

Contents

Introduction.....106
 Materials106
 Procedure108
 Notes for the Instructor111
 Literature Cited.....112
 Appendix A: Preparation of Bone Staining and Clearing Solutions114
 Appendix B: Suppliers for Equipment, Chemicals, and Eggs.....115

Introduction

This method, referred to by students as “chicks-in-plastic,” “chick-in-a-cup,” or “the omelette lab,” allows continuous observation of living chick embryos from day 3 to day 18 of incubation. Differentiation of organ systems and extraembryonic structures as well as pre-hatching behavior can be studied by students from grade school through college. For very young students, a demonstration should be set up by the instructor for the class to observe. In high school or college introductory biology or embryology courses, students can study developmental processes and also practice in vitro culture techniques. For advanced developmental biology courses, project-oriented studies of growth (Dunn and Boone, 1976), teratology (Pearson, 1983), calcium metabolism (Burke et al., 1979; Dunn et al, 1981; Narbaitz, 1979; Narbaitz and Jande, 1978; Tuan, 1980), angiogenesis (Castellot et al. 1986; Dobson et al., 1990; Sherer and Dostal, 1982), or osteogenesis can be devised.

Early attempts to grow chick embryos outside their shells (Boone, 1963; Quisenberry and Dillon, 1962) met with limited success. Bruce Dunn (1974), a high school student working in an improvised basement laboratory, devised a method for growing chick embryos in plastic slings. Working with Boone, Ramsey and Dunn improved shell-less embryo culture methods (Dunn and Boone, 1976; Ramsey and Boone, 1972). Castellot et al. (1982) simplified the method further by substituting disposable hot cups (“chick-in-a-cup”) for plastic tripods.

Since Dunn's publication of his method in 1974, it has fascinated grade school children, won high school Science Fair Awards, and served as the experimental model for published scientific research. The simplicity, adaptability, and elegance of this method make it a pedagogical delight. In a world of rapidly changing technology and expensive equipment, the success of this method demonstrates that ingenuity and brains are more productive than button-pushing for good research and teaching.

Materials

Fertile Chicken Eggs

- 48–72 hours of incubation (6 per pair of students)
- 12- to 14-day-old embryos (2 per pair of students)

Equipment

For each pair of students:

Plastic bag to discard shells and eggs (1)
 Curved forceps (1)
 Straight forceps (1)
 Scissors (1)
 Sterile pasteur pipets (2)
 Glass rod (1)
 Styrofoam hot cups (6)
 Disposable, sterile petri dishes (100 × 15 mm, 3)
 Disposable petri dishes (60 × 15 mm, 1)
 Plastic wrap (1 roll)
 Rubber bands, 5.5" (6)
 150-ml sterile beaker (1)
 Sterile cotton balls (6)
 Finger bowl (about 4" in diameter) (1)
 Small foam rubber pad to set eggs on (1)
 Wax pencil or waterproof marking pen (1)
 Small screw-top bottles (1.5–2" in diameter such as baby food jars) (2)
 Tray, 9" × 12" (1)

To be shared by 20 students:

UV hoods (5)
 Egg candler (5)
 Hot plate (1)
 Thermometer (1)
 Incubator (electric cabinet) for incubating at least 60 eggs at 38°C (1)
 Large glass-topped incubator or oven-type incubator (preferably with inside glass door) (1)
 Paper tape (1 roll)

Solutions

For each pair of students:

Ethyl alcohol, 70%
 Betadine solution
 Saline, 0.85% (warm)

To be shared by 20 students:

Saline, 0.85% (2 liters)
 Betadine skin cleanser, 4-oz bottles (2)
 Ethyl alcohol, 70% (2 liters)

Ethyl alcohol, 95% (2 liters)
Ethyl alcohol, 100% (1 liter)
Toluene (500 ml)

For bone staining:

KOH, 1% (1 liter)
Working Alizarine red S solution (500 ml)

For storing wet mounts:

Clearing Solution A (200 ml)
Clearing Solution B (200 ml)
Clearing Solution C (200 ml)
Glycerin and thymol (200 ml)

For embedding chicks in plastic:

Caroplastic (2 pounds)
Catalyst (2 units)
Mold release (1 can)
Embedding in Caroplastic manual

Procedure

Preparation of culture chambers (Total time: 35 minutes)

1. Cut 2 holes in the sides of styrofoam hot cups.
2. Cut an 8" long piece of plastic wrap and make a "sling" over the top of the hot cup. Secure sling in place with a rubber band (3–6 chambers/student).
3. Place on trays under UV lamp to sterilize for 20 minutes.

Observation of living chick embryos and extraembryonic membranes and fixation of embryos for bone-staining (Total time: 15 minutes)

1. While chambers are being sterilized (see above), candle 12-day-old embryos and discard dead or unfertilized eggs.
2. Crack shells and gently drop into a finger bowl filled with warm 0.85% saline.
3. Observe chorion, amnion, and yolk sac with extraembryonic circulation and external features of embryo.
4. Dissect away membranes and drop embryo into small screw-top bottle filled with 95% alcohol.

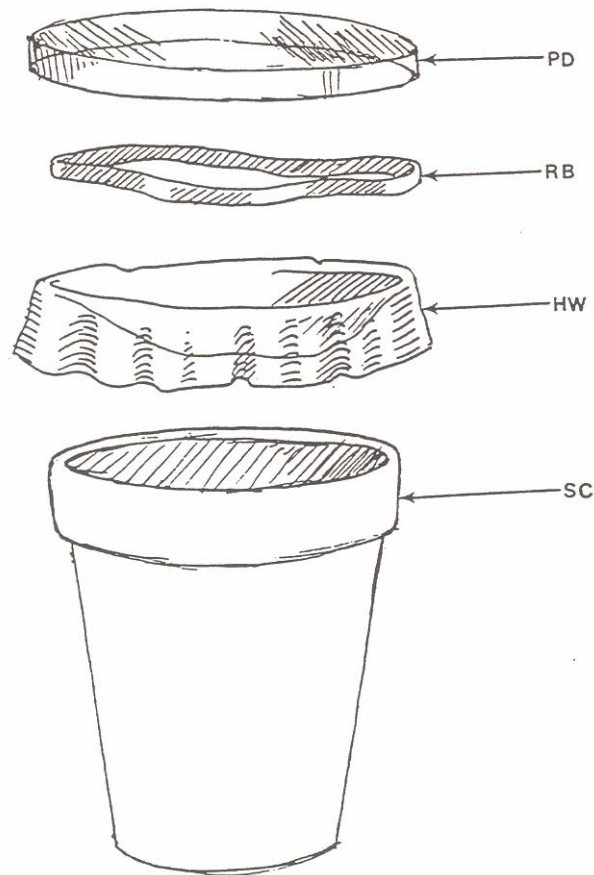


Figure 8.1. Construction of chick-in-a-cup culture chamber. Styrofoam hot cup (SC) supports a sling of plastic wrap (HW) held in place with a rubber band (RB) which is covered with a 100 × 15-mm petri dish cover (PD). Adapted from Dunn et al. (1981).

Transfer of 72-hour-old chick embryos from shell to prepared sterile chambers

(Total time: 45 minutes)

1. Candle 72-hour-old eggs, wash with Betadine solution and 70% ethyl alcohol and place on rubber pad for 2–3 minutes to allow chick to rotate to top of egg. Caution: Do not allow eggs to cool for more than 5 or 10 minutes or the embryo may die.
2. Wash hands, especially thumbs, with Betadine.
3. Gently crack the bottom side of the egg on the edge of the sterile glass beaker, pull shell open with both thumbs and gently drop egg contents into prepared chamber. The embryo should be on top of the yolk. If it is not and if it doesn't rotate to the top of the egg, use the wide, sterile ends of two pasteur pipets to gently rotate the embryo to the top surface. Discard any eggs in which the yolk is broken or the chick is not on top of the yolk.
4. Cover with the top of a 100 × 15-mm petri dish. Write the date and your name on the top of the petri dish and place immediately in a 38°C incubator.

Bone staining (Humason, 1979) and preparation of wet or plastic-embedded embryos

These procedures take short periods of time over a period of 5–7 days. Wet mounts require much less time, expense, and skill to prepare. An alternate method of embedding in plastic is also given here.

A – Bone staining

1. Remove embryos (12–19 days) from shell and membranes. Pluck feathers if present. Drop into jar of 95% ethyl alcohol for 1–2 days.
2. Discard alcohol and cover embryo with 1% KOH for 1 hour to 1 day (depending on age of embryo) until skeleton shows through muscles.
3. Discard KOH and cover embryo with Working Alizarine red S solution for 3–24 hours until bone is stained red.
4. Discard Alizarine solution and destain with 1% KOH for 5–10 minutes. Embryos can then be cleared and stored wet in bottles for display (follow procedure B below) or embedded in plastic (follow procedure C below).

B – Clearing and storing of wet mounts

1. Transfer through three clearing solutions (solutions A, B, and C): 24 hours each.
2. Transfer to pure glycerin with thymol added as a preservative.
3. Store in sealed tubes or bottles.

C – Clearing and embedding in plastic

See *Embedding in Caroplastic* manual (Edgerton, 1981).

1. Transfer embryos to 70% alcohol with 0.5% sodium carbonate. Soak specimens, changing solution several times, until no yellow color remains.
2. Soak in 75% ethyl alcohol for 10 minutes.
3. Dehydrate and clear according to the following schedule:
 - (a) 80% ethyl alcohol for 1 hour
 - (b) 95% ethyl alcohol for 1 hour
 - (c) Absolute ethyl alcohol for 30 minutes
 - (d) Absolute ethyl alcohol for 30 minutes
 - (e) Absolute alcohol-butanol (1:1 v/v) for 1 hour
 - (f) Butanol for 30 minutes
 - (g) Butanol-xylol (1:1 v/v) for 30 minutes
 - (h) Xylol for 1 hour
 - (i) Xylol overnight or until transparentCaution: Xylol fumes are toxic. Waste xylol should not be poured down sink but should be saved in a waste bottle for proper disposal according to accepted safety rules.
4. Infiltration: Transfer cleared embryo to uncatalyzed plastic for 1 day to 1 week.
5. Embedding: A mold is selected and coated with a thin coat of mold release compound. For small embryos, the bottom of a 60-mm petri dish makes a good mold. For larger specimens

small glass or metal dishes may be used. A quarter-inch thick layer of catalyzed plastic (64 drops of catalyst/cup of caroplastic) is poured into mold and allowed to set.

6. The stained, infiltrated embryo (see step 4 above) is placed in the mold on top of the hardened bottom plastic layer. Fresh catalyzed plastic (56 drops of catalyst/cup of caroplastic) is poured over embryo and is allowed to cool slowly until set. After gelatin is complete, cover with wax paper and allow to cool overnight.
7. Curing the mount: Place mold in oven or water bath at room temperature and slowly raise temperature over a 3-hour period to 120°F. Maintain temperature for 1 or 2 hours. Turn off heat and allow to cool slowly. Avoid sudden changes in temperature. An alternate method is to leave the mount at room temperature for 2–7 days.
8. Finishing: Sand rough surfaces with increasingly fine sand paper. Start with 220-grit paper. Repeat with 400-grit and then 600-grit paper. The final buffing can be done with buffing compound.
9. For further details see Embedding in Caroplastic manual (Carolina Biological Supply Co.).

Notes for the Instructor

Preparation of culture chambers

Chambers used in shell-less egg culture have been modified by different laboratories: Dunn used plastic tripods (Dunn and Boone, 1976, 1977); Sawyer (1979) used an even simpler plastic ring; Castellot's (183) "chick-in-a-cup" method substituted inexpensive, disposable hot cups for plastic tripods. The latter method is described here (Figure 8.1).

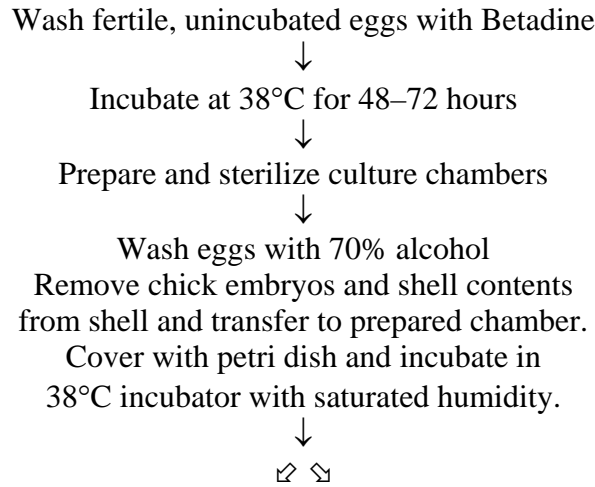
Originally Handi-Wrap was used to make the plastic slings. Manufacturing changes in Handi-Wrap II made this product less permeable to gases and hence less favorable for survival of chick embryos. The thinnest, most permeable plastic wraps with the least additives, such as Grand Union plastic wrap, are better than "improved" varieties (J. Castellot, Jr., personal communication). Before setting up a student exercise or research project, it is advisable to do a pilot study using several brands to determine the best, currently available plastic wrap. Then purchase a 1- or 2-year supply of that brand.

Transfer of 72-hour-old chick embryos from shell to prepared sterile chambers

It is essential that high humidity be maintained in the incubator to prevent desiccation and death of the embryos. Trays of distilled water with paper towel wicks are a simple way to increase humidity. If CO₂ is available, 1–2% CO₂ improves survival. Opening and shutting of incubator doors should be limited to once or twice a day to minimize jarring the fragile embryos in their slings.

After a little practice in setting up chicks-in-a-cup, 80–90% of the embryos will survive until 8 days of total incubation. Survival decreases with length of incubation but a few will live up to 21 days of total incubation (Dunn and Boone, 1976). If contamination of cultures by mold or bacteria becomes a problem, the incubator should be thoroughly cleaned and fumigated overnight with formaldehyde gas produced by adding 2 g of potassium permanganate to 4 ml of 37% formaldehyde solution. Careful attention to aseptic technique will also eliminate contamination. Forceps and scissors should be soaked in 70% alcohol prior to use. Plastic wrap does not have to be sterilized if the first 3–4 turns of wrap are discarded prior to each day's use (Dunn et al., 1981).

Flow Chart of Procedures



Observational projects for introductory classes:

1. Observe growth and differentiation of organs and extraembryonic structures (day 3–18).
2. Observe prehatching behavior.

Research projects for advanced classes:

1. Quantitative growth measurements.
2. Teratology: Effect of vitamins, steroids, nicotine, caffeine, etc., on survival and differentiation.
3. Bone staining: Calcification of developing skeleton (day 12–18).

Literature Cited

Boone, M. A. 1963. A method of growing chick embryos in vitro. *Poultry Science*, 42:916–921.

Burke, B. R., R. Narbaitz, and S. Tolnai. 1979. Abnormal characteristics of blood from chick embryos maintained in shell-less culture. *Revue Canadienne de Biologie*, 63-66.

Castellot, J. J., Jr., A. M. Kambe, D. E. Dobson, and D. M. Spiegelman. 1986. Heparin potentiation of 3-T-3-adipocyte stimulated angiogenesis: Mechanism of actions on endothelial cells. *Journal of Cell Physiology*, 127:323–329.

Castellot, J. J., Jr., M. T. Karnovsky, and B. M. Spiegelman. 1982. Differentiation-dependent stimulation of neo-vascularization and endothelial cell chemotaxis by 3-T-3-adipocytes. *Proceedings of the National Academy of Sciences*, 79:5597–5601.

- Dobson, D. E., A. M. Kambe, E. Block, T. Dion, H. Lu, J. J. Castellot, Jr., and D. M. Spiegelman. 1990. 1-Butyryl-glycerol: A novel angiogenesis factor secreted by differentiating adipocytes. *Cell*, 61:223–230.
- Dunn, B. E. 1974. Technique for shell-less culture of the 72-hour avian embryo. *Poultry Science*, 53:409–412.
- Dunn, B. E., and M. A. Boone. 1976. Growth of the chick embryo in vitro. *Poultry Science*, 55:1967–1981.
- . 1977. Growth and mineral content of cultured chick embryos. *Poultry Science*, 56:662–672.
- . 1978. Photographic study of chick embryo development in vitro. *Poultry Science*, 57:370–377.
- Dunn, B. E., T. P. Fitzharris, and B. D. Barnett. 1981. Effects of varying chamber construction and embryo pre-incubation age on survival and growth of chick embryos in shell-less culture. *The Anatomical Record*, 199:33–43.
- Dunn, B. E., J. S. Graves, and T. P. Fitzharris. 1981. Active calcium transport in the chick chorioallantoic membrane requires interaction with the shell membranes and/or shell calcium. *Developmental Biology*, 88:259–268.
- Edgerton, H. H. 1981. Embedding in caroplastic. Carolina Biological Supply Co., Burlington, North Carolina.
- Humason, G. 1979. Alizarine red S method for embryos (bone formation). Pages 156–158, in *Animal tissue techniques* (Fourth edition). W. H. Freeman, San Francisco, California.
- Narbaitz, R. 1979. Response of shell-less cultured chick embryos to exogenous parathyroid hormone and 1,25-dihydroxycholecalciferol. *General and Comparative Endocrinology*, 37:440–442.
- Narbaitz, R., and S. S. Jande. 1978. Ultrastructural observations on the chorionic epithelium, parathyroid glands and bones from chick embryos developed in shell-less culture. *Journal of Embryology and Experimental Morphology*, 45:1–12.
- Pearson, S. 1983. Effect of nicotine and caffeine on development of chicks in shell-less culture. Prize-winning research in Poughkeepsie High School and Dutchess County Science Fairs. Poughkeepsie High School, Poughkeepsie, New York. (Unpublished.)
- Quisenberry, J. H., and E. J. Dillon. 1962. Growing chick embryos in plastic shells. *Poultry Science*, 41:1675.
- Ramsey, J. B., Jr., and M. A. Boone. 1972. Incubator for growing chick embryos in vitro and in ova. *Poultry Science*, 51:707–709.
- Sawyer, R. H. 1979. Modification of chick in shell-less culture procedure. Biology Department, U. of South Carolina, Columbia, South Carolina. (Personal Communication).
- Sherer, G. K., and G. H. Dostal. 1982. Influence of medroxy-progesterone, an inhibitor of vascularization, on hepatic morphogenesis. *Journal of Cell Biology*, 95:187a.
- Tuan, R. S. 1980. Calcium transport and related functions in the chorioallantoic membrane of cultured shell-less chick embryos. *Developmental Biology*, 74: 196–204.

APPENDIX A

Preparation of Bone Staining and Clearing Solutions (Humason, 1979)

1% Potassium hydroxide:

10 g potassium hydroxide
1 liter distilled water

Stock Alizarine red S solution:

5 ml Alizarine red S (C.I. 58005), saturated in 50% acetic acid
10 ml glycerin
60 ml chloral hydrate, 1% aqueous (a drug)

Working Alizarine red S solution:

1.0 ml alizarine stock solution
1 liter 1% potassium hydroxide
(Make up at least 500 ml; use at room temperature.)

Clearing solutions:

Solution A:

Glycerin: 4% potassium hydroxide: distilled water (in a 20:3:77 v/v/v ratio)

Solution B:

Glycerin: 4% potassium hydroxide: distilled water (in a 50:3:47 v/v/v ratio)

Solution C:

Glycerin: distilled water (in a 3:1 v/v ratio)

APPENDIX B
Suppliers for Equipment, Chemicals, and Eggs

Incubator:

Glass-topped incubator custom made by Gene Brigman:

White Oak Digital, Route 2
Box 40, Winnsboro, SC 29180
(803) 635-4180
Cost: \$350 US

Instructions for building an inexpensive styrofoam incubator can be obtained free of charge from:

“Incubating of Eggs of Domestic Birds”
Circular #530
Cooperative Extension Service
U.S. Department of Agriculture
Clemson University
Clemson, SC 29631

Alizarine red S (C.I. 58005):

J. T. Baker Reagents and Lab Products
222 Red School Lane
Phillipsburg, NJ 08865
(908) 859-2151

Chloral hydrate:

J. T. Baker Reagents and Lab Products

Caroplastic, catalyst, mold release, and embedding manual:

Carolina Biological Supply
Burlington, NC 27215
1-800-334-5551

Betadine skin cleanser:

Any drug store

Fertile eggs:

Large, commercial hatcheries are the best source of reliable, fertile (hatching) eggs. If there are no hatcheries in your area, locating a year-round source of fertile eggs may require considerable perseverance and detective work. You may be able to locate a local poultry farmer or breeder who sells them. Call your County Agent, check exhibitors at local County Fairs, or call university agricultural stations. “Who’s Who in the Egg and Poultry Industry” publishes a Directory of Hatcheries in the U.S. and Canada.