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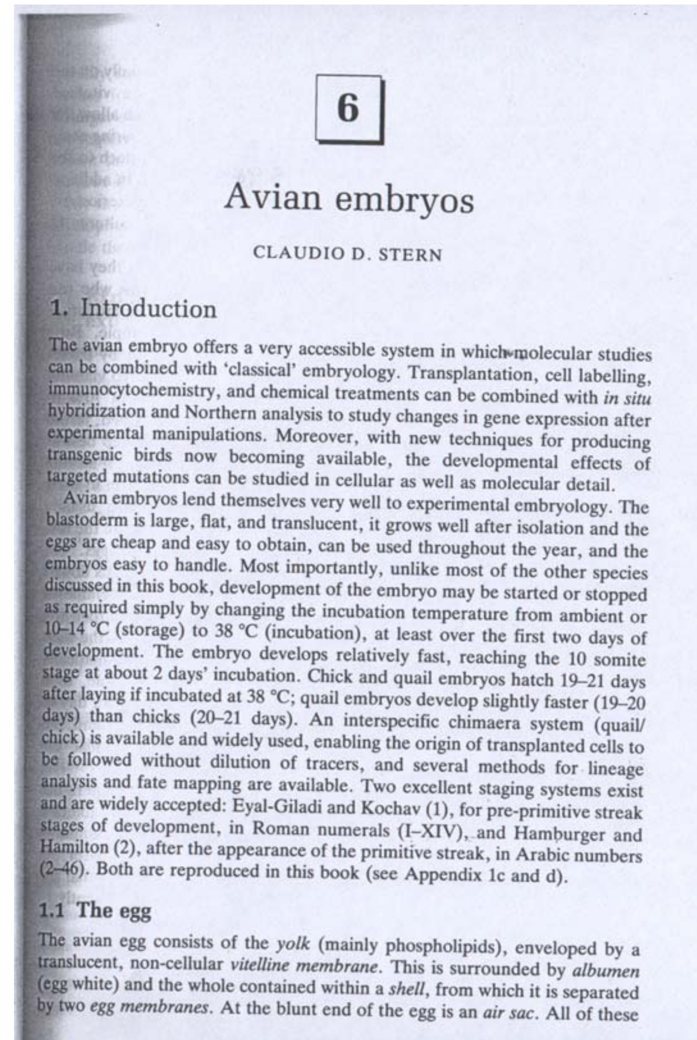

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Avian embryos

CLAUDIO D. STERN

1. Introduction

The avian embryo offers a very accessible system in which molecular studies can be combined with 'classical' embryology. Transplantation, cell labelling, immunocytochemistry, and chemical treatments can be combined with *in situ* hybridization and Northern analysis to study changes in gene expression after experimental manipulations. Moreover, with new techniques for producing transgenic birds now becoming available, the developmental effects of targeted mutations can be studied in cellular as well as molecular detail.

Avian embryos lend themselves very well to experimental embryology. The blastoderm is large, flat, and translucent, it grows well after isolation and the eggs are cheap and easy to obtain, can be used throughout the year, and the embryos easy to handle. Most importantly, unlike most of the other species discussed in this book, development of the embryo may be started or stopped as required simply by changing the incubation temperature from ambient or 10–14 °C (storage) to 38 °C (incubation), at least over the first two days of development. The embryo develops relatively fast, reaching the 10 somite stage at about 2 days' incubation. Chick and quail embryos hatch 19–21 days after laying if incubated at 38 °C; quail embryos develop slightly faster (19–20 days) than chicks (20–21 days). An interspecific chimaera system (quail/chick) is available and widely used, enabling the origin of transplanted cells to be followed without dilution of tracers, and several methods for lineage analysis and fate mapping are available. Two excellent staging systems exist and are widely accepted: Eyal-Giladi and Kochav (1), for pre-primitive streak stages of development, in Roman numerals (I–XIV), and Hamburger and Hamilton (2), after the appearance of the primitive streak, in Arabic numbers (2–46). Both are reproduced in this book (see Appendix 1c and d).

1.1 The egg

The avian egg consists of the *yolk* (mainly phospholipids), enveloped by a translucent, non-cellular *vitelline membrane*. This is surrounded by *albumen* (egg white) and the whole contained within a *shell*, from which it is separated by two *egg membranes*. At the blunt end of the egg is an *air sac*. All of these

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components are secreted by the mother. The embryo itself lies initially on the surface of the yolk, just under the vitelline membrane. The vitelline membrane is attached to two glycoprotein threads (*chalazae*), which allow the yolk to rotate so that the embryo always faces the top of the egg. During early stages of development, the edges of the single-cell thick embryo attach to the inner (yolk) face of the vitelline membrane, on which it expands. In addition to nutritive components, the albumen contains *lysozyme*, a bacteriostatic agent. For this reason, egg albumen is often used in embryo cultures to prevent the growth of microorganisms as well as for nutrition.

Eggs bought in shops do not usually contain viable embryos as they have not been fertilized. Many areas have local breeders/poultry farms who can supply suitable numbers of fertile hens' eggs, and in addition there are larger suppliers who can deliver country-wide. In the UK, for example, Ross Poultry will deliver quantities of fertile hens' eggs by courier or by parcel post, but this will increase the cost of the eggs. Fertility and embryo viability should be at least 90% in winter months but may drop to about 70% in summer. You should insist on this from your supplier. Once obtained, fertile eggs may be stored for up to 5–7 days before incubation; they keep best at 10–14 °C and should in any case not be allowed to fall below 6 °C. If storing eggs for a number of days before use, it may be useful to put them in their trays with the pointed end upwards (unlike the way they are usually delivered) and to stack the trays up tightly, in a box, so as to minimize the loss of CO_2. This is reported to improve the keeping properties of the eggs.

To start the incubation, simply place the eggs horizontally (long axis down) in a 38 °C incubator kept humid by inclusion of a suitably large volume of distilled water. If the incubation period desired is more than 48 h, then the eggs should be turned to prevent adhesion of the yolk membranes to the shell membranes, which impairs development. Incubators that do this automatically several times a day are available. If no such incubator is available, then turning may be done by hand, an odd number (e.g. three) of times per day so that they spend alternate nights on a different side. To turn them by hand, flip them over by 180° about the long axis of the egg so as to maximize the movement.

After incubation, the eggs can be allowed to cool to room temperature before use for up to a few hours, although younger embryos are more tolerant of this than older ones. After the third day of incubation, embryos should be used for most purposes within 2–4 h of removing them from the incubator. As eggs cool, the heart will slow down or even stop; this does not imply that the embryo is dead. Check by warming up the egg or embryo to 38 °C gently.

2. The embryo

By the time of laying, hens' eggs have spent some 20 h developing *in utero* (see Appendix 1). For this reason, freshly laid eggs are at the 'blastula' stage

(usually stages IX–X in winter). In summer, eggs are retained inside the mother for longer and may be at later stages (typically stages XII–XIII). Younger embryos can be obtained by extraction from the mother, which may not require killing the bird. It is possible to stroke the belly of the bird at a known time in oviposition to coax it to drop its egg prematurely, but this requires some skill and, of course, access to suitable birds.

For the techniques described below, I will concentrate on young stages of development, where the embryo is small and some guidance is needed to handle the embryos successfully.

2.1 Explanting embryos for fixation or protein/RNA extraction

To explant embryos, you will need the following materials:

- dissecting microscope, preferrably one with transmitted light
- saline (either Tyrode's, CMF-Tyrode's, PBS or Pannett–Compton; see Section 4)
- two pairs of watchmakers' forceps, number 4 or 5
- one pair of coarse forceps, about 15 cm (6 in) long
- one pair of small, fine scissors, with straight blades about 2 cm (0.75 in) long.
- for young embryos (less than 2 days), a spoon/spatula
- Petri dish to collect embryos
- sylgard-coated Petri dish in which to dissect embryos[a] and fine steel insect pins, size A1 or D1.
- container for egg waste
- Pasteur pipette with the end cut off at the shoulder, stump flamed to remove sharp edges, and rubber teat
- as required, materials and solutions for fixation[b], protein extraction[c], or RNA extraction[d].

[a] Sylgard 184 (Dow Corning) is clear silicone rubber polymerized by mixing two components (9 parts rubber solution : 1 part of accelerator/catalyst). Mix the two well and pour to the desired depth (2–5 mm) into suitable containers, e.g. plastic Petri dishes. Allow the dishes to stand for about 1 h at room temperature for air bubbles to leave, then cure at about 55 °C until polymerized (3 h to overnight). The dishes can be stored indefinitely. Black Sylgard is also available.

[b] Fixatives. The most commonly used fixatives are: absolute ethanol (fix 48 h), absolute methanol (fix overnight at −20 °C) and buffered formol saline (mix 9 parts of phosphate buffered saline at pH 7–7.4 with 1 part of formaldehyde solution, to give 4%, final, of formaldehyde) (fix 30 min to overnight). Further deails can be found in appropriate chapters for each method. It is a good idea first to pin the embryo in the desired position through the extraembryonic membranes whilst submerged in saline in a Sylgard dish, then remove the saline and replace with fixative. After a few minutes the pins can be removed and the embryo transferred to a vial containing either fixative or saline for storage.

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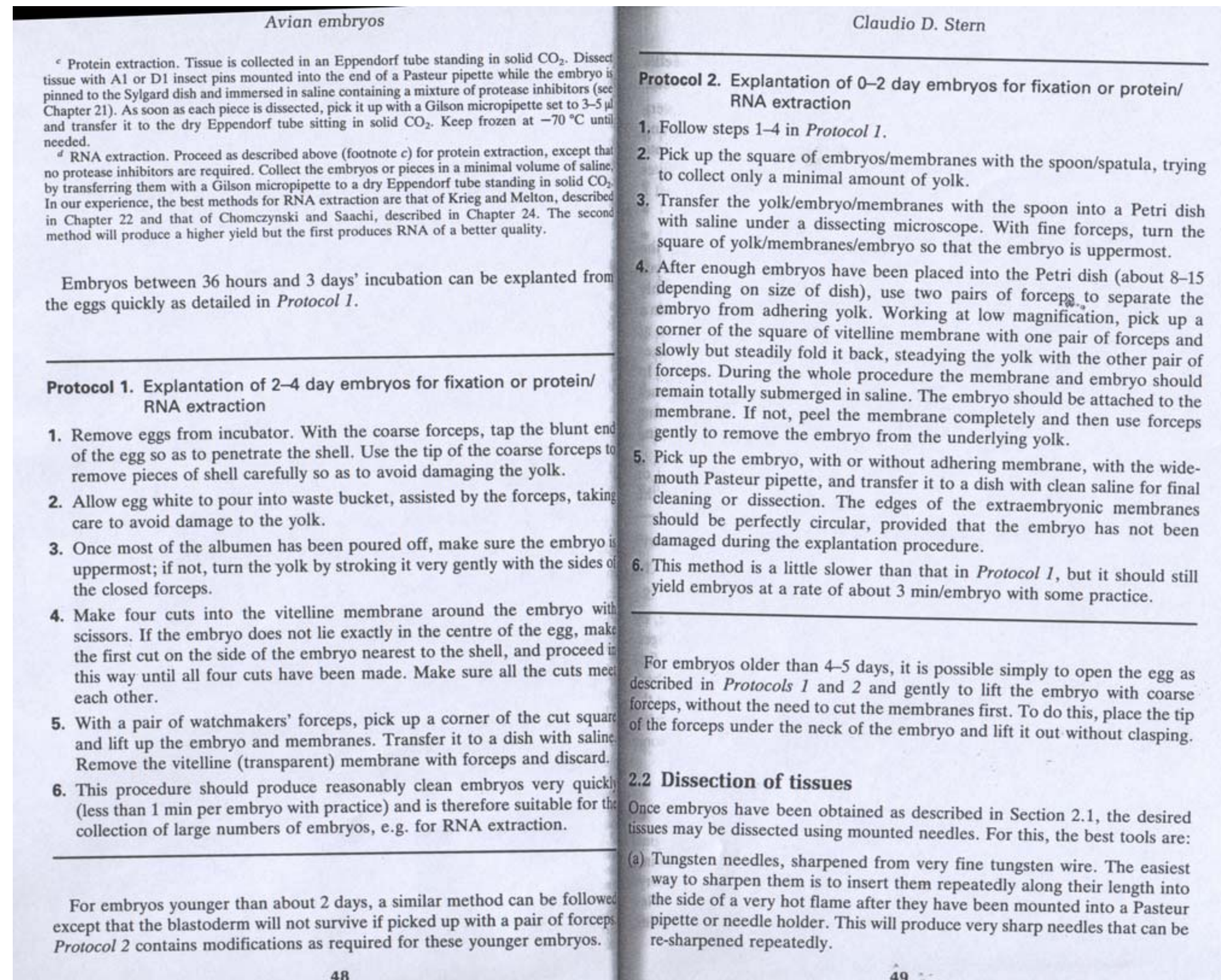

[c] Protein extraction. Tissue is collected in an Eppendorf tube standing in solid CO_2. Dissect tissue with A1 or D1 insect pins mounted into the end of a Pasteur pipette while the embryo is pinned to the Sylgard dish and immersed in saline containing a mixture of protease inhibitors (see Chapter 21). As soon as each piece is dissected, pick it up with a Gilson micropipette set to 3–5 µl and transfer it to the dry Eppendorf tube sitting in solid CO_2. Keep frozen at −70 °C until needed.

[d] RNA extraction. Proceed as described above (footnote *c*) for protein extraction, except that no protease inhibitors are required. Collect the embryos or pieces in a minimal volume of saline, by transferring them with a Gilson micropipette to a dry Eppendorf tube standing in solid CO_2. In our experience, the best methods for RNA extraction are that of Krieg and Melton, described in Chapter 22 and that of Chomczynski and Saachi, described in Chapter 24. The second method will produce a higher yield but the first produces RNA of a better quality.

Embryos between 36 hours and 3 days' incubation can be explanted from the eggs quickly as detailed in *Protocol 1*.

Protocol 1. Explantation of 2–4 day embryos for fixation or protein/RNA extraction

1. Remove eggs from incubator. With the coarse forceps, tap the blunt end of the egg so as to penetrate the shell. Use the tip of the coarse forceps to remove pieces of shell carefully so as to avoid damaging the yolk.
2. Allow egg white to pour into waste bucket, assisted by the forceps, taking care to avoid damage to the yolk.
3. Once most of the albumen has been poured off, make sure the embryo is uppermost; if not, turn the yolk by stroking it very gently with the sides of the closed forceps.
4. Make four cuts into the vitelline membrane around the embryo with scissors. If the embryo does not lie exactly in the centre of the egg, make the first cut on the side of the embryo nearest to the shell, and proceed in this way until all four cuts have been made. Make sure all the cuts meet each other.
5. With a pair of watchmakers' forceps, pick up a corner of the cut square and lift up the embryo and membranes. Transfer it to a dish with saline. Remove the vitelline (transparent) membrane with forceps and discard.
6. This procedure should produce reasonably clean embryos very quickly (less than 1 min per embryo with practice) and is therefore suitable for the collection of large numbers of embryos, e.g. for RNA extraction.

For embryos younger than about 2 days, a similar method can be followed except that the blastoderm will not survive if picked up with a pair of forceps. *Protocol 2* contains modifications as required for these younger embryos.

Protocol 2. Explantation of 0–2 day embryos for fixation or protein/RNA extraction

1. Follow steps 1–4 in *Protocol 1*.
2. Pick up the square of embryos/membranes with the spoon/spatula, trying to collect only a minimal amount of yolk.
3. Transfer the yolk/embryo/membranes with the spoon into a Petri dish with saline under a dissecting microscope. With fine forceps, turn the square of yolk/membranes/embryo so that the embryo is uppermost.
4. After enough embryos have been placed into the Petri dish (about 8–15 depending on size of dish), use two pairs of forceps to separate the embryo from adhering yolk. Working at low magnification, pick up a corner of the square of vitelline membrane with one pair of forceps and slowly but steadily fold it back, steadying the yolk with the other pair of forceps. During the whole procedure the membrane and embryo should remain totally submerged in saline. The embryo should be attached to the membrane. If not, peel the membrane completely and then use forceps gently to remove the embryo from the underlying yolk.
5. Pick up the embryo, with or without adhering membrane, with the wide-mouth Pasteur pipette, and transfer it to a dish with clean saline for final cleaning or dissection. The edges of the extraembryonic membranes should be perfectly circular, provided that the embryo has not been damaged during the explantation procedure.
6. This method is a little slower than that in *Protocol 1*, but it should still yield embryos at a rate of about 3 min/embryo with some practice.

For embryos older than 4–5 days, it is possible simply to open the egg as described in *Protocols 1* and *2* and gently to lift the embryo with coarse forceps, without the need to cut the membranes first. To do this, place the tip of the forceps under the neck of the embryo and lift it out without clasping.

2.2 Dissection of tissues

Once embryos have been obtained as described in Section 2.1, the desired tissues may be dissected using mounted needles. For this, the best tools are:

(a) Tungsten needles, sharpened from very fine tungsten wire. The easiest way to sharpen them is to insert them repeatedly along their length into the side of a very hot flame after they have been mounted into a Pasteur pipette or needle holder. This will produce very sharp needles that can be re-sharpened repeatedly.

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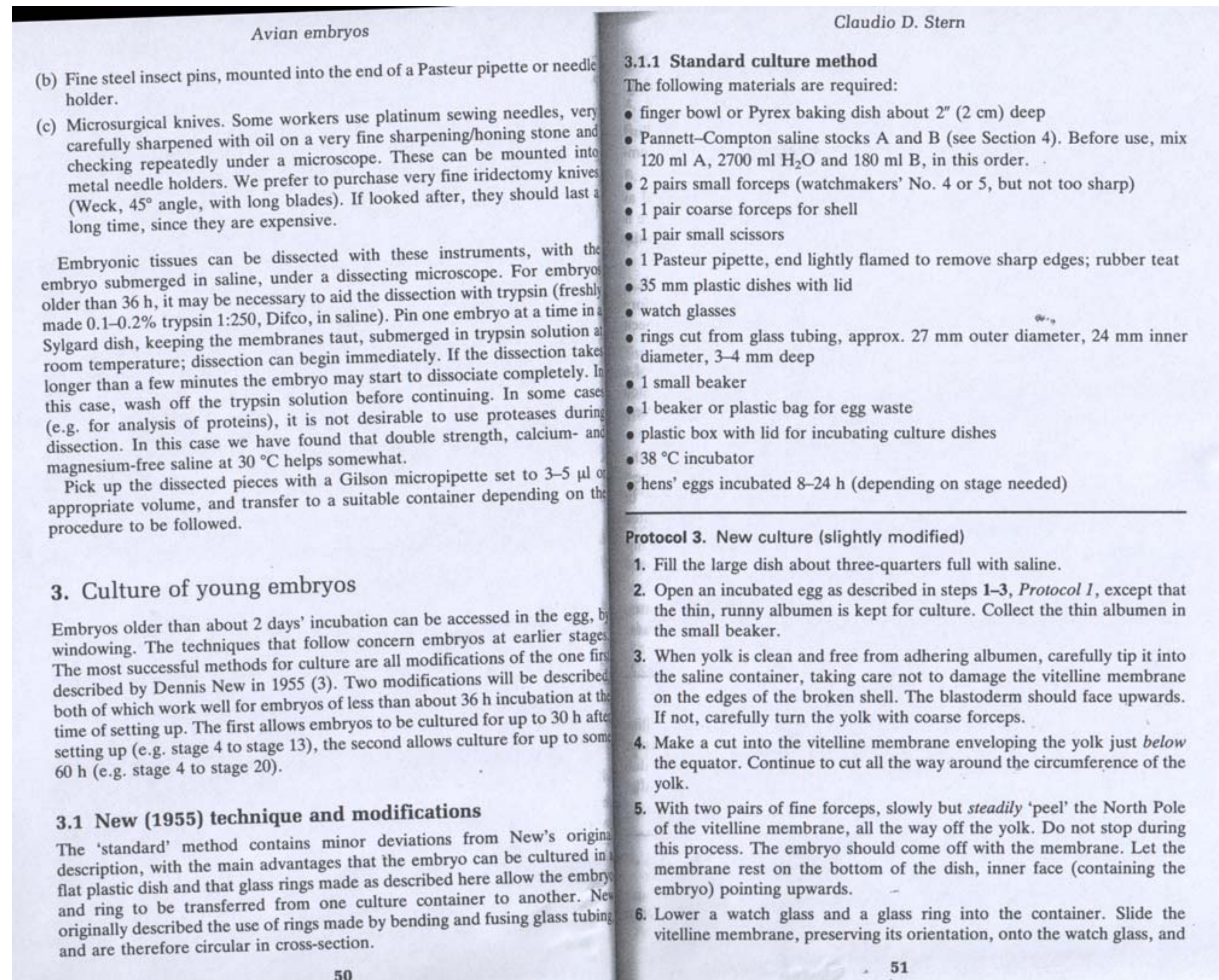

(b) Fine steel insect pins, mounted into the end of a Pasteur pipette or needle holder.

(c) Microsurgical knives. Some workers use platinum sewing needles, very carefully sharpened with oil on a very fine sharpening/honing stone and checking repeatedly under a microscope. These can be mounted into metal needle holders. We prefer to purchase very fine iridectomy knives (Weck, 45° angle, with long blades). If looked after, they should last a long time, since they are expensive.

Embryonic tissues can be dissected with these instruments, with the embryo submerged in saline, under a dissecting microscope. For embryos older than 36 h, it may be necessary to aid the dissection with trypsin (freshly made 0.1–0.2% trypsin 1:250, Difco, in saline). Pin one embryo at a time in a Sylgard dish, keeping the membranes taut, submerged in trypsin solution at room temperature; dissection can begin immediately. If the dissection takes longer than a few minutes the embryo may start to dissociate completely. In this case, wash off the trypsin solution before continuing. In some cases (e.g. for analysis of proteins), it is not desirable to use proteases during dissection. In this case we have found that double strength, calcium- and magnesium-free saline at 30 °C helps somewhat.

Pick up the dissected pieces with a Gilson micropipette set to 3–5 μl or appropriate volume, and transfer to a suitable container depending on the procedure to be followed.

3. Culture of young embryos

Embryos older than about 2 days' incubation can be accessed in the egg, by windowing. The techniques that follow concern embryos at earlier stages. The most successful methods for culture are all modifications of the one first described by Dennis New in 1955 (3). Two modifications will be described, both of which work well for embryos of less than about 36 h incubation at the time of setting up. The first allows embryos to be cultured for up to 30 h after setting up (e.g. stage 4 to stage 13), the second allows culture for up to some 60 h (e.g. stage 4 to stage 20).

3.1 New (1955) technique and modifications

The 'standard' method contains minor deviations from New's original description, with the main advantages that the embryo can be cultured in a flat plastic dish and that glass rings made as described here allow the embryo and ring to be transferred from one culture container to another. New originally described the use of rings made by bending and fusing glass tubing and are therefore circular in cross-section.

3.1.1 Standard culture method

The following materials are required:

- finger bowl or Pyrex baking dish about 2″ (2 cm) deep
- Pannett–Compton saline stocks A and B (see Section 4). Before use, mix 120 ml A, 2700 ml H_2O and 180 ml B, in this order.
- 2 pairs small forceps (watchmakers' No. 4 or 5, but not too sharp)
- 1 pair coarse forceps for shell
- 1 pair small scissors
- 1 Pasteur pipette, end lightly flamed to remove sharp edges; rubber teat
- 35 mm plastic dishes with lid
- watch glasses
- rings cut from glass tubing, approx. 27 mm outer diameter, 24 mm inner diameter, 3–4 mm deep
- 1 small beaker
- 1 beaker or plastic bag for egg waste
- plastic box with lid for incubating culture dishes
- 38 °C incubator
- hens' eggs incubated 8–24 h (depending on stage needed)

Protocol 3. New culture (slightly modified)

1. Fill the large dish about three-quarters full with saline.
2. Open an incubated egg as described in steps **1–3**, *Protocol 1*, except that the thin, runny albumen is kept for culture. Collect the thin albumen in the small beaker.
3. When yolk is clean and free from adhering albumen, carefully tip it into the saline container, taking care not to damage the vitelline membrane on the edges of the broken shell. The blastoderm should face upwards. If not, carefully turn the yolk with coarse forceps.
4. Make a cut into the vitelline membrane enveloping the yolk just *below* the equator. Continue to cut all the way around the circumference of the yolk.
5. With two pairs of fine forceps, slowly but *steadily* 'peel' the North Pole of the vitelline membrane, all the way off the yolk. Do not stop during this process. The embryo should come off with the membrane. Let the membrane rest on the bottom of the dish, inner face (containing the embryo) pointing upwards.
6. Lower a watch glass and a glass ring into the container. Slide the vitelline membrane, preserving its orientation, onto the watch glass, and

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Protocol 3. *Continued*

arrange the ring over it so that membrane protrudes around the ring. Pull out the assembly from the saline.

7. With fine forceps, work carefully to fold the cut edges of the vitelline membrane over the edge of the ring, all the way around its circumference.
8. Place the watch glass over a black surface. Suck off as much fluid as possible from the outside of the ring with the Pasteur pipette. If there is much yolk remaining over and/or around the embryo, wash it carefully with clean saline.
9. Remove any remaining saline, both inside and outside the ring. It is important that the embryo remains dry during incubation. Therefore discard any embryos in which the vitelline membrane has been damaged.
10. Put some thin albumen (about 1–2 mm thick layer) on the bottom of a 35 mm plastic dish. Slide the ring with vitelline membrane off the watch glass, and transfer it to the dish, over the pool of egg albumen. Press lightly on the ring with two forceps to allow it to adhere to the dish.
11. If the level of albumen comes close to the edge of the ring, remove the excess. Also aspirate any remaining fluid from inside the ring. It is best if the vitelline membrane bulges upwards, above a good pool of albumen. This will also help to drain off further fluid accumulated during culture to the edges of the ring.
12. Wet the lid of the plastic dish with albumen. Discard the excess, and seal.
13. Place the dish in a plastic box containing a piece of tissue paper or cotton wool wetted in distilled water, seal the box, and place it in an incubator at 38 °C.

3.1.2 A modification allowing longer culture periods

This method is based on a technique described recently (4). Here the vitelline membrane is draped *over* the glass ring, which allows the extraembryonic tissues to spread more freely. It also helps to use a larger (50 mm) plastic dish for culture. In this way, embryos may be cultured for up to 60 h after explantation, or stage 20, whichever occurs sooner.

Protocol 4. Modification for extended culture

1. Follow steps **1–5**, *Protocol 3*.
2. Submerge a 50 mm plastic dish directly into the large saline container, and place the glass ring into it.

3. Carefully slide the vitelline membrane, still with the embryo uppermost, onto the glass ring inside the plastic dish.
4. Remove this assembly from the saline container.
5. Pipette off the excess saline, allowing the vitelline membrane gradually to come to rest over the glass ring.
6. Working in stages, replace the saline under the ring with a pool of albumen, making sure that the ring contains this pool. The vitelline membrane should bulge up slightly at its centre.
7. Seal dish by smearing albumen on the lid and proceed as in steps **12–13**, *Protocol 3*.

This method has not yet been fully perfected and may be modified further for better results. It takes a little longer than the standard method to set up, mainly because of the difficulty in replacing the saline with albumen. It may help if the ring is first secured onto the bottom of the plastic dish using a thin film of silicon grease.

4. Salines for avian embryos

Two salines are most widely used: Pannett–Compton saline[a], used mainly for procedures in which the yolk is submerged completely in a large volume, and Tyrode's saline[b], used for dissection of parts of embryo. The reason for using either of these is probably mainly historical. American laboratories tend to use Howard–Ringer's saline, again for historical reasons. Alternatively, standard phosphate-buffered saline, containing calcium and magnesium, may be used. In my experience, embryos do grow better if either Pannett–Compton or Tyrode's are used rather than Howard–Ringer's.

[a] Pannett–Compton saline stocks (can be autoclaved for storage):
- solution A: 121 g NaCl, 15.5 g KCl, 10.42 g $CaCl_2.2H_2O$, 12.7 g $MgCl_2.6H_2O$, H_2O to 1 litre;
- solution B: 2.365 g $Na_2HPO_4.2H_2O$, 0.188 g $NaH_2PO_4.2H_2O$, H_2O to 1 litre.

Before use, mix (in order): 40 ml A, 900 ml H_2O, and 60 ml B.

[b] Tyrode's saline stock (10×; can be autoclaved for storage):
- dissolve: 80 g NaCl, 2 g KCl, 2.71 g $CaCl_2.2H_2O$, 0.5 g $NaHPO_4.2H_2O$, 2 g $MgCl_2.6H_2O$ and 10 g glucose in 1 litre H_2O.

Before use, dilute 1:10 with distilled water. The working solution may be buffered with bicarbonate, but we usually omit this.

For Ca^{2+} and Mg^{2+}-free Tyrode's (CMF), omit the Ca^{2+} and Mg^{2+} salts.

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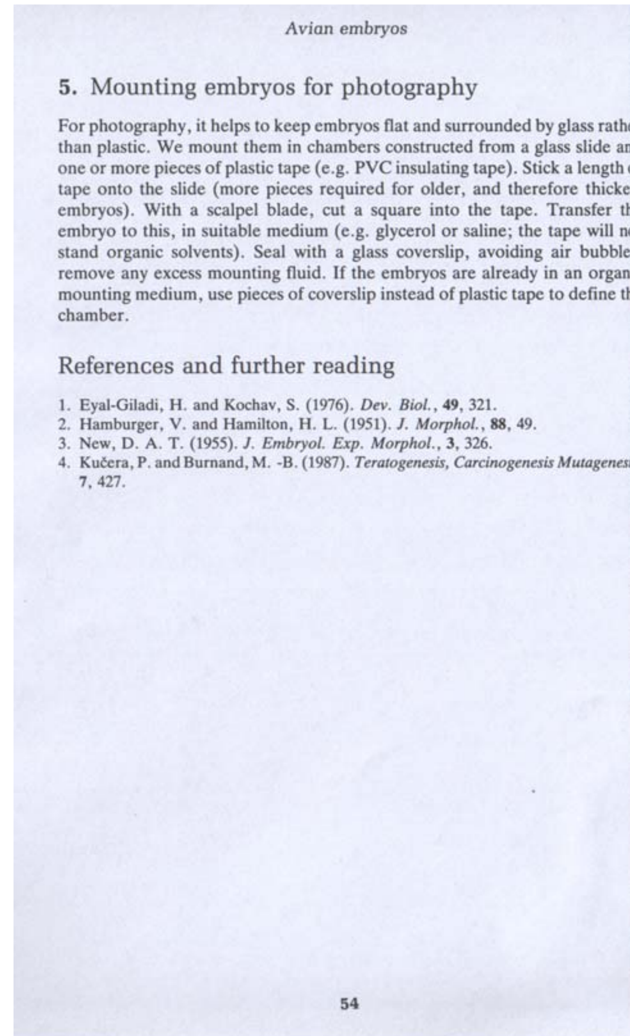

5. Mounting embryos for photography

For photography, it helps to keep embryos flat and surrounded by glass rather than plastic. We mount them in chambers constructed from a glass slide and one or more pieces of plastic tape (e.g. PVC insulating tape). Stick a length of tape onto the slide (more pieces required for older, and therefore thicker, embryos). With a scalpel blade, cut a square into the tape. Transfer the embryo to this, in suitable medium (e.g. glycerol or saline; the tape will not stand organic solvents). Seal with a glass coverslip, avoiding air bubbles; remove any excess mounting fluid. If the embryos are already in an organic mounting medium, use pieces of coverslip instead of plastic tape to define the chamber.

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