large holes drilled into both the lid and the base. A 32 mm diameter coverslip is glued to the inside of both the lid and the base using silicon grease. This leaves a space of approximately 3 mm between both coverslips. The vitelline membrane is stretched around a ring like that in Figure 1D, but much thinner (1-1.5 mm thick). (Reproduced with permission from Stern, 1990).*

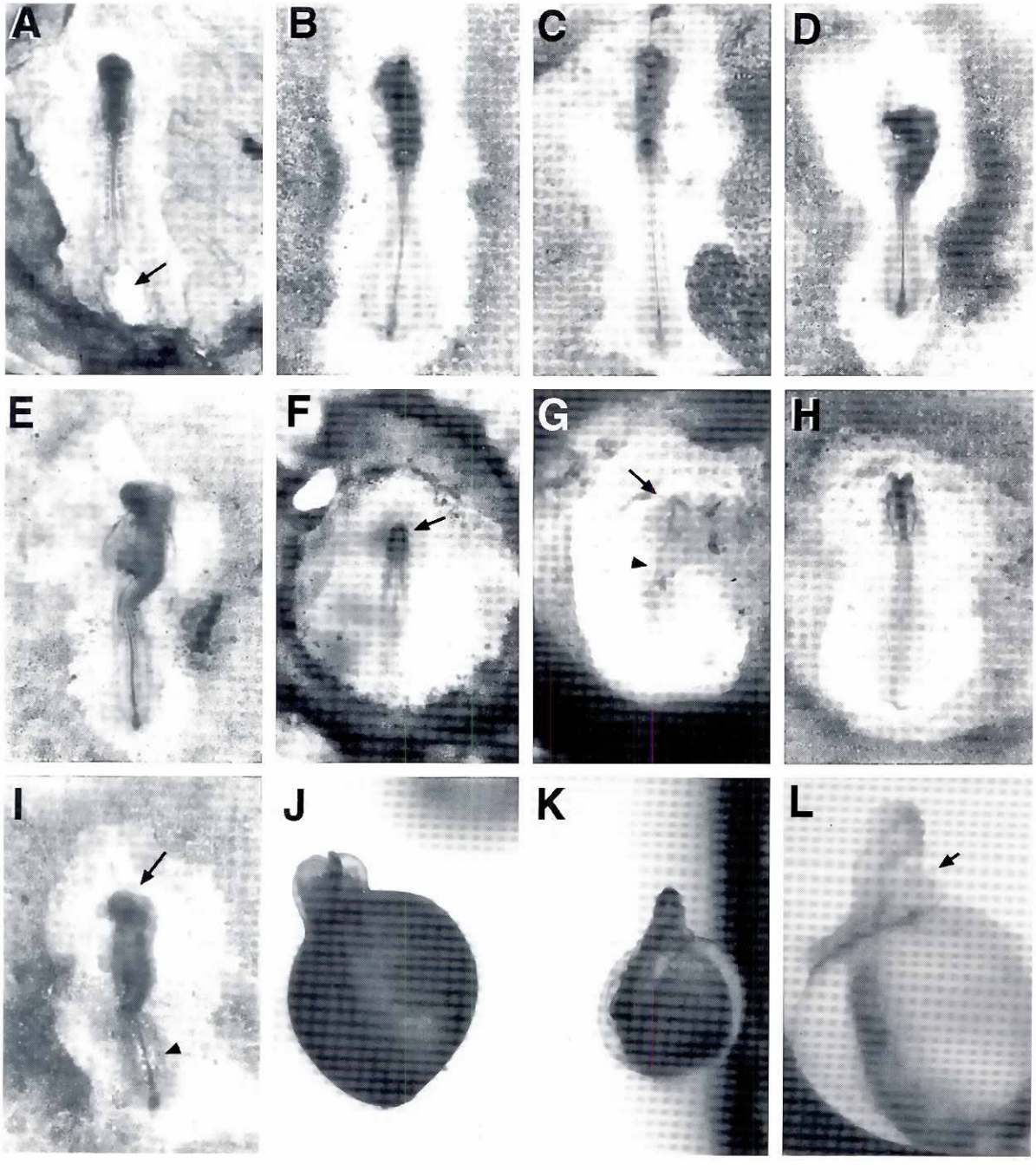

Fig. 8. Embryos cultured by different methods from stage 4. **(A)** *Embryo (stage 11) cultured in a 35 mm dish using a small (27 mm) ring of rectangular section, after 38 hours' culture, showing splitting of the caudal part of the axis (arrow) that often precedes death.* **(B)** *Embryo (about stage 13) cultured in a watch glass using a small ring of rectangular section, after 48 hours' culture. The somites have started to disintegrate.* **(C)** *Embryo (stage 12) cultured in a watch glass using a larger ring (30 mm) after 48 h. The somites appear narrow and the axis thin, but angiogenesis has started in peripheral regions.* **(D)** *Embryo (stage 13/14) cultured in a 35 mm dish using a large ring after 48 h. Morphology is fairly normal in this case, but angiogenesis is delayed. The head has started to turn.* **(E)** *Embryo (stage 13/14) cultured as in D. Several anomalies are apparent, including a degree of microcephaly.* **(F)** *Embryo (stage 7) cultured by the inverted double-ring method of Nicolet and Gallera, 19 h after explantation. Formation of the head fold (arrow) is delayed.* **(G)** *Embryo (stage 7?) cultured by the De Carli vial method, after 19 hours' culture. Gross abnormalities can be seen, including a malformed head fold (arrow) and thin, wiggly and malformed axial mesoderm (arrowhead).* **(H)** *Embryo cultured in a Kucera and Burnand chamber, after 19 h. The embryo is now at stage 8⁺ and has an abnormally thick neural tube in the head region.* **(I)** *Embryo cultured as in H, 48 h after explantation (now stage 14/15). Severe microcephaly (arrow) and splitting of the caudal part of the embryonic axis as well as loss of structure in the paraxial mesoderm (arrowhead) can be seen at around the time of death.* **(J-L)** *Embryos grown by the Cornish pasty method of Connolly et al., after 19, 27 and 50 hours' culture, respectively. They appear to be, respectively, at stages 8, 13 and 16, although these embryos are difficult to stage because of their unusual morphology. The embryo in* **(L)** *has a very well developed heart (arrow), which appears unusually large in comparison with the relatively stunted axis and small head. Note that at early stages* **(J)** *the head appears to be too large, while at later stages* **(K,L)** *embryos are microcephalic.*

dish, by aiming small jets of saline tangentially towards the edges of the blastodisc using a Pasteur pipette. In both cases, the embryo is dissected from the yolk still attached to an underlying plug of white yolk, which can then be removed carefully with forceps. After cleaning of the embryo, it can be placed (epiblast side down) onto a clean vitelline membrane stretched around a glass ring as in New (1955) culture. If all else fails, it is possible to obtain membranes and embryos separately. Survival of pre-primitive streak embryos in New (1955) culture and its simple modifications (e.g. Stern and Ireland, 1981) is nearly as successful as for older embryos. Invariably they develop a primitive streak within about 12 h and nearly always develop to at least stage 9-10. Occasionally we have been able to maintain these embryos in culture up to stage 14 (about 50 h).

It is also possible to use the Connolly *et al.* (1995) roller-bottle technique for pre-primitive streak stage embryos. But these young embryos, which have a very narrow *area opaca*, cannot be cut in the same way as older ones. It suffices, however, simply to fold the whole blastodisc (hypoblast facing inwards) and to crimp the edges in several places with a pair of forceps before placing the embryo in a tube for roller culture. In our laboratory we have successfully cultured embryos in this way to at least stage 8, using pure egg albumen as the culture medium.

Culture of avian blastoderms other than chick

Quail embryos can be cultured successfully on chick vitelline membranes. For this, a chick vitelline membrane (without the embryo) is set up as described, a quail embryo collected separately and freed from its membrane, and placed onto the chick membrane. Chick albumen is placed under the membrane. Survival is as good as for chick embryos.

Recently, we have also attempted to culture very early (pre-primitive streak) turkey embryos on either turkey or chick vitelline membranes. However, we were not successful. We do not yet know whether this is due to the young age of the embryos (which appear to be radially symmetrical at this stage) or to an incompatibility of these embryos with the chick albumen or membrane. Given Waddington's (1932) success with both duck and rabbit embryos in plasma clot cultures, it is not inconceivable that both of these species can be cultured on chick vitelline membranes, but to our knowledge this has not yet been attempted.

A simple experimental comparison of several techniques

To compare more directly the rate of development and survival of the different techniques described above, we explanted 53 embryos at stages 3^{+}-4^{-} in Pannett-Compton saline and established cultures of the following types (at least 5 of each):

(a) original New (1955) culture using rounded 30 mm rings, in watch glasses (n= 6)
(b) the same but using 30 mm rings of rectangular cross-section, in watch glasses (n= 7)
(c) the same but cultured in 35 mm plastic dishes (n= 8)
(d) 27 mm rings of rectangular cross-section, in watch glasses (n= 7)
(e) the same but cultured in 35 mm plastic dishes (n= 8)
(f) Nicolet and Gallera (1963) culture, using a 30 mm outer ring and a 25 mm inner ring (n= 5)
(g) Kucera and Burnand (1987) culture (n= 7)
(h) De Carli (1965) culture, using a 12 mm plastic lid and tube (n= 5)
(i) Connolly *et al.* (1995) culture, but in a 20 ml scintillation vial with 5 ml thin egg albumen (n= 12)

The stage of development attained was recorded every 12 h for up to 4 days, and photographs taken of each embryo (Fig. 8). About 12 h following explantation, all the embryos cultured by the ring techniques were very similar to one another, regardless of size of ring or type of container, and were at stages 5-8. One of the 5 embryos cultured by the De Carli (1965) method was lost due to leakage of culture medium between the slide and chamber. The 12 embryos cultured by the Connolly *et al.* (1995) technique were the most advanced; 10/12 had reached stage 9.

About 24 h after explantation, three more of the De Carli (1965) type embryos had died, by longitudinal splitting of the axis; the remaining embryo was at stage 8^{-} (3 somites) but the somites were greatly elongated laterally and looked abnormal. All 5 embryos cultured by the Nicolet and Gallera (1963) method had abnormal heads (see Fig. 8F) and were at stage 7^{+}-8 (2-4 somites). All the embryos cultured by the ring methods were still comparable to one another and had reached stages 10-11 and all looked normal except for the embryos in the Kucera and Burnand chambers, which were microcephalic. Those cultured by the Connolly *et al.* (1995) method tended to be macrocephalic (see Fig. 8J), three had open neural tubes and many vacuolated areas; all were at about stage 10 but the somites appeared weakly defined and elongation of the axis stunted.

By 38 hours' incubation, the embryos in 27 mm rings (regardless of container) had slowed down their development in comparison with those in 30 mm rings (stage 11-12 as compared to 13-14, respectively). In the case of all those in smaller rings, the *area opaca* had expanded to reach the inner edge of the ring. One of the embryos in Kucera and Burnand (1987) culture had disintegrated, and the remaining 6 were still microcephalic and at stages 13-14. The remaining embryo cultured by the De Carli method had now also died (apparently shortly after the previous observation) by splitting of the neural tube. All the embryos in inverted double-ring culture had also split. Of the 12 embryos in roller tube culture, 7 were very small and had no recognisable structures. The remaining 5 were stunted (short trunks) and appeared to be at about stage 12.

By 48 h, 7/15 of the embryos in smaller rings were still alive (beating hearts), morphologically normal and at stages 14-16; the remaining ones had died at about stages 11-13. Their neural tubes were split or the whole embryo had disintegrated. Ten of the 21 embryos in larger rings were also alive and normal, at stages 16-17; the others had died at stages 13-14. The remaining 6 embryos in Kucera chambers were now dead, at stages 13-16, and markedly microcephalic (Fig. 8I). In the roller bottles, only two embryos remained, which appeared to be at stage 14-15 and the heart was beating vigorously, but the axis was stunted and embryos very microcephalic (Fig. 8K).

By the third day, only one living embryo remained, in the roller bottle culture. While the heart was still beating vigorously, it did not appear to have changed markedly since the previous day. It was found to be dead on the fourth day (80 h after explantation), still without appreciable changes in morphology but had remained intact.

In conclusion (Table 1), it appears that the speed of development is not affected by the size of the rings or the shape of the

TABLE 1

COMPARISON OF DIFFERENT TECHNIQUES FOR CULTURING PRIMITIVE STREAK STAGE EMBRYOS

Technique	Maximum survival (stage)	Usual survival (stage)	Usual cause of death/ Comments
large ring/watch glass	17	14-15	splitting; starvation?
large ring/35 mm dish	17	14-15	splitting; starvation?
small ring/watch glass	15-16	13-14	splitting; filling of ring; starvation?
small ring/35 mm dish	15-16	13-14	splitting; filling of ring; starvation?
inverted ring culture	14-15	12-13	splitting; filling of ring; starvation?
Kucera chamber	17	15-16	splitting; starvation?
De Carli chamber	11	8-9	splitting; other anomalies
Cornish pasty method	16-17?	?	developmental arrest - cause unknown; stunting; starvation?

TABLE 2

COMPARISON OF DIFFERENT TECHNIQUES FOR CULTURING PRE-PRIMITIVE STREAK EMBRYOS

Technique	Maximum survival (stage)	Usual survival (stage)	Usual cause of death/ Comments
large ring/watch glass	14	9-11	splitting; starvation?
large ring/35 mm dish	14	9-11	splitting; starvation?
small ring/watch glass	14	9-11	splitting; starvation?
small ring/35 mm dish	14	9-11	splitting; starvation?
inverted ring culture	–	–	not suitable
Kucera chamber	?	?	not attempted
De Carli chamber	?	?	not attempted
Cornish pasty method	14?	?	?
Spratt agar/albumen	12	9-11	vacuolation; severe anomalies

culture vessel, but larger rings appear to allow the embryo to grow for a little longer than do smaller rings. Neither the De Carli (1965) nor the Nicolet and Gallera (1963) methods appeared to offer any advantage. The Kucera and Burnand (1987) chambers did support normal development as well as conventional rings, but not for a longer period and the head abnormalities described by the original authors were apparent early on during culture. The Connolly *et al.* (1995) method did succeed in keeping embryos alive for the longest period, but development also appeared to cease at about the same stage as in those maintained in New (1955) culture. An assessment of these techniques when used for culturing pre-primitive streak embryos, based on several years' experience with these techniques, is shown for comparison as Table 2.

Conclusion: the next 40 years

It is conceivable that, with some modification, the Connolly *et al.* (1995) method might be made to allow development to continue for longer than the original ring method, and if so, applications other than culture in the presence of antisense oligonucleotides will be possible. For example, it may be possible to perform a microsurgical operation in New (1955) culture, allow it to heal for some hours, and then set up a Cornish pasty culture of the embryo for another 1-3 days. The above discussion and the small experiment performed suggest that the New (1955) technique still compares favourably with its modifications by other authors and with other culture methods. For most applications, and with judicious control of the various parameters considered above that appear to enhance wound healing or the growth of embryos explanted at earlier stages of development, it seems most likely that this method will continue to contribute to experimental embryology for at least another 40 years.

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