[redacted]

Cell Bio Lab

Primary Culture

A primary culture is that stage of the culture after isolation of the cells but before the first subculture after which it becomes a cell line (*see* Section 2.7 and Appendix IV). Although there is formal acceptance for this definition of "primary culture" and "cell line" [Schaeffer, 1990], the advent of commercial supplies of early passage culture of specialized cells (*see* Appendix II) has seen the increasing use of the phrase "primary cells" or "primary cell lines." One has to be clear what is meant when purchasing these cells. In some cases they may be dissociated cells from a primary culture that will become a cell line when the recipient seeds them into culture, but in other cases they may be growing cultures already passaged from a primary culture and technically a cell line (usually a finite cell line) or frozen cells from a primary culture, or, more likely, from the first or second passage. This distinction may seem a little pedantic, but it is important to know the stage of the culture as it will affect its uniformity (*see* Section 2.7) and the number of generations left to it before senescence, namely the life span of the culture. If subcultured from the primary culture, the correct term is *early passage cell line* and not "primary cell line," which is a contradiction in terms.

11.1 INITIATION OF A PRIMARY CELL CULTURE

There are four stages to consider: (1) acquisition of the sample, (2) isolation of the tissue, (3) dissection and/or disaggregation, and (4) culture after seeding into the culture vessel. After isolation, a primary cell culture may be obtained either by allowing cells to migrate out from fragments of tissue adhering to a suitable substrate or by disaggregating the tissue mechanically or enzymatically to produce a suspension of cells, some of which will ultimately attach to the substrate. It appears to be essential for most normal untransformed cells, with the exception of hematopoietic cells and stem cells, to attach to a flat surface in order to survive and proliferate with maximum efficiency. Transformed cells, however, particularly cells from transplantable animal tumors, are often able to proliferate in suspension (*see* Section 17.5.1).

11.1.1 Enzymes Used in Disaggregation

The enzymes used most frequently for tissue disaggregation are crude preparations of trypsin, collagenase, elastase, pronase, Dispase, DNase, and hyaluronidase, alone or in various combinations, such as Elastase and DNase for type II alveolar cell isolation [Dobbs & Gonzalez, 2002], collagenase with Dispase [Booth & O'Shea, 2002], and collagenase with hyaluronidase [Berry & Friend, 1969; Seglen, 1975]. There are other, nonmammalian enzymes, such as Trypzean (Sigma), a recombinant, maize-derived, trypsin, TrypLE (Invitrogen), recombinant microbial, and Accutase and Accumax (Innovative Cell Technologies), and Liberase Blendzyme 3 or Liberase TM (Roche), also available for primary disaggregation. Crude preparations are often more successful than purified enzyme preparations. This is because the former contain other proteases as contaminants; the latter are nevertheless generally less toxic and more specific in their action. Trypsin and pronase give the most complete disaggregation but may damage the cells. Collagenase and Dispase, in contrast, give incomplete disaggregation, but are less harmful. Hyaluronidase can be used in conjunction with

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collagenase to digest the extracellular matrix, and DNase is used to disperse DNA released from lysed cells; DNA tends to impair proteolysis and promote reaggregation (*see* Table 12.5). Care should be taken when combining enzymes as some may inactivate others. For example DNase should be added after trypsin has been removed, as the trypsin may degrade the DNase.

As the specific activity of enzymes may vary make sure that each batch has the same activity or adjust the concentration to achieve the same activity (see Appendix I).

11.1.2 Common Features of Disaggregation

Although each tissue may require a different set of conditions, certain requirements are shared by most of them:

(1) Fat and necrotic tissues are best removed during dissection.
(2) The tissue should be chopped finely with sharp scalpels to cause minimum damage.
(3) Enzymes used for disaggregation should be removed subsequently by gentle centrifugation.
(4) The concentration of cells in the primary culture should be much higher than that normally used for subculture because the proportion of cells from the tissue that survives in primary culture may be quite low.
(5) A rich medium, such as Ham's F12, is preferable to a simple medium, such as Eagle's MEM, and if serum is required, fetal bovine often gives better survival than does calf or horse. Isolation of specific cell types will probably require selective serum-free media (*see* Section 9.2.2; Chapter 22).
(6) Embryonic tissue disaggregates more readily, yields more viable cells, and proliferates more rapidly in primary culture than does adult tissue.

11.2 ISOLATION OF THE TISSUE

Before attempting to work with human or animal tissue, make sure that your work fits within medical ethical rules or current legislation on experimentation with animals (*see* Section 6.9.1). For example, in the United Kingdom, the use of embryos or fetuses beyond 50% gestation or incubation is regulated under the Animal Experiments (Scientific Procedures) Act of 1986. Work with human biopsies or fetal material usually requires the consent of the local ethical committee and the patient and/or his or her relatives (*see* Section 6.9.2).

Δ ***Safety Note.*** Work with human tissue should be carried out at Containment Level 2 in a Class II biological safety cabinet (*see* Section 6.8.3).

An attempt should be made to sterilize the site of the resection with 70% alcohol if the site is likely to be contaminated (e.g., skin). Remove the tissue aseptically and transfer it to the tissue culture laboratory in dissection BSS (DBSS) or collection medium (*see* Appendix I) as soon as possible. Do not dissect animals in the tissue culture laboratory, as the animals may carry microbial contamination. If a delay in transferring the tissue is unavoidable, it can be held at 4°C for up to 72 h, although a better yield will usually result from a quicker transfer.

Embryonic or fetal animals that are more than half term may require specified methods of humane killing before dissection. In the United Kingdom, guidelines are available from the Home Office [Home Office, 2005] and in the United States the Office of Laboratory Animal Welfare [OLAW, 2002]. Where guidelines are not available, seek advice from your local animal ethics committee. Usually separating the embryo from the fetal membranes or placenta followed by decapitation is regarded as humane, particularly if the gravid uteri or eggs are placed on ice beforehand.

11.2.2 Chick Embryo

Chick embryos are easier to dissect, as they are larger than mouse embryos at the equivalent stage of development. Like mouse embryos, chick embryos are used to provide predominantly mesenchymal cell primary cultures for cell proliferation analysis, to provide feeder layers, and as a substrate for viral propagation. Because of their larger size, it is easier to dissect out individual organs to generate specific cell types, such as hepatocytes, cardiac muscle, and lung epithelium. As with mouse embryos, the use of chick embryos may be subject to animal legislation and working with embryos that are more than half-term may require a license.

PROTOCOL 11.2. ISOLATION OF CHICK EMBRYOS

Outline

Remove embryo aseptically from the egg and transfer to dish.

Materials

Sterile: PBS

- ❑ ~~DBSS~~: dissection BSS (BSS with a high concentration of antibiotics; *see* Appendix I) in 25- to 50-mL screw-capped tube or universal container
- ❑ BSS, 50 mL in a sterile beaker (used to cool instruments after flaming)
- ❑ Small beaker, 20 to 50 mL or egg cup
- ❑ Forceps, straight and curved
- ❑ Petri dishes, 9 cm

Nonsterile:

- ❑ Embryonated eggs, 10th day of incubation
- ❑ Alcohol, 70%
- ❑ Swabs
- ❑ Humid incubator (no additional CO_2 above atmospheric level)

Procedure

1. Incubate the eggs at 38.5°C in a humid atmosphere, and turn the eggs through 180° daily. Although hens' eggs hatch at around 20 to 21 days, the lengths of their developmental stages are different from those of mouse embryos. For a culture of dispersed cells from the whole embryo, the egg should be taken at about 8 days, and for isolated-organ rudiments, at about 10 to 13 days.
2. Swab the egg with 70% alcohol, and place it with its blunt end facing up in a small beaker (Fig. 11.4*a*).
3. Crack the top of the shell (Fig. 11.4*b*), and peel the shell off to the edge of the air sac with sterile forceps (Fig. 11.4*c*).
4. Resterilize the forceps (i.e., dip them in alcohol, burn off the alcohol, and cool the forceps in sterile BSS), and then use the forceps to peel off the white shell membrane to reveal the chorioallantoic membrane (CAM) below, with its blood vessels (Fig. 11.4*d*, *e*).
5. Pierce the CAM with sterile curved forceps (Fig. 11.4*f*), and lift out the embryo by grasping it gently under the head (Fig. 11.4*g*, *h*). Do not close the forceps completely, or else the neck will sever; place the middle digit under the forceps and use the finger pad to restrict the pressure of the forefinger (*see* Fig. 11.4*g*).
6. Transfer the embryo to a 9-cm Petri dish containing 20-mL ~~DBSS~~ PBS (Fig. 11.4*i*). (For subsequent dissection and culture, *see* Protocol 11.7.)

3.

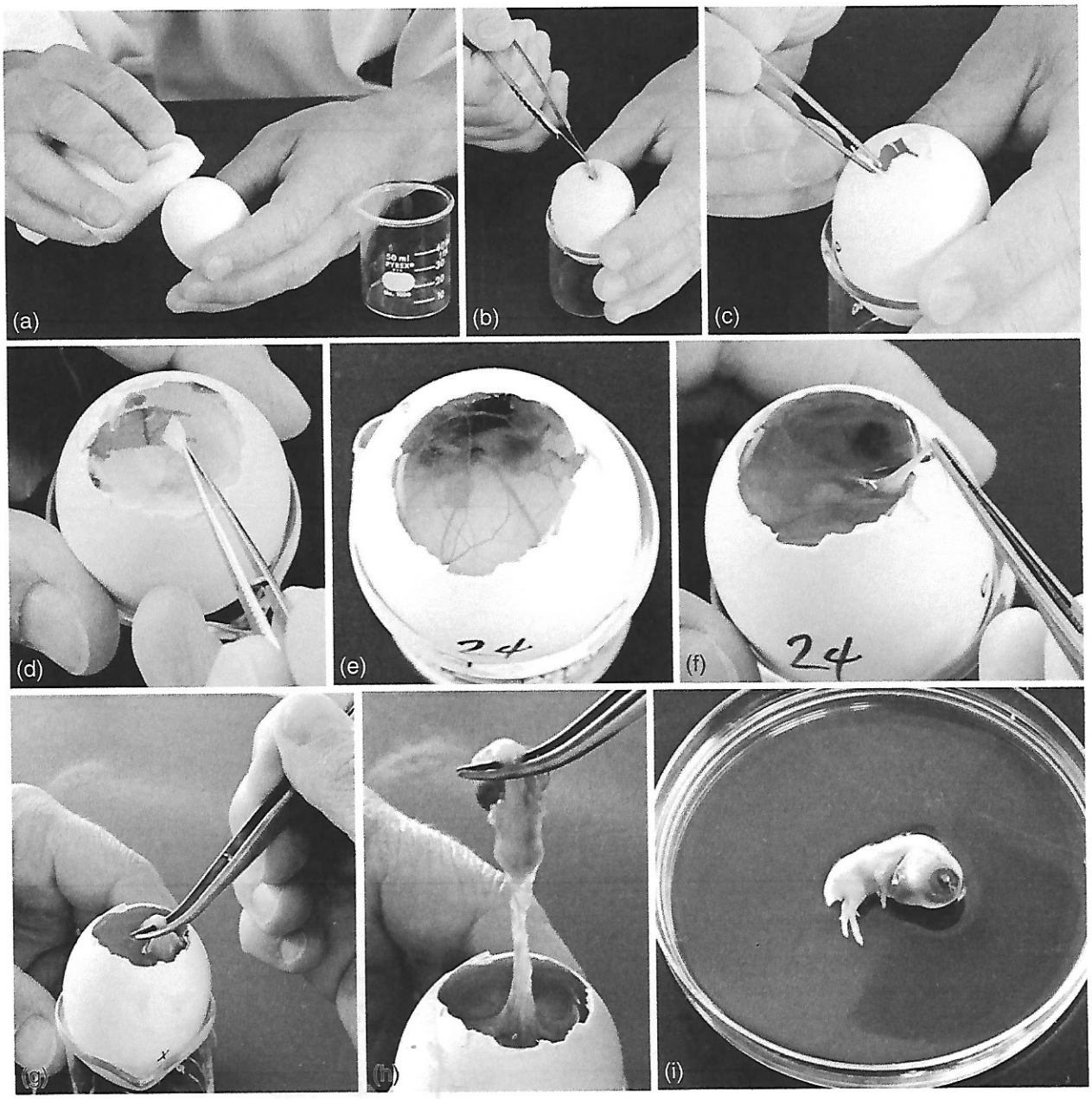

Fig. 11.4. Removing a Chick Embryo from an Egg. Stages in the extraction of the whole chick embryo from an egg. (*a*) Swabbing the egg with alcohol. (*b*) Cracking the shell. (*c*) Peeling off the shell. (*d*) Peeling off the shell membrane. (*e*) Chorioallantoic membrane (CAM) and vasculature revealed. (*f*) Removing CAM with forceps. (*g*) Grasping the embryo round the neck. (*h*) Withdrawing the embryo from the egg. (*i*) Isolated 10-day embryo in Petri dish.

4.

PROTOCOL 11.4. PRIMARY EXPLANTS

Outline

The tissue is chopped finely and rinsed, and the pieces are seeded onto the surface of a culture flask or Petri dish in a small volume of medium with a high concentration (i.e., 40–50%) of serum, such that surface tension holds the pieces in place until they adhere spontaneously to the surface (Fig. 11.6*a*). Once this is achieved, outgrowth of cells usually follows (Fig. 11.6*b*, *c*; Plates 1*a*, 2*b*).

Materials

Sterile or aseptically prepared:

- ❑ Tissue sample
- ❑ Growth medium (e.g., 50:50 DMEM:F12 with 20% fetal bovine serum)
- ❑ ~~DBSS~~ PBS, 100 mL
- ❑ Petri dishes, 9 cm, non–tissue–culture grade
- ❑ Forceps
- ❑ Scalpels
- ❑ Pipettes, 10 mL with wide tips
- ❑ Centrifuge tubes, 15 or 20 mL, or universal containers
- ❑ Culture flasks, 25 cm^2, or tissue-culture-grade Petri dishes, 5 to 6 cm. The size of flasks and volume of growth medium depend on the amount of tissue: roughly five 25-cm^2 flasks per 100 mg of tissue.

Procedure

1. Transfer tissue to fresh, sterile DBSS, and rinse.
2. Transfer the tissue to a second dish; dissect off unwanted tissue, such as fat or necrotic material, and transfer to a third dish.

Note. Clean healthy tissue with little blood may not need these two transfers and can be dissected in the first dish, after transfer from the transport medium, which will have acted as the first wash.

3. Chop finely with crossed scalpels (*see* Fig. 11.6*a*, top) into about 1-mm cubes.
4. Transfer by pipette (10–20 mL, with wide tip) to a 15- or 50-mL sterile centrifuge tube or universal container. (Wet the inside of the pipette first with BSS or medium, or else the pieces will stick.)
5. Allow the pieces to settle.
6. Wash by resuspending the pieces in DBSS, allowing the pieces to settle, and removing the supernatant fluid. Repeat this step two more times. This step may be omitted if there is little blood or necrotic tissue.
7. Transfer the pieces (remember to wet the pipette) to a culture flask, with about 20 to 30 pieces per 25-cm^2 flask.
8. Remove most of the fluid, and add 1-mL growth medium per 25-cm^2 growth surface. Tilt the flask gently to spread the pieces evenly over the growth surface.
9. Cap the flask, and place it in an incubator or hot room at 37°C for 18 to 24 h.
10. Add 1 mL of medium the following day.
11. Make up the medium volume gradually to 5 mL per 25 cm^2 over the next 3 to 5 days.
12. Change the medium weekly until a substantial outgrowth of cells is observed (*see* Fig. 11.6*b*).
13. Once an outgrowth has formed, the remaining explant may be picked off with a scalpel (Fig. 11.6c) and transferred by prewetted pipette to a fresh culture vessel. (Then return to step 7.)
14. Replace the medium in the first flask until the outgrowth has spread to cover at least 50% of the growth surface, at which point the cells may be subcultured (*see* Protocol 12.3).

* There are different protocols depending on desired cell type (different trypsin times, etc.)

Chicken Myocyte Primary Culture

1. Using sterile equipment, remove heart from embryo and place in dish with PBS. (add a couple hearts to one dish)
2. Cut heart into small pieces
3. Using sterile forceps, place heart pieces in 50ml centrifuge tube.
4. Add 3-5 ml 0.025% Trypsin
5. Incubate in warm water bath (37°C) for 5 min
6. Discard supernatant from first trypsinization.
7. Repeat # 4 and #5
8. Collect supernatant (carefully) and place in new 50ml centrifuge tube.
9. In tube w/ tissue, again repeat #4 and #5, collect supernatant again and place in same tube as #8 (you are collecting cells that are coming off of the tissue)
10. Centrifuge 50 ml tube w/ supernatant at 1200 rpm for 5 minutes.
11. Resuspend pellet in culture medium and put in petri dish (incubate in CO_2 incubator, 37°C)

Culture medium - DMEM w/ 10% FBS (fetal bovine serum) (w/ antibiotics)

Materials Needed:

- ☐ Fertilized Chicken Eggs
- ☐ sterile large petri dishes to open eggs
- ☐ 70% alcohol
- ☐ forceps and scalpels individually wrapped in foil and autoclaved
- ☐ beakers that have been wrapped in foil and autoclaved (to hold forceps and scalpels while in use)
- ☐ PBS (sterile)
- ☐ DMEM (sterile) with antibiotics
- ☐ 50ml centrifuge tubes
- ☐ warm water bath (37°C)
- ☐ sterile pipettes (5 ml)
- ☐ micropipettes w/ sterile tips
- ☐ culture flasks (25 cm^2)
- ☐ centrifuge
- ☐ CO_2 incubator