CHAPTER 12

Primary Culture

12.1 TYPES OF PRIMARY CELL CULTURE

A primary culture is that stage of the culture after isolation of the cells but before the first subculture. 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, to attach to a flat surface in order to survive and proliferate with maximum efficiency. Transformed cells (*see* Section 18.5.1), on the other hand, particularly cells from transplantable animal tumors, are often able to proliferate in suspension.

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, e.g., 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), also available for primary disaggregation. Crude preparations are often more successful than purified enzyme preparations, because the former contain other proteases as contaminants, although the latter are 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, on the other hand, give incomplete disaggregation, but are less harmful. Hyaluronidase can be used in conjunction with collagenase to digest the intracellular matrix, and DNase is used to disperse DNA released from lysed cells; DNA tends to impair proteolysis and promote reaggregation (*see* Table 13.4).

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

(1) Fat and necrotic tissue are best removed during dissection.
(2) The tissue should be chopped finely with sharp instruments 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 media (*see* Section 10.2.1 and Chapter 23).
(6) Embryonic tissue disaggregates more readily, yields more viable cells, and proliferates more rapidly in primary culture than does adult tissue.

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12.2 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 7.9.1). 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 7.9.2).

Δ ***Safety Note.*** Work with human tissue should be carried out at Containment Level 2 in a Class II biological safety cabinet (*see* Section 7.8.3).

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 transport 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.

12.2.1 Mouse Embryo

Mouse embryos are a convenient source of cells for undifferentiated fibroblastic cultures. They are often used as feeder layers (*see* Fig. 14.2.3).

PROTOCOL 12.1. ISOLATION OF MOUSE EMBRYOS

Outline

Remove uterus aseptically from a timed pregnant mouse and dissect out embryos.

Materials

Sterile:

- ❑ 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)
- ❑ Petri dishes, 9 cm
- ❑ Pointed forceps
- ❑ Pointed scissors

Nonsterile:

- ❑ Small laminar-flow hood
- ❑ Timed pregnant mice (*see* Step 1 of this protocol)
- ❑ Alcohol, 70%, in wash bottle
- ❑ Alcohol, 70%, to sterilize instruments (*see* Fig. 7.4)
- ❑ Bunsen burner

Δ ***Safety Note.*** When sterilizing instruments by dipping them in alcohol and flaming them, take care not to return the instruments to alcohol while they are still alight!

Protocol

1. *Induction of estrus.* If males and females are housed separately, then put together for mating, estrus will be induced in the female 3 days later, when the maximum number of successful matings will occur. This process enables the planned production of embryos at the appropriate time. The timing of successful matings may be determined by examining the vaginas each morning for a hard, mucous plug.
2. *Dating the embryos.* The day of detection of a vaginal plug, or the ''plug date,'' is noted as day zero, and the development of the embryos is timed from this date. Full term is about 19–21 days. The optimal age for preparing cultures from a whole disaggregated embryo is around 13 days, when the embryo is relatively large (*Figs.* 12.1, 12.2) but still contains a high proportion of undifferentiated mesenchyme, which is the main source of the culture. However, isolation and handling embryos beyond 50% full-term may require a license (e.g., in the United Kingdom) so 9- or 10-day embryos may be preferable. Although the amount of tissue recovered from these embryos will be substantially less, a higher proportion of the cells will grow. Most individual organs, with the exception of the brain and the heart, begin to form at about the 9th day of gestation, but are difficult to isolate until about the 11th day. Dissection of individual organs is easier at 13–14 days, and most of the organs are completely formed by the 18th day.
3. Kill the mouse by cervical dislocation (U.K. Schedule I procedure), and swab the ventral surface liberally with 70% alcohol (Fig. 12.3a).
4. Tear the ventral skin transversely at the median line just over the diaphragm (Fig. 12.3b), and, grasping the skin on both sides of the tear, pull in opposite directions to expose the untouched ventral surface of the abdominal wall (Fig. 12.3c).
5. Cut longitudinally along the median line of the exposed abdomen with sterile scissors, revealing the viscera (Fig. 12.3d). At this stage, the uterus, filled with embryos, is obvious in the posterior abdominal cavity (Fig. 12.3e).
6. Dissect out the uteri into a 25-mL or 50-mL screw-capped vial containing 10 or 20 mL DBSS (Fig. 12.3f).

Note. All of the preceding steps should be done outside the tissue culture laboratory; a small laminar-flow hood

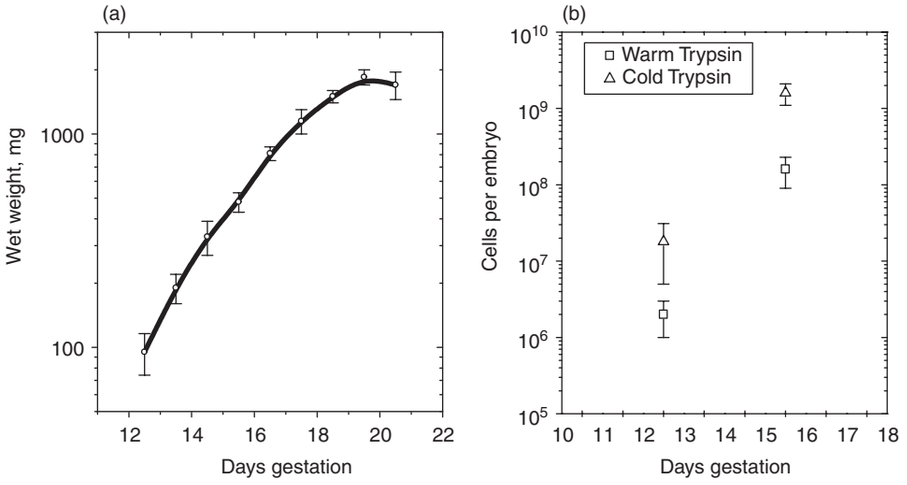

Fig. 12.1. Total Wet Weight and Yield of Cells per Mouse Embryo. (a) Total wet weight of embryo without placenta or membranes, mean ± standard deviation [from Paul et al., 1969]. (b) Cell yield per embryo after incubation in 0.25% trypsin at 37°C for 4 h with no intermediate harvesting (squares) or after soaking in 0.25% trypsin at 4°C for 5 h and incubation at 37°C for 30 min (triangles; Protocol 12.6).

and rapid technique will help to maintain sterility. Do not take live animals into the tissue culture laboratory, as the animals may carry contamination. If an animal carcass must be handled in the tissue culture area, make sure that the carcass is immersed in alcohol briefly, or thoroughly swabbed, and disposed of quickly after use.

7. Take the intact uteri to the tissue culture laboratory, and transfer them to a fresh Petri dish of sterile DBSS (Fig. 12.3g).
8. Dissect out the embryos:
   (a) Tear the uterus with two pairs of sterile forceps, keeping the points of the forceps close together to avoid distorting the uterus and bringing too much pressure to bear on the embryos (Fig. 12.3g,h).
   (b) Free the embryos from the membranes (Fig. 12.3i) and placenta and place them to one side of the dish to bleed.
9. Transfer the embryos to a fresh Petri dish. If a large number of embryos is required (i.e., more than four or five litters), it may be helpful to place the dish on ice (for subsequent dissection and culture; *see* Protocols 12.4–12.8).

12.2.2 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 (e.g., in the United Kingdom) and working with embryos that are more than half-term may require a license.

PROTOCOL 12.2. ISOLATION OF CHICK EMBRYOS

Outline

Remove embryo aseptically from the egg and transfer to dish.

Materials

Sterile:

- ❑ 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, 20–50 mL or egg cup
- ❑ Forceps, straight and curved
- ❑ Petri dishes, 9 cm

Nonsterile:

- ❑ Embryonated eggs, 10th day of incubation
- ❑ Alcohol, 70%

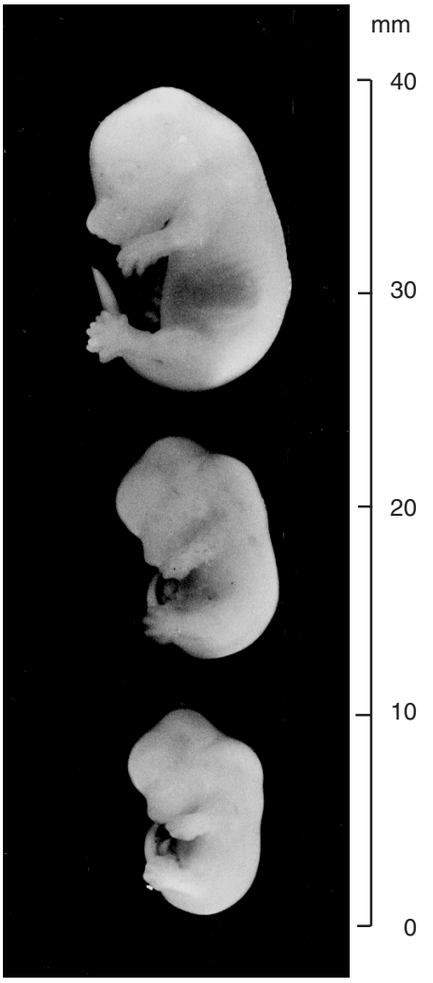

Fig. 12.2. Mouse Embryos. Embryos from the 12th, 13th, and 14th days of gestation. The 12-day embryo (bottom) came from a small litter (three) and is larger than would normally be found at this stage.

- ❑ Swabs
- ❑ Humid incubator (no additional CO_2 above atmospheric level)

Protocol

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 day, and for isolated-organ rudiments, at about 10–13 day. (10 days is the maximum in the United Kingdom without a license.)
2. Swab the egg with 70% alcohol, and place it with its blunt end facing up in a small beaker (Fig. 12.4a).
3. Crack the top of the shell (Fig. 12.4b), and peel the shell off to the edge of the air sac with sterile forceps (Fig. 12.4c).
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. 12.4d,e).
5. Pierce the CAM with sterile curved forceps (Fig. 12.4f), and lift out the embryo by grasping it gently under the head (Fig. 12.4g,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. 12.4g).
6. Transfer the embryo to a 9-cm Petri dish containing 20 mL DBSS (Fig. 12.4i). (For subsequent dissection and culture, *see* Protocol 12.7.)

12.2.3 Human Biopsy Material

Handling human biopsy material presents certain problems that are not encountered with animal tissue. It usually is necessary to obtain consent (1) from the hospital ethical committee, (2) from the attending physician or surgeon, and (3) from the donor or patient or the patient's relatives (*see* Section 7.9.2). Furthermore, biopsy sampling is usually performed for diagnostic purposes, and hence the needs of the pathologist must be met first. This factor is less of a problem if extensive surgical resection or nonpathological tissue (e.g., placenta or umbilical cord) is involved.

The operation is often performed by one of the resident staff at a time that is not always convenient to the tissue culture laboratory, so some formal collection or storage system must be employed for times when you or someone on your staff cannot be there. If delivery to your lab is arranged, then there must be a system for receiving specimens, recording details of the source, tissue of origin, pathology, etc. (*see* Section 12.3.11), and alerting the person who will perform the culture that the specimens have arrived; otherwise, valuable material may be lost or spoiled.

Δ ***Safety Note.*** Human biopsy material carries a risk of infection (*see* Section 7.8.3), so it should be handled under Containment Level 2 in a Class II biohazard cabinet, and all media and apparatus must be disinfected after use by autoclaving or immersion in a suitable disinfectant (*see* Section 7.8.5). The tissue should be screened for adventitious infections such as hepatitis, HIV, and tuberculosis [in the United States, U.S. Department of Health and Human

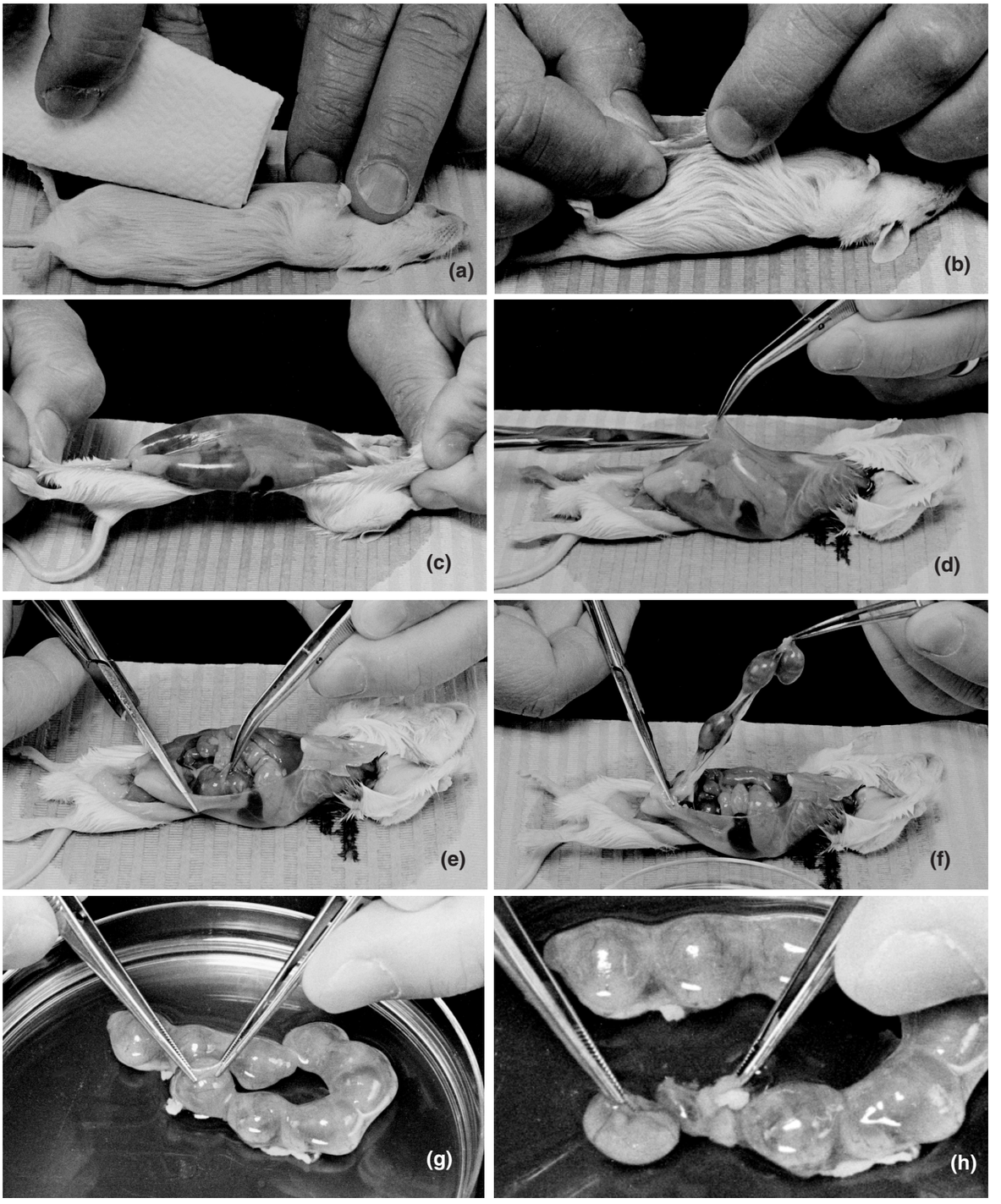

Fig. 12.3. Mouse Dissection. Stages in dissection of a pregnant mouse for the collection of embryos (*see* Protocol 12.1). (a) Swabbing the abdomen. (b), (c) Tearing the skin to expose the abdominal wall. (d) Opening the abdomen. (e) Revealing the uterus *in situ*. (f) Removing the uterus. (g), (h) Dissecting the embryos from the uterus. (i) Removing the membranes. (j) Removing the head (optional). (k) Chopping the embryos. (l) Transferring pieces to trypsinization flask (for warm trypsinization; *see* Protocol 12.5). (m) Transferring the pieces to a small Erlenmeyer flask (for cold trypsinization; *see* Protocol 12.6). (n) Flask on ice.

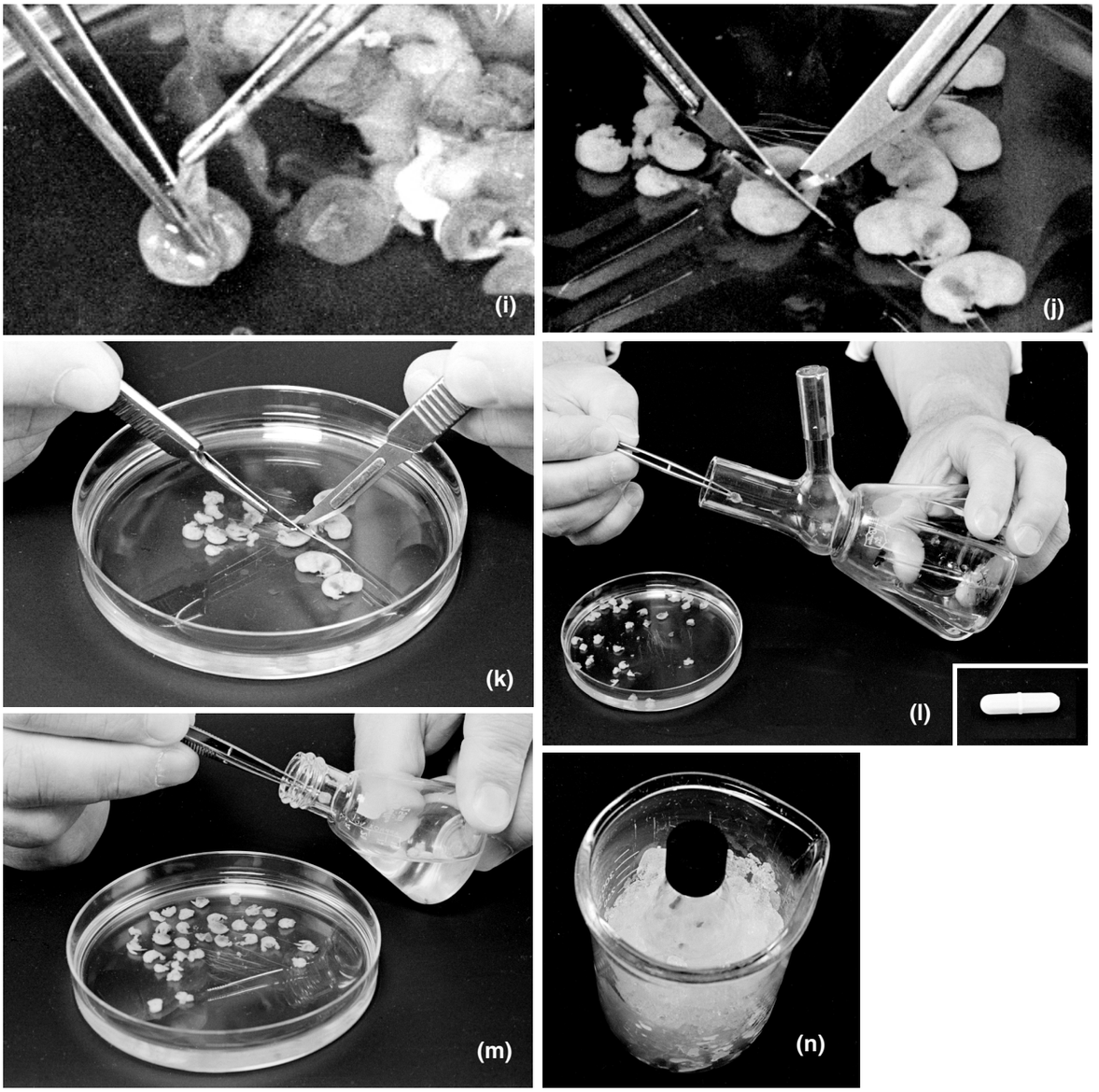

Fig. 12.3. (*Continued*)

Services, 1993; in the UK, Advisory Committee on Dangerous Pathogens, 1995b] unless the patient has already been tested for these infections.

PROTOCOL 12.3. HUMAN BIOPSIES

Outline

Consult with hospital staff, provide labeled container(s) of medium, and arrange for collection of samples from operating room or pathologist.

Materials

- Specimen tubes (15–30 mL) with leakproof caps about one-half full with culture medium containing antibiotics (*see* Appendix I: Collection Medium) and labeled with your name, address, and telephone number.

Protocol

1. Provide containers of collection medium, clearly labeled, to the anteroom of the operating theater or to the pathology laboratory.

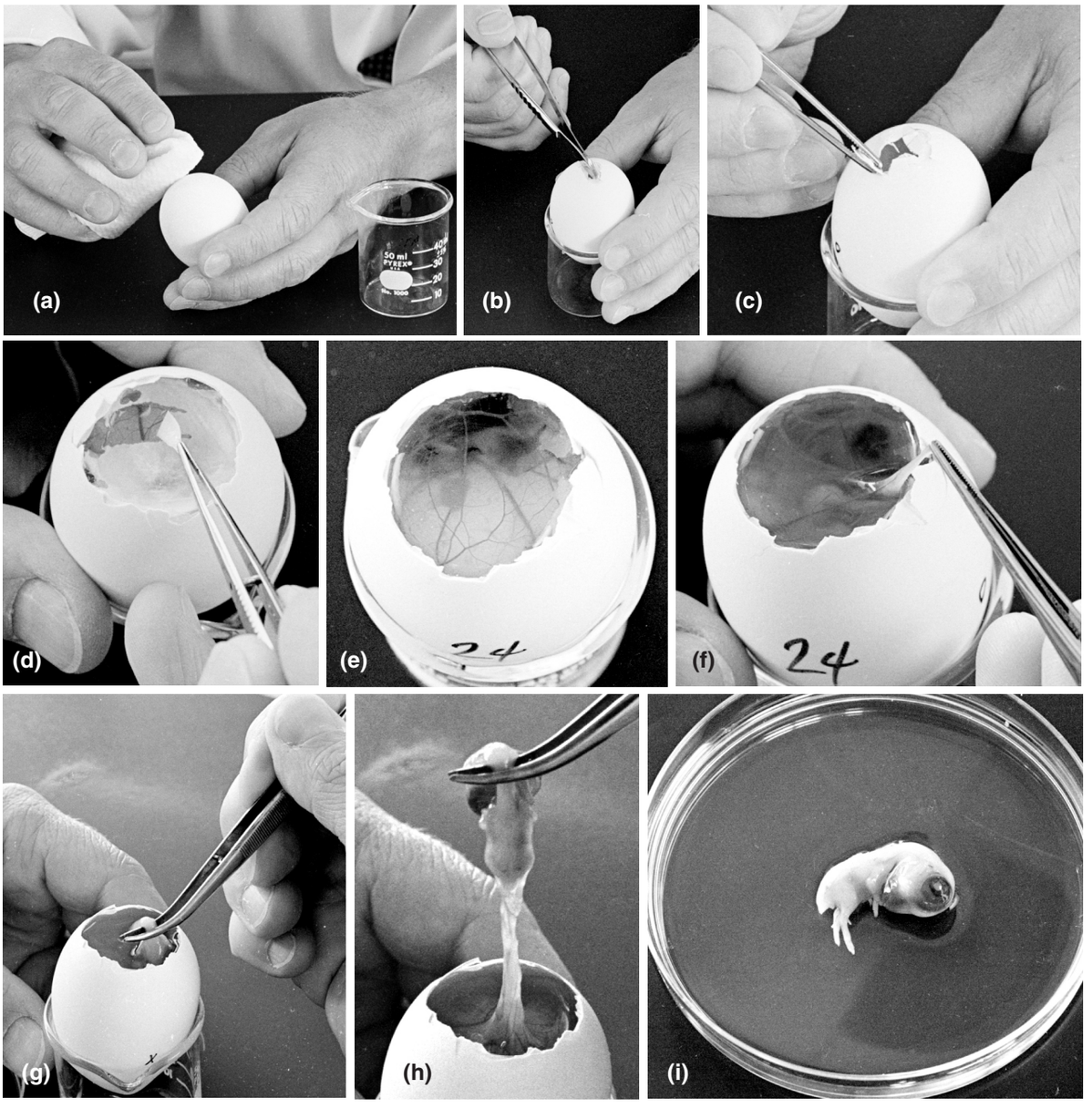

Fig. 12.4. Removing a Chick Embryo from an Egg. Stages in the extraction of the whole chick embryo from an egg. (a) Swabbing the egg with alcohol. (b) Cracking the shell. (c) Peeling off the shell. (d) Peeling off the shell membrane. (e) Chorioallantoic membrane (CAM) and vasculature revealed. (f) Removing 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.

2. Make arrangements to be alerted when the material is ready for collection.
3. Collect the containers after surgery, or have someone send them to you immediately after collection and inform you when they have been dispatched.
4. Transfer the sample to the tissue culture laboratory. The sample should be triple wrapped (e.g., in a sealed tube within a sealed plastic bag full of absorbent tissue, in case of leakage, within a padded envelope with your name, address, and telephone number on it; *see* Section 20.4).

Usually, if kept at 4°C, biopsy samples survive for at least 24 h and even up to 3 or 4 days, although the longer the time from surgery to culture, the more the samples are likely to deteriorate.

5. Log receipt of sample as a numbered entry in a hand-written record book for subsequent transfer to a computerized database, or key into database directly on receipt.
6. *Decontamination*. Although most surgical specimens are sterile when removed, problems may arise with subsequent handling. Superficial specimens (e.g., skin biopsies, melanomas, etc.) and gastrointestinal tract specimens are particularly prone to contamination even when a disinfectant wash is given before skin biopsy and a parenteral antibiotic is given before gastrointestinal surgery. It may be advantageous to consult a medical microbiologist to determine which flora to expect in a given tissue and then choose your antibiotics for collection and dissection accordingly. If the surgical sample is large enough (i.e., 200 mg or more), then a brief dip (i.e., 30 s–1 min) in 70% alcohol will help to reduce superficial contamination without harming the center of the tissue sample.

12.3 PRIMARY CULTURE

Several techniques have been devised for the disaggregation of tissue isolated for primary culture. These techniques can be divided into (1) purely mechanical techniques, involving dissection with or without some form of maceration, and (2) techniques utilizing enzymatic disaggregation (Fig. 12.5). Primary explants are suitable for very small amounts of tissue; enzymatic disaggregation gives a better yield when more tissue is available, and mechanical disaggregation works well with soft tissues, and some firmer tissues when the size of the viable yield is not important.

12.3.1 Primary Explant

The primary explant technique was the original method developed by Harrison [1907], Carrel [1912], and others for initiating a tissue culture. As originally performed, a fragment of tissue was embedded in blood plasma or lymph, mixed with heterologous serum and embryo extract, and placed on a coverslip that was inverted over a concavity slide. The clotted plasma held the tissue in place, and the explant could be examined with a conventional microscope. The heterologous serum induced clotting of the plasma, and the embryo extract and serum, together with the plasma, supplied nutrients and growth factors and stimulated cell migration from the explant. This technique is still used but has been largely replaced by the simplified method described in Protocol 12.4.

PROTOCOL 12.4. 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. 12.6a). Once this is achieved, outgrowth of cells usually follows (Fig. 12.6b; Plates 1a, 2b).

Materials

Sterile:

- ❑ Growth medium (e.g., 50:50 DMEM:F12 with 20% fetal bovine serum)
- ❑ 100 mL DBSS
- ❑ 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, 5–6 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.

Protocol

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.
3. Chop finely with crossed scalpels (*see* Fig. 12.6a, 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.
7. Transfer the pieces (remember to wet the pipette) to a culture flask, with about 20–30 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 18–24 h.

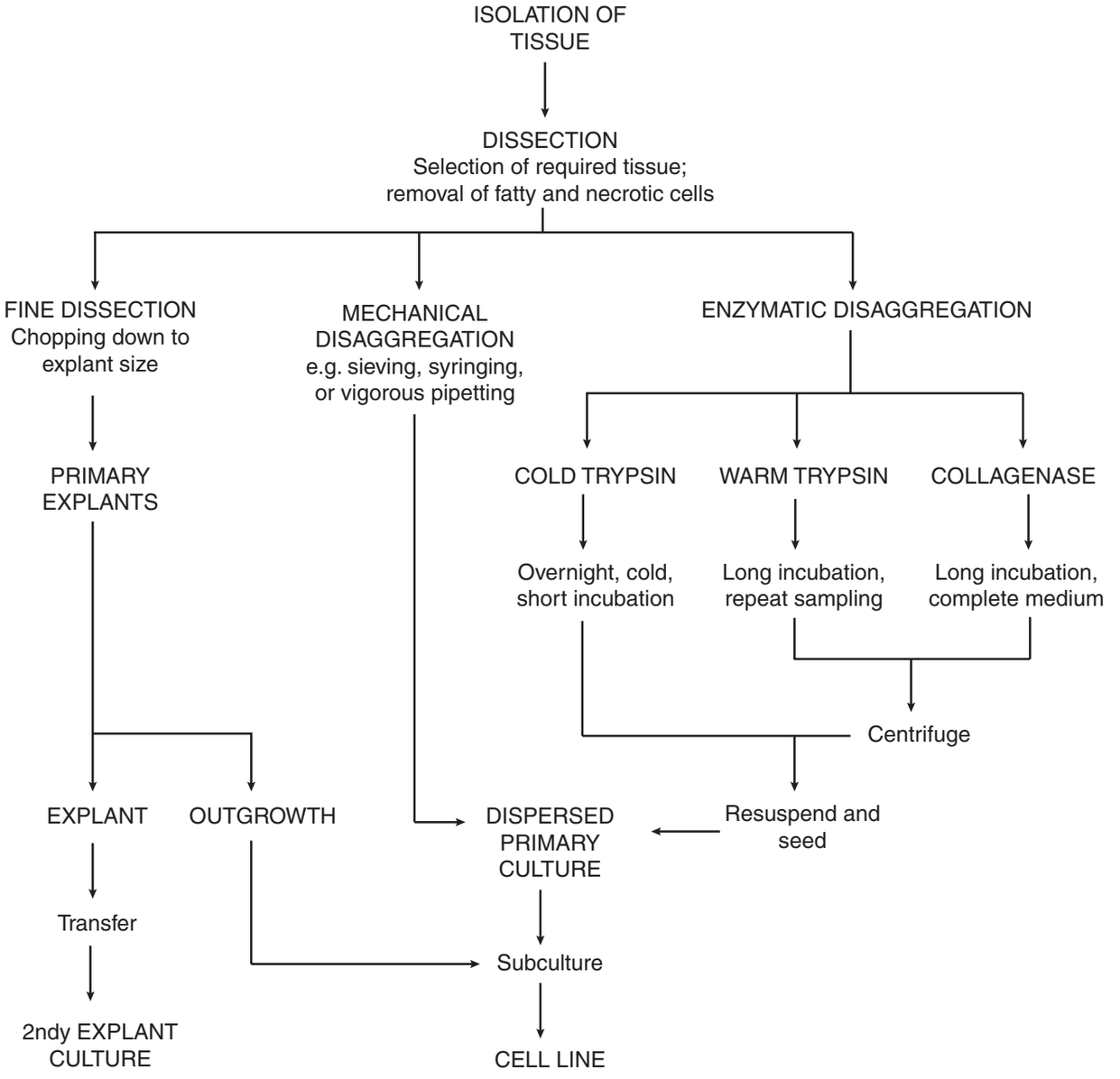

Fig. 12.5. Options for Primary Culture. Multiple paths to obtaining a cell line; center and left, by mechanical disaggregation, right, by enzymatic disaggregation. An explant may be transferred to allow further outgrowth to form, while the outgrowth from the explant may be subcultured to form a cell line.

10. If the pieces have adhered, then the medium volume may be made up gradually over the next 3–5 days to 5 mL per 25 cm^2 and then changed weekly until a substantial outgrowth of cells is observed (*see* Fig. 12.6b).

11. Once an outgrowth has formed, the remaining explant may be picked off with a scalpel (Fig. 12.6c) and transferred by prewetted pipette to a fresh culture vessel. (Then return to step 7.)

12. 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 13.2).

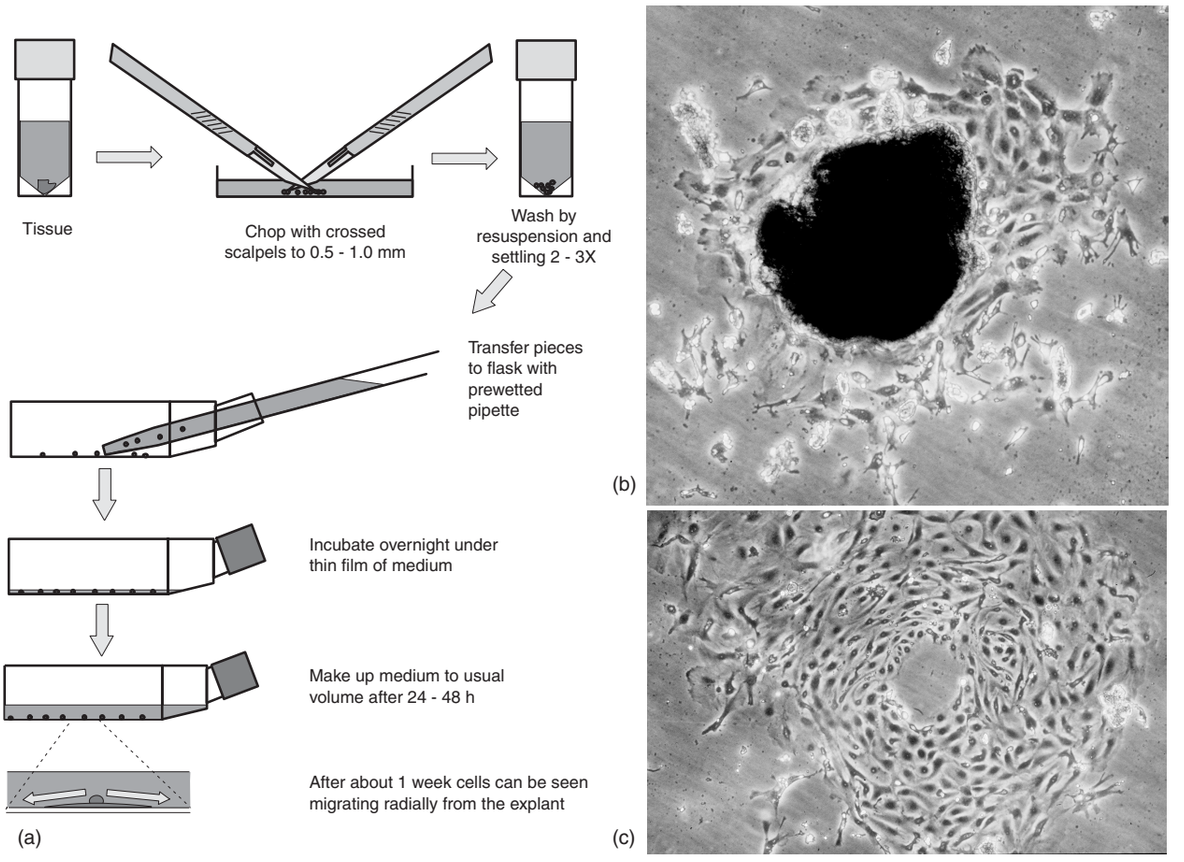

Fig. 12.6. Primary Explant Culture. (a) Schematic diagram of stages in dissection and seeding primary explants. (b) Primary explant culture from mouse squamous skin carcinoma; explant and early stage of outgrowth about 3 days after explantation (*see also* Plate 2b). (c) Outgrowth after removal of explant, about 7 days after explantation. 10× objective.

This technique is particularly useful for small amounts of tissue, such as skin biopsies, for which there is a risk of losing cells during mechanical or enzymatic disaggregation. Its disadvantages lie in the poor adhesiveness of some tissues and the selection of cells in the outgrowth. In practice, however, most cells, particularly embryonic, migrate out successfully.

Attaching explants. Both adherence and migration may be stimulated by placing a glass coverslip on top of the explant, with the explant near the edge of the coverslip, or the plastic dish may be scratched through the explant to attach the tissue to the flask [Elliget & Lechner, 1992] (*see* Protocol 23.9). Attachment may also be promoted by treating the plastic with polylysine or fibronectin (*see* Sections 8.4.11, 10.4.5, 14.2.1), extracellular matrix (*see* Protocol 8.1), or feeder layers (*see* Protocol 14.3). Historically, plasma clots have been used to promote attachment. Place a drop of plasma on the plastic surface, and embed the explant in it. This should induce the plasma to clot in a few minutes, whereupon medium can be added. Alternatively, purified fibrinogen and thrombin can be used [Nicosia & Ottinetti, 1990].

12.3.2 Enzymatic Disaggregation

Cell–cell adhesion in tissues is mediated by a variety of homotypic interacting glycopeptides (cell adhesion molecules, or CAMs) (*see* Section 3.2.1), some of which are calcium dependent (cadherins) and hence are sensitive to chelating agents such as EDTA or EGTA. Integrins, which bind to the arginine-glycine-aspartic acid (RGD) motif in extracellular matrix, also have Ca^{2+}-binding domains and are affected by Ca^{2+} depletion. Intercellular matrix and basement membranes contain other glycoproteins, such as fibronectin and laminin, which are protease sensitive, and proteoglycans, which are less so but can sometimes be degraded by glycanases, such as hyaluronidase or heparinase. The easiest approach is to proceed from a simple disaggregation solution to a more complex solution (*see* Table 13.4) with trypsin alone or trypsin/EDTA as a starting point, adding other proteases to improve disaggregation, and deleting trypsin if necessary to increase viability. In general, increasing the purity of an enzyme will give better control and less toxicity with increased specificity but may result in less disaggregation activity.

Mechanical and enzymatic disaggregation of the tissue avoids problems of selection by migration and yields a higher number of cells that are more representative of the whole tissue in a shorter time. However, just as the primary explant technique selects on the basis of cell migration, dissociation techniques will select protease- and mechanical stress-resistant cells.

Embryonic tissue disperses more readily and gives a higher yield of proliferating cells than newborn or adult tissue. The increasing difficulty in obtaining viable proliferating cells with increasing age is due to several factors, including the onset of differentiation, an increase in fibrous connective tissue and extracellular matrix, and a reduction of the undifferentiated proliferating cell pool. When procedures of greater severity are required to disaggregate the tissue (e.g., longer trypsinization or increased agitation), the more fragile components of the tissue may be destroyed. In fibrous tumors, for example, it is very difficult to obtain complete dissociation with trypsin while still retaining viable carcinoma cells.

The choice of which trypsin grade to use has always been difficult, as there are two opposing trends: (1) The purer the trypsin, the less toxic it becomes, and the more predictable its action; (2) the cruder the trypsin, the more effective it may be, because of other proteases. In practice, a preliminary test experiment may be necessary to determine the optimum grade for viable cell yield, as the balance between sensitivity to toxic effects and disaggregation ability may be difficult to predict.

Crude trypsin is by far the most common enzyme used in tissue disaggregation [Waymouth, 1974], as it is tolerated quite well by many cells and is effective for many tissues. Residual activity left after washing is neutralized by the serum of the culture medium, or by a trypsin inhibitor (e.g., soya bean trypsin inhibitor) when serum-free medium is used.

12.3.3 Warm Trypsin

It is important to minimize the exposure of cells to active trypsin in order to preserve maximum viability. Hence, when whole tissue is being trypsinized at 37°C, dissociated cells should be collected every half hour, and the trypsin should be removed by centrifugation and neutralized with serum in medium.

PROTOCOL 12.5. TISSUE DISAGGREGATION IN WARM TRYPSIN

Outline

The tissue is chopped and stirred in trypsin for a few hours. The dissociated cells are collected every half hour, centrifuged, and pooled in medium containing serum (Fig. 12.7).

Materials

Sterile or aseptically prepared:

- ❑ Tissue, 1–5 g
- ❑ DBSS, 50 mL (*see* Appendix I)
- ❑ Trypsin (crude), 2.5% in D-PBSA or normal saline
- ❑ D-PBSA, 200 mL
- ❑ Growth medium with serum (e.g., DMEM/F12 with 10% fetal bovine serum)
- ❑ Culture flasks, 5–10 flasks per g tissue (varies depending on cellularity of tissue)
- ❑ Petri dishes, 9 cm, non-tissue-culture grade
- ❑ Preweighed vials, 50-mL centrifuge tubes, or universal containers, 2
- ❑ Trypsinization flask: 250-mL Erlenmeyer flask (preferably indented as in Fig. 12.9) or stirrer flask (*see* Fig. 13.5)
- ❑ Magnetic follower, autoclaved in a test tube
- ❑ Curved forceps
- ❑ Pipettes (Pasteur, 2 mL, 10 mL)

Nonsterile:

- ❑ Magnetic stirrer
- ❑ Hemocytometer or cell counter

Protocol

1. Transfer the tissue to fresh, sterile DBSS in 9-cm Petri dish, 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.
3. Chop with crossed scalpels (*see* Fig. 12.7) into about 3-mm cubes.
4. Transfer the tissue with curved forceps to the preweighed vial or tube.
5. Allow the pieces to settle.
6. Wash the tissue by resuspending the pieces in DBSS, allowing the pieces to settle, and removing the supernatant fluid. Repeat this step two more times.
7. Drain the vial or tube and reweigh.
8. Transfer all the pieces to the empty trypsinization flask, flushing the vial or tube with DBSS.
9. Remove most of the residual fluid, and add 180 mL of D-PBSA.
10. Add 20 mL of 2.5% trypsin. (Other enzymes, e.g., collagenase, hyaluronidase, or DNase, may be added at this stage as well, if required.)
11. Add the magnetic follower to the flask.
12. Cap the flask, and place it on the magnetic stirrer in an incubator or hot room at 37°C.
13. Stir at about 100 rpm for 30 min at 37°C.
14. After 30 min, collect disaggregated cells as follows:
    (a) Allow the pieces to settle.

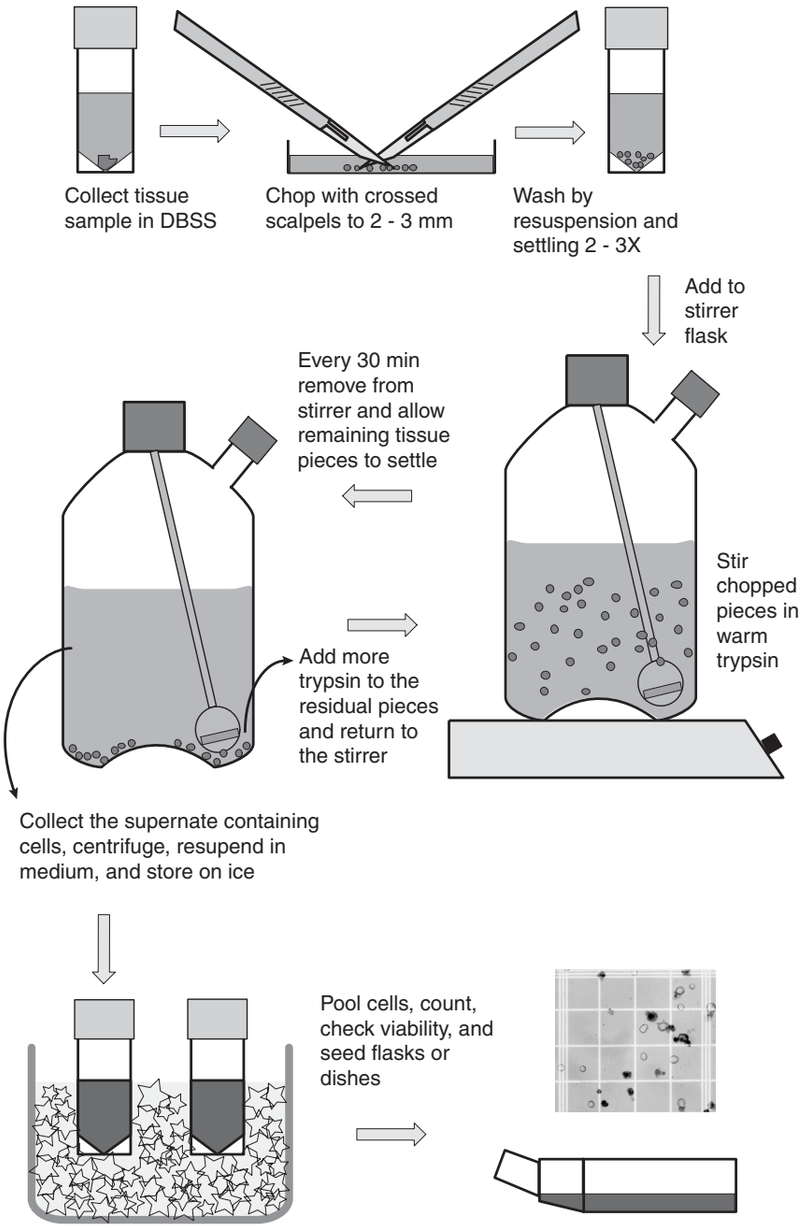

Fig. 12.7. Warm Trypsin Disaggregation.

(b) Pour off the supernatant into a centrifuge tube and place it on ice.

(c) Add fresh trypsin to the pieces remaining in the flask, and continue to stir and incubate for a further 30 min.

(d) Centrifuge the harvested cells from step 11(b) at approximately 500 *g* for 5 min.

(e) Resuspend the resulting pellet in 10 mL of medium with serum, and store the suspension on ice.

15. Repeat step 11 until complete disaggregation occurs or until no further disaggregation is apparent (usually 3–4 h).

16. Collect and pool chilled cell suspensions, count the cells by hemocytometer or electronic cell

counter (*see* Section 21.1), and check viability (*see* Section 22.3.1).

17. As the cell population will be very heterogeneous, electronic cell counting will require confirmation with a hemocytometer, because calibration can be difficult.
18. Remove any large remaining aggregates by filtering through sterile muslin or a proprietary sieve (*see*, e.g., Fig. 12.8).
19. Dilute the cell suspension to 1×10^6/mL in growth medium, and seed as many flasks as are required, with approximately 2×10^5 cells/cm^2. When the survival rate is unknown or unpredictable, a cell count is of little value (e.g., in tumor biopsies, in which the proportion of necrotic cells may be quite high). In this case, set up a range of concentrations from about 5 to 25 mg of tissue per mL.
20. Change the medium at regular intervals (2–4 days as dictated by depression of pH). Check the supernate for viable cells before discarding it, as some cells can be slow to attach or may even prefer to proliferate in suspension.

This technique is useful for the disaggregation of large amounts of tissue in a relatively short time, particularly for whole mouse embryos or chick embryos. It does not work as well with adult tissue, in which there is a lot of fibrous connective tissue, and mechanical agitation can be damaging to some of the more sensitive cell types, such as epithelium. If reaggregation is found after centrifugation and resuspension, incubate in DNase, 10–20 μg/mL, for 10–20 min, and recentrifuge.

12.3.4 Trypsinization with Cold Preexposure

One of the disadvantages of using trypsin to disaggregate tissue is the damage that may result from prolonged exposure to the tissue to trypsin at 37°C; hence the need to harvest cells after 30-min incubations in the warm trypsin method rather than have them exposed for the full time (i.e., 3–4 h) required to disaggregate the whole tissue. A simple method of minimizing damage to the cells during exposure is to soak the tissue in trypsin at 4°C for 6–18 h to allow penetration of the enzyme with little tryptic activity (Table 12.1). Following this procedure, the tissue will only require 20–30 min at 37°C for disaggregation [Cole & Paul, 1966].

Fig. 12.8. Cell Strainer. Disposable polypropylene filter and tube for straining aggregates from primary suspensions (BD Biosciences). Can also be used for disaggregating soft tissues (*see also* Fig. 12.13).

PROTOCOL 12.6. TISSUE DISAGGREGATION IN COLD TRYPSIN

Outline

Chop tissue and place in trypsin at 4°C for 6–18 h. Incubate after removing the trypsin, and disperse the cells in warm medium (Fig. 12.9).

Materials

Sterile or aseptically prepared:

- ❑ Tissue, 1–5 g, preweighed
- ❑ Growth medium (e.g., DMEM/F12 with 10% FBS)
- ❑ DBSS

TABLE 12.1. Cell Yield by Warm and Cold Trypsinization

Duration and temperature of trypsinization		After trypsinization			After 24 h in culture	
		Cells recovered per embryo $\times 10^{-7}$	% Viability by dye exclusion (Trypan blue)	Total no. of viable cells $\times 10^{-7}$	Recovered, % of total seeded	Viability, % of viable cells seeded
4°C	37°C					
0 h	4 h	1.69	86	1.45	47.2	54.9
5.5 h	0.5 h	3.32	60	1.99	74.5	124
24 h	0.5 h	3.40	75	2.55	60.3	80.2

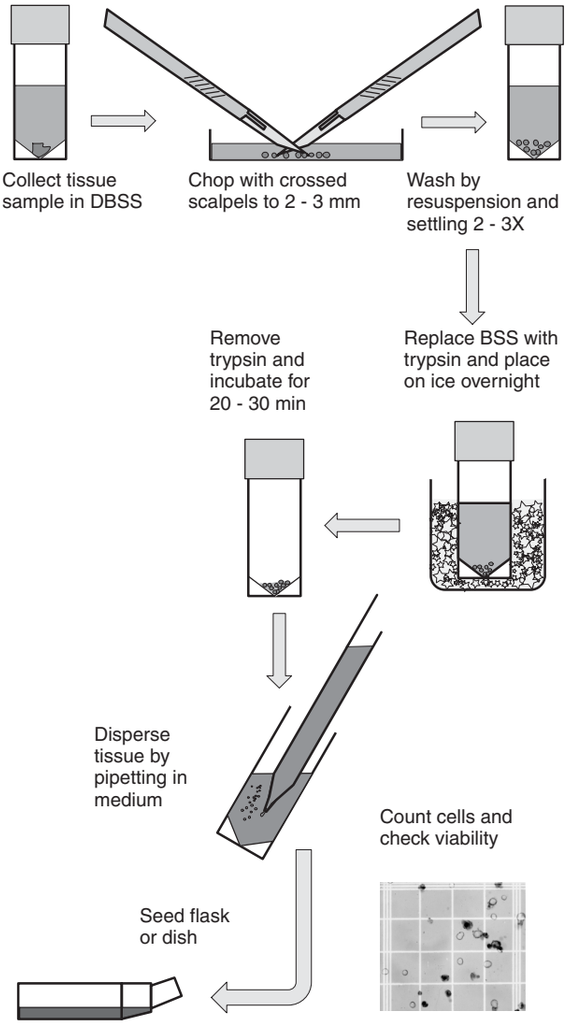

Fig. 12.9. Cold Trypsin Disaggregation. *See also* Plate 2a, d, e and Plate 3.

- ❑ 0.25% crude trypsin in serum-free RPMI 1640 or MEM/Stirrer Salts (S-MEM)
- ❑ Petri dishes, 9 cm, non-tissue-culture grade
- ❑ Forceps, straight and curved
- ❑ Scalpels
- ❑ Erlenmeyer flask, 25 or 50 mL, screw capped, preweighed (or glass vial or universal container)
- ❑ Culture flasks, 25 or 75 cm^2
- ❑ Pipettes (Pasteur, 2 mL, 10 mL)

Nonsterile:

- ❑ Ice bath

Protocol

1. Transfer the tissue to fresh, sterile DBSS in a 9-cm Petri dish, and rinse.
2. Transfer the tissue to a second dish and dissect off unwanted tissue, such as fat or necrotic material.
3. Transfer to a third dish and chop with crossed scalpels (*see* Fig. 12.9) into about 3-mm cubes. Embryonic organs, if they do not exceed this size, are better left whole.
4. Transfer the tissue with curved forceps to a 15- or 50-mL preweighed sterile vial.
5. Allow the pieces to settle.
6. Wash the tissue by resuspending the pieces in DBSS, allowing the pieces to settle, and removing the supernatant fluid. Repeat this step two more times.
7. Carefully remove the residual fluid and reweigh the vial.
8. Add 10 mL/g of tissue of 0.25% trypsin in RPMI 1640 or S-MEM at 4°C.
9. Place the mixture at 4°C for 6–18 h.
10. Remove and discard the trypsin carefully, leaving the tissue with only the residual trypsin. (Other enzymes, e.g., collagenase, hyaluronidase, or DNase, may be added in 1- to 2-mL amounts at this stage, if required.)
11. Place the tube at 37°C for 20–30 min.
12. Add warm medium, approximately 1 mL for every 100 mg of original tissue, and gently pipette the mixture up and down until the tissue is completely dispersed.
13. If some tissue does not disperse, then the cell suspension may be filtered through sterile muslin or stainless steel mesh (100–200 μm), or a disposable plastic mesh strainer (Fig. 12.10), or the larger pieces may simply be allowed to settle. When there is a lot of tissue, increasing the volume of suspending medium to 20 mL for each gram of tissue will facilitate settling and subsequent collection of supernatant fluid. Two to three minutes should be sufficient to get rid of most of the larger pieces.
14. Determine the cell concentration in the suspension by hemocytometer or electronic cell counter (*see* Section 21.1), and check viability (*see* Protocol 22.1).
15. The cell population will be very heterogeneous; electronic cell counting will initially require confirmation with a hemocytometer, as calibration can be difficult.
16. Dilute the cell suspension to 1×10^6/mL in growth medium, and seed as many flasks as are required, with approximately 2×10^5 cells/cm^2. When the survival rate is unknown or unpredictable, a cell count is of little value (e.g., in tumor biopsies, for which the proportion of necrotic cells may be quite high). In this case,

set up a range of concentrations from about 5 to 25 mg of tissue per mL.

17. Change the medium at regular intervals (2–4 days as dictated by depression of pH). Check the supernate for viable cells before discarding it, as some cells can be slow to attach or may even prefer to proliferate in suspension.

The cold trypsin method usually gives a higher yield of viable cells, with improved survival after 24 h culture (*see* Figs. 12.1, 12.10 and Table 12.1), and preserves more different cell types than the warm method (*see* Plates 2d,e and 3). Cultures from mouse embryos contain more epithelial cells when prepared by the cold method, and erythroid cultures from 13-day fetal mouse liver respond to erythropoietin after this treatment, but not after the warm trypsin method or mechanical disaggregation [Cole & Paul, 1966; Conkie, personal communication]. The cold trypsin method is also convenient, as no stirring or centrifugation is required and the incubation at 4°C may be done overnight.

12.3.5 Chick Embryo Organ Rudiments

The cold trypsin method is particularly suitable for small amounts of tissue, such as embryonic organs. Protocol 12.7 gives good reproducible cultures from 10- to 13-day chick embryos with evidence of several different cell types characteristic of the tissue of origin. This protocol forms a good exercise for teaching purposes.

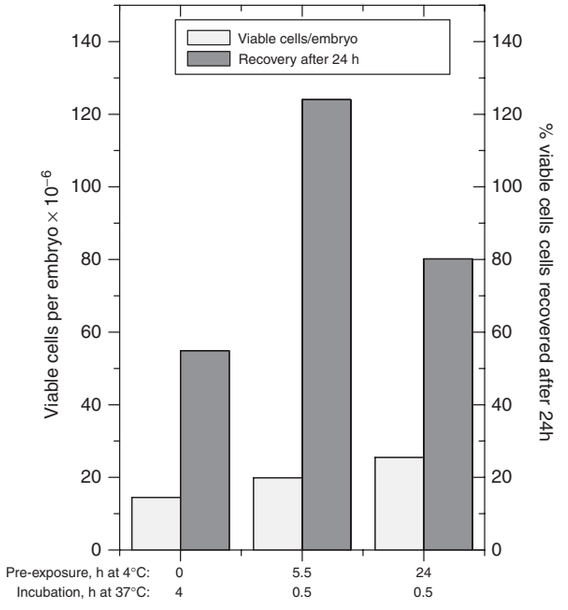

Fig. 12.10. Warm and Cold Trypsinization. Yield of viable cells per embryo by warm and cold trypsinization methods. Yield of viable cells per embryo increases by cold trypsinization up to 24 h at 4°C, but recovery after 24 h culture is greatest with shorter cold trypsinization (>100%, implying cell proliferation), perhaps because some of the cells released by longer cold trypsinization are not proliferative.

PROTOCOL 12.7. CHICK EMBRYO ORGAN RUDIMENTS

Outline

Dissect out individual organs or tissues, and place them, preferably whole, in cold trypsin overnight. Remove the trypsin, incubate the organs or tissue briefly, and disperse them in culture medium. Dilute and seed the cultures.

Materials

Sterile:

- ❑ DBSS
- ❑ Crude trypsin (Difco 1:250 or equivalent) 0.25% in RPMI 1640 or S-MEM on ice; lower concentrations may be used with purer grades of trypsin, e.g., 0.05–0.1% Sigma crystalline or Worthington Grade IV
- ❑ Culture medium (e.g., DMEM/F12 with 10% FBS), minimum of 12 mL per tissue
- ❑ Petri dishes, 9 cm, non-tissue-culture grade
- ❑ Culture flasks, 25 cm^2 (2 per tissue)
- ❑ Scalpels (No. 11 blade for most steps)
- ❑ Iridectomy knives for fine dissection
- ❑ Curved and straight fine forceps
- ❑ Pipettes (Pasteur, 2 mL, 10 mL)
- ❑ Test tubes, preferably glass, 10–15 mL, with screw caps

Nonsterile:

- ❑ Embryonated hen's eggs, 10- to 13-days incubation (>10 days requires license in the United Kingdom)
- ❑ Ice bath
- ❑ Binocular dissecting microscope

Protocol

1. Remove the embryo from the egg as described previously (*see* Protocol 12.2), and place it in sterile DBSS.
2. Remove the head (Fig. 12.11a,b).
3. Remove an eye and open it carefully, releasing the lens and aqueous and vitreous humors (Fig. 12.11c,d).
4. Grasp the retina in two pairs of fine forceps and gently peel the pigmented retina off the neural retina and connective tissue (Fig. 12.11e). (This step requires a dissection microscope for 10-day embryos. A brief exposure to 0.25% trypsin in

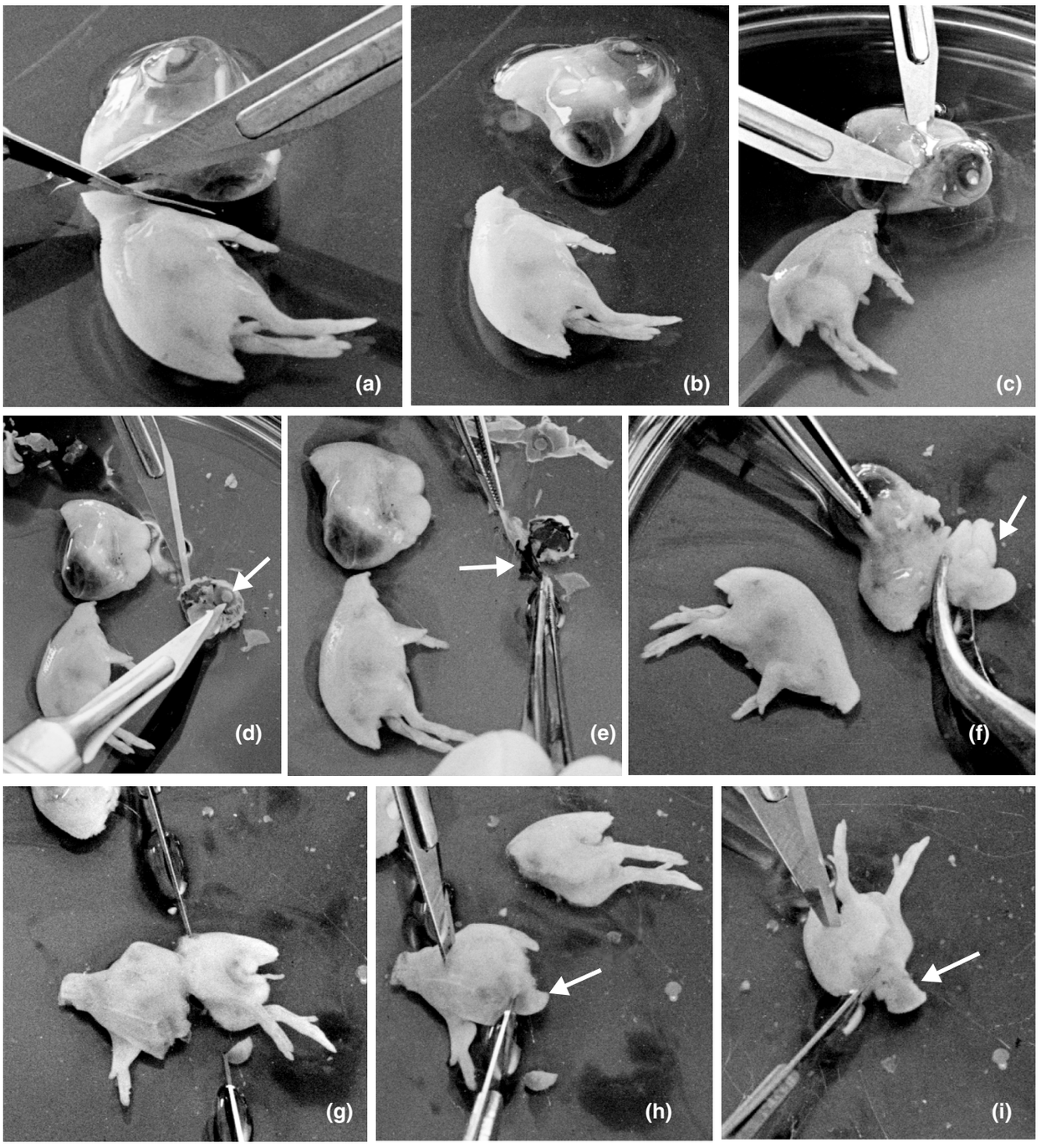

Fig. 12.11. Dissection of a Chick Embryo. (a), (b) Removing the head. (c) Removing the eye. (d) Dissecting out the lens. (e) Peeling off the retina. (f) Scooping out the brain. (g) Halving the trunk. (h) Teasing out the heart and lungs from the anterior half. (i) Teasing out the liver and gut from the posterior half. (j) Inserting the tip of the scalpel between the left kidney and the dorsal body wall. (k) Squeezing out the spinal cord. (l) Peeling the skin off the back of the trunk and hind leg. (m) Slicing muscle from the thigh. (n) Organ rudiments arranged around the periphery of the dish. From the right, clockwise, we have the following organs: brain, heart, lungs, liver, gizzard, kidneys, spinal cord, skin, and muscle.

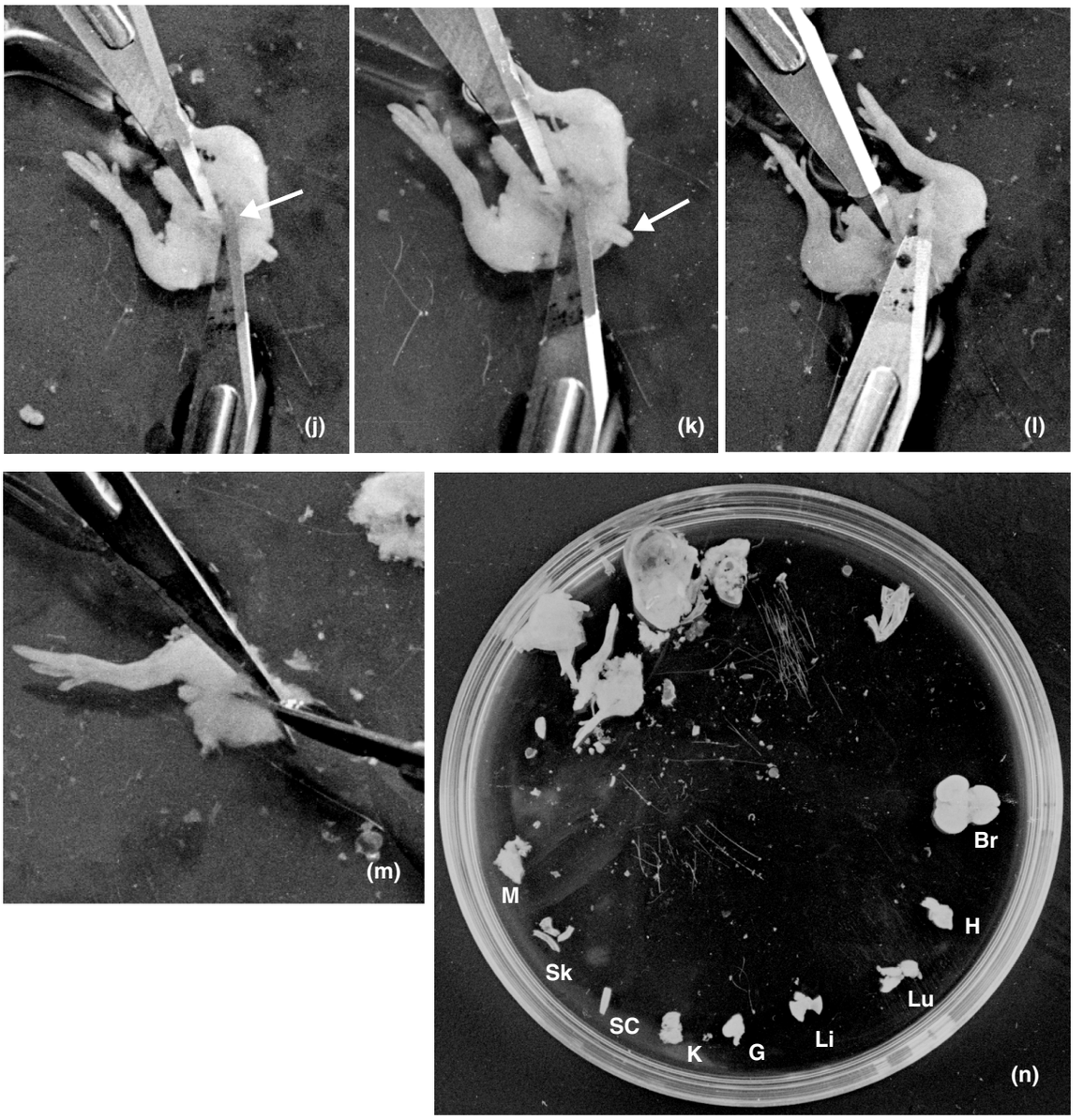

Fig. 12.11. (*Continued*)

1 mM EDTA will allow the two tissues to separate more easily.) Put the tissue to one side.

5. Pierce the top of the head with curved forceps, and scoop out the brain (Fig. 12.11f). Place the brain with the retina at the side of the dish.
6. Halve the trunk transversely where the pink color of the liver shows through the ventral skin (Fig. 12.11g). If the incision is made on the line of the diaphragm, then it will pass between the heart and the liver; but sometimes the liver will go to the anterior instead of the posterior half.
7. Gently probe into the cut surface of the anterior half, and draw out the heart and lungs (Fig. 12.11h; tease the organs out, and do not cut until you have identified them). Separate the heart and lungs and place at the side of the dish.
8. Probe the posterior half, and draw out the liver, with the folds of the gut enclosed in between the lobes (Fig. 12.11i). Separate the liver from the gut and place each at the side of the dish.
9. Fold back the body wall to expose the inside of the dorsal surface of the body cavity in the

posterior half. The elongated lobulated kidneys should be visible parallel to and on either side of the midline.

10. Gently slide the tip of the scalpel under each kidney and tease the kidneys away from the dorsal body wall (Fig. 12.11j). (This step requires a dissection microscope for 10-day embryos.) Carefully cut the kidneys free, and place them on one side.
11. Place the tips of the scalpels together on the midline at the posterior end, and, advancing the tips forward, one over the other, express the spinal cord as you would express toothpaste from a tube (Fig. 12.11k). (This step may be difficult with 10-day embryos.)
12. Turn the posterior trunk of the embryo over, and strip the skin off the back and upper part of the legs (Fig. 12.11l). Collect and place this skin on one side.
13. Dissect off muscle from each thigh, and collect this muscle together (Fig. 12.11m).
14. Transfer all of these tissues, and any others you may want, to separate test tubes containing 1 mL of 0.25% trypsin, and place these tubes on ice. Make sure that the tissue slides right down the tube into the trypsin.
15. Leave the test tubes for 6–18 h at 4°C.
16. Carefully remove the trypsin from the test tubes without disturbing the tissue; tilting and rolling the tube slowly will help.
17. Incubate the tissue in the residual trypsin for 15–20 min at 37°C.
18. Add 4 mL of medium to each of two 25-cm^2 flasks for each tissue to be cultured.
19. Add 2 mL of medium to tubes containing tissues and residual trypsin, and pipette up and down gently to disperse the tissue.
20. Allow any large pieces of tissue to settle.
21. Pipette off the supernatant fluid into the first flask, mix, and transfer 1 mL of diluted suspension to the second flask. This procedure gives two flasks at different cell concentrations and avoids the need to count the cells. Experience will determine the appropriate cell concentration to use in subsequent attempts.
22. Change the medium as required (e.g., for brain, it may need to be changed after 24 h, but pigmented retina will probably last 5–7 days), and check for characteristic morphology and function.

Analysis. After 3–5 days, contracting cells may be seen in the heart cultures, colonies of pigmented cells in the pigmented retina culture, and the beginning of myotubes in skeletal muscle cultures. Culture may be fixed and stained (*see* Protocol 16.2) for future examination.

12.3.6 Other Enzymatic Procedures

Disaggregation in trypsin can be damaging (e.g., to some epithelial cells) or ineffective (e.g., for very fibrous tissue, such as fibrous connective tissue), so attempts have been made to utilize other enzymes. Because the extracellular matrix often contains collagen, particularly in connective tissue and muscle, collagenase has been the obvious choice [Freshney, 1972 (colon carcinoma); Speirs et al., 1996 (breast carcinoma); Chen et al., 1989 (kidney); Booth & O'Shea, 2002 (gut); Heald et al., 1991 (pancreatic islet cells)] (*see* Sections 12.3.6, 23.2.6–23.2.8). Other bacterial proteases, such as pronase [Schaffer et al., 1997; Glavin et al., 1996] and dispase (Boehringer-Mannheim) [Compton et al., 1998; Inamatsu et al., 1998], have also been used with varying degrees of success. The participation of carbohydrate in intracellular adhesion has led to the use of hyaluronidase [Berry & Friend, 1969] and neuraminidase in conjunction with collagenase. Other proteases continue to appear on the market (*see* Section 12.1). With the selection now available, screening available samples is the only option if trypsin, collagenase, dispase, pronase, hyaluronidase, and DNase, alone and in combinations, do not prove to be successful.

12.3.7 Collagenase

This technique is very simple and effective for many tissues: embryonic, adult, normal, and malignant. It is of greatest benefit when the tissue is either too fibrous or too sensitive to allow the successful use of trypsin. Crude collagenase is often used and may depend, for some of its action, on contamination with other nonspecific proteases. More highly purified grades are available if nonspecific proteolytic activity is undesirable, but they may not be as effective as crude collagenase.

PROTOCOL 12.8. TISSUE DISAGGREGATION IN COLLAGENASE

Outline

Place finely chopped tissue in complete medium containing collagenase and incubate. When tissue is disaggregated, remove collagenase by centrifugation, seed cells at a high concentration, and culture (Fig. 12.12).

Materials

Sterile:

- ❑ Collagenase (2000 units/mL), Worthington CLS or Sigma 1A

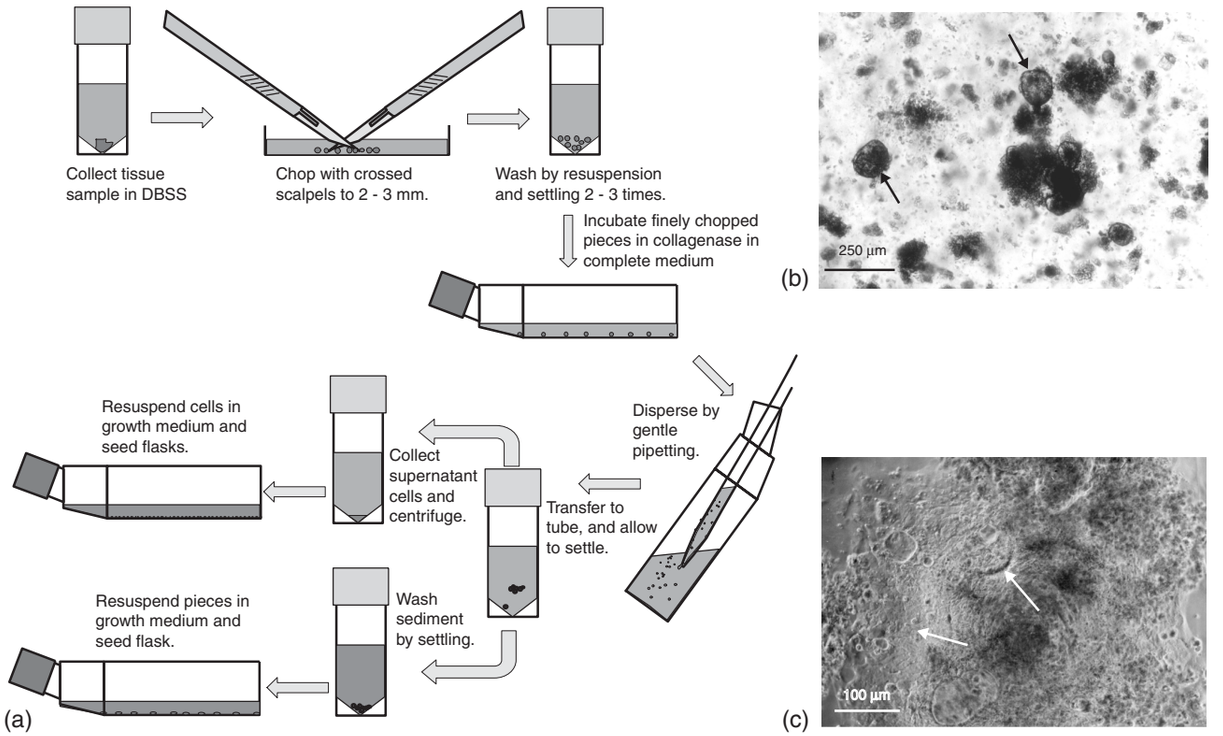

Fig. 12.12. Tissue Disaggregation by Collagenase. (a) Schematic diagram of dissection followed by disaggregation in collagenase. (b) Cell clusters from human colonic carcinoma after 48 h dissociation in crude collagenase (Worthington CLS grade); before removal of collagenase. (c) As (b), but after removal of collagenase, further disaggregation by pipetting, and culture for 48 h. The clearly defined rounded clusters (black arrows) in (b) form epithelium-like sheets (white arrows) in (c), some still three-dimensional, some spreading as a sheet, and the more irregularly shaped clusters produce fibroblasts. (*See also* Plate 2b).

- ❑ Culture medium, e.g., DMEM/F12 with 10% FBS
- ❑ DBSS
- ❑ Pipettes, 1 mL, 10 mL
- ❑ Petri dishes, 9 cm, non-tissue-culture grade
- ❑ Culture flasks, 25 cm^2
- ❑ Centrifuge tubes or universal containers, 15–50 mL, depending on the amount of tissue being processed
- ❑ Scalpels

Nonsterile:

- ❑ Centrifuge

Protocol

1. Transfer the tissue to fresh, sterile DBSS, and rinse.
2. Transfer the tissue to a second dish and dissect off unwanted tissue, such as fat or necrotic material.
3. Transfer to a third dish and chop finely with crossed scalpels (*see* Fig. 12.12) into about 1-mm cubes.
4. Transfer the tissue 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 DBSS, or else the pieces will stick.)
5. Allow the pieces to settle.
6. Wash the tissue by resuspending the pieces in DBSS, allowing the pieces to settle, and removing the supernatant fluid. Repeat this step two more times.
7. Transfer 20–30 pieces to one 25-cm^2 flask and 100–200 pieces to a second flask.
8. Drain off the DBSS, and add 4.5 mL of growth medium with serum to each flask.
9. Add 0.5 mL of crude collagenase, 2000 units/mL, to give a final concentration of 200 units/mL collagenase.
10. Incubate at 37°C for 4–48 h without agitation. Tumor tissue may be left up to 5 days or more if disaggregation is slow (e.g., in scirrhous carcinomas of the breast or the colon), although it may be necessary to centrifuge the tissue and resuspend it in fresh medium and collagenase

before that amount of time has passed if an excessive drop in pH is observed (i.e., <pH 6.5).

11. Check for effective disaggregation by gently moving the flask; the pieces of tissue will ''smear'' on the bottom of the flask and, with gentle pipetting, will break up into single cells and small clusters (Fig. 12.13).
12. With some tissues (e.g., lung, kidney, and colon or breast carcinoma), small clusters of epithelial cells can be seen to resist the collagenase and may be separated from the rest by allowing them to settle for about 2 min. If these clusters are further washed with DBSS by resuspension and settling and the sediment is resuspended in medium and seeded, then they will form islands of epithelial cells. Epithelial cells generally survive better if they are not completely dissociated.
13. When complete disaggregation has occurred, or when the supernatant cells are collected after removing clusters by settling, centrifuge the cell suspension from the disaggregate and any washings at 50–100 *g* for 3 min.
14. Discard the supernatant DBSS or medium, resuspend and combine the pellets in 5 mL of medium, and seed in a 25-cm^2 flask. If the pH fell during collagenase treatment (to pH 6.5 or less by 48 h), then dilute the suspension two- to threefold in medium after removing the collagenase.
15. Replace the medium after 48 h.

Some cells, particularly macrophages, may adhere to the first flask during the collagenase incubation. Transferring the cells to a fresh flask after collagenase treatment (and subsequent removal of the collagenase) removes many of the macrophages from the culture. The first flask may be cultured as well, if required. Light trypsinization will remove any adherent cells other than macrophages.

Disaggregation in collagenase has proved particularly suitable for the culture of human tumors [Pfragner & Freshney, 2004], mouse kidney, human adult and fetal brain, liver (*see* Protocol 23.6), lung, and many other tissues, particularly epithelium [Freshney & Freshney, 2002]. The process is gentle and requires no mechanical agitation or special equipment. With more than 1 g of tissue, however, it becomes tedious at the dissection stage and can be expensive, because of the amount of collagenase required. It will also release most of the connective tissue cells, accentuating the problem of fibroblastic outgrowth, so it may need to be followed by selective culture (*see* Section 10.2.1) or cell separation (*see* Chapter 15).

The discrete clusters of epithelial cells produced by disaggregation in collagenase (*see* Step 9 of Protocol 12.8) and

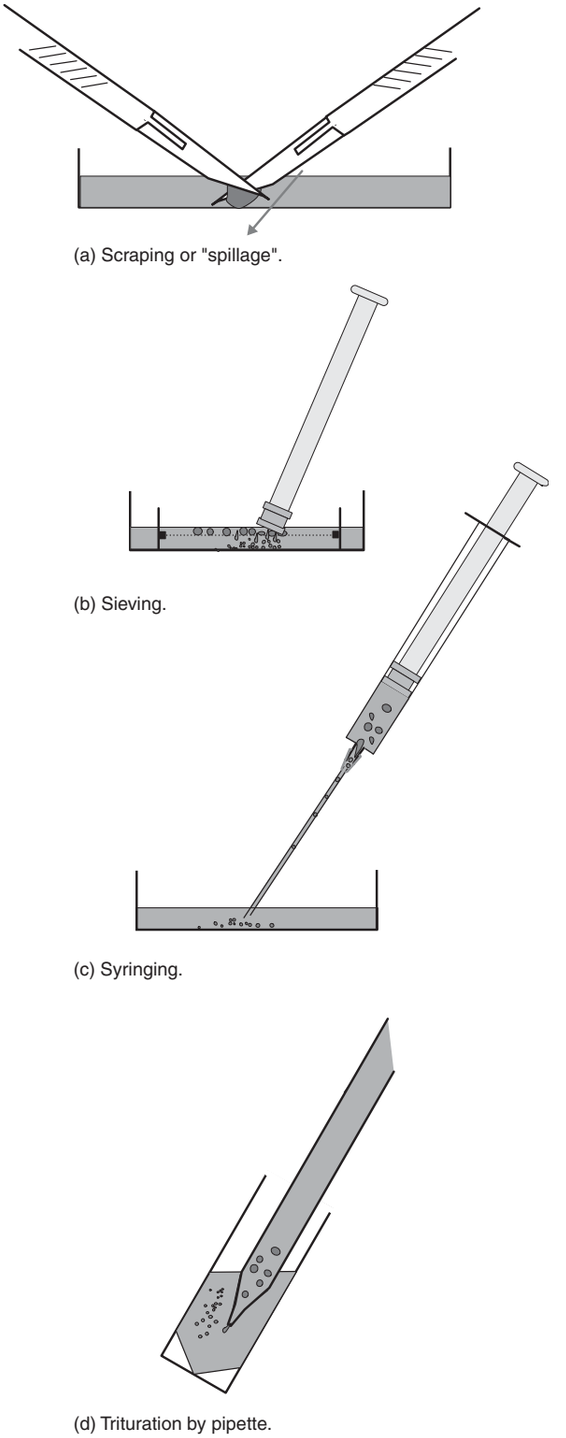

Fig. 12.13. Mechanical Disaggregation. (a) Scraping or ''spillage''. Cutting action, or abrasion of cut surface, releases cells. (b) Sieving. Forcing tissue through sieve with syringe piston. (Falcon Cell Strainer can be used; *see* Fig. 12.8.) (c) Syringing. Drawing tissue into syringe through wide bore needle or canula and expressing. (d) Trituration by pipette. Pippetting tissue fragments up and down through wide bore pipette.

by the cold trypsin method (*see* Plates 2, 3) can be selected under a dissection microscope and transferred to individual wells in a microtitration plate, alone or with irradiated or mitomycin C-treated feeder cells (*see* Sections 14.2.3, 23.1.1, 23.1.4).

12.3.8 Mechanical Disaggregation

The outgrowth of cells from primary explants is a relatively slow process and can be highly selective. Enzymatic digestion is rather more labor intensive, although, potentially, it gives a culture that is more representative of the tissue. As there is a risk of proteolytic damage to cells during enzymatic digestion, many people have chosen to use the alternative of mechanical disaggregation, e.g., collecting the cells that spill out when the tissue is carefully sliced [Lasfargues, 1973], pressing the dissected tissue through a series of sieves for which the mesh is gradually reduced in size, or, alternatively forcing the tissue fragments through a syringe (with or without a wide-gauge needle) [Zaroff et al., 1961] or simply pipetting it repeatedly (*see* Fig. 12.13). This procedure gives a cell suspension more quickly than does enzymatic digestion but may cause mechanical damage. Scraping (''spillage'') (Fig. 12.13a) and sieving (Fig. 12.13b) are probably the gentlest mechanical methods while pipetting (Fig. 12.13d) and, particularly, syringing (Fig. 12.13c), are most likely to generate shear. Protocol 12.9 is one method of mechanical disaggregation that has been found to be moderately successful with soft tissues, such as brain.

PROTOCOL 12.9. MECHANICAL DISAGGREGATION BY SIEVING

Outline

The tissue in culture medium is forced through a series of sieves for which the mesh is gradually reduced in size until a reasonable suspension of single cells and small aggregates is obtained. The suspension is then diluted and cultured directly.

Materials

Sterile:

- ❑ Growth medium, e.g., DMEM/F12 with 10% FBS
- ❑ Forceps
- ❑ Sieve (Fig. 12.13b), or graded series of sieves from 100 μm down to 20 μm, or Falcon ''Cell Strainer'' (*see* Fig. 12.8)
- ❑ Petri dishes, 9 cm
- ❑ Scalpels
- ❑ Disposable plastic syringes (2 mL or 5 mL)
- ❑ Culture flasks

Protocol

1. After washing and preliminary dissection of the tissue (*see* Steps 1 and 2 of Protocol 12.5), chop the tissue into pieces about 3–5 mm across, and place a few pieces at a time into a stainless steel or polypropylene sieve of 1-mm mesh in a 9-cm Petri dish (*see* Fig. 12.13b) or centrifuge tube (*see* Fig. 12.8).
2. Force the tissue through the mesh into medium by applying gentle pressure with the piston of a disposable plastic syringe. Pipette more medium through the sieve to wash the cells through it.
3. Pipette the partially disaggregated tissue from the Petri dish into a sieve of finer porosity, perhaps 100-μm mesh, and repeat Step 2.
4. The suspension may be diluted and cultured at this stage, or it may be sieved further through 20-μm mesh if it is important to produce a single-cell suspension. In general, the more highly dispersed the cell suspension, the higher the sheer stress required and the lower the resulting viability.
5. Seed the culture flasks at 2×10^5, 1×10^6, and 2×10^6 cells/mL by diluting the cell suspension in medium.

Only soft tissues, such as spleen, embryonic liver, embryonic and adult brain, and some human and animal soft tumors, respond well to this technique. Even with brain, for which fairly complete disaggregation can be obtained easily, the viability of the resulting suspension is lower than that achieved with enzymatic digestion, although the time taken may be very much less. When the availability of tissue is not a limitation and the efficiency of the yield is unimportant, it may be possible to produce, in a shorter amount of time, as many viable cells with mechanical disaggregation as with enzymatic digestion, but at the expense of very much more tissue.

12.3.9 Separation of Viable and Nonviable Cells

When an adherent primary culture is prepared from dissociated cells, nonviable cells are removed at the first change of medium. With primary cultures maintained in suspension, nonviable cells are gradually diluted out when cell proliferation starts. If necessary, however, nonviable cells may be removed from the primary disaggregate by centrifuging the cells on a mixture of Ficoll and sodium metrizoate (e.g., Hypaque or Triosil) [Vries et al., 1973]. This technique is similar to the preparation of lymphocytes from peripheral blood (*see* Protocol 27.1). The viable cells collect at the interface between the medium and the Ficoll/metrizoate, and the dead cells form a pellet at the bottom of the tube.

TABLE 12.2. Data Record for Primary Culture

Date . . . . . Time . . . . . Operator . . . . . .

		Record
Origin of tissue	Species	
	Race or strain	
	Age	
	Sex	
	Path. no. or animal tag no.	
	Tissue	
	Site	
	Stored tissue/DNA location	
Pathology		
Disaggregation agent	Trypsin, collagenase, etc.	
	Concentration	
	Duration	
	Diluent	
Cell count	Concentration after resuspension (C_I)	
	Volume (V_I)	
	Yield ($Y = C_I \times V_I$)	
	Yield per g (wet weight of tissue)	
Seeding	Number (N) and type of vessel (flask, dish, or plate wells)	
	Final concentration (C_F)	
	Volume per flask, dish, or well (V_F)	
Medium	Type	
	Batch no.	
	Serum type and concentration	
	Batch no.	
	Other additives	
	CO_2 concentration	
Matrix coating	e.g., fibronectin, Matrigel, collagen	
Subculture	Recovery at 1st subculture, cell/flask	
	% (cells recovered ÷ cells seeded)	
	Cell line designation	

PROTOCOL 12.10. ENRICHMENT OF VIABLE CELLS

Outline

Up to 2×10^7 cells in 9 mL of medium may be layered on top of 6 mL of Ficoll-Hypaque in a 25-mL screw-capped centrifuge bottle. The mixture is then centrifuged, and viable cells are collected from the interface.

Materials

Sterile:

- ❑ Cell suspension with as few aggregates as possible
- ❑ Clear centrifuge tubes or universal containers
- ❑ D-PBSA
- ❑ Ficoll Hypaque or equivalent (*see* Appendix II) (Ficoll/metrizoate, adjusted to 1.077 g/cc)
- ❑ Growth medium
- ❑ Syringe with blunt cannula or square-cut needle, Pasteur pipettes, or pipettor

Nonsterile:

- ❑ Hemocytometer or cell counter
- ❑ Centrifuge

Protocol

1. Allow major aggregates in the cell suspension to settle.
2. Layer 9 mL of the cell suspension onto 6 mL of the Ficoll-paque mixture. This step should be done in a wide, transparent centrifuge tube with a cap, such as the 25-mL Sterilin or Nunclon universal container, or in the clear plastic Corning 50-mL tube, using double the aforementioned volumes.
3. Centrifuge the mixture for 15 min at 400 *g* (measured at the center of the interface).
4. Carefully remove the top layer without disturbing the interface.
5. Collect the interface carefully with a syringe, Pasteur pipette, or pipettor.

Δ ***Safety Note.*** If you are using human or other primate material, do not use a sharp needle or glass Pasteur pipette.

6. Dilute the mixture to 20 mL in medium (e.g., DMEM/F12/10FB).
7. Centrifuge the mixture at 70 *g* for 10 min.
8. Discard the supernatant fluid, and resuspend the pellet in 5 mL of growth medium.
9. Repeat Steps 7 and 8 in order to wash cells free of density medium.
10. Count the cells with a hemocytometer or an electronic counter.
11. Seed the culture flask(s).

This procedure can be scaled up or down and works with lower ratios of density medium to cell suspension (e.g., 5 mL of cell suspension over 1 mL of density medium).

12.3.10 Primary Culture in Summary

The disaggregation of tissue and preparation of the primary culture make up the first, and perhaps most vital, stage in the culture of cells with specific functions. If the required cells are lost at this stage, then the loss is irrevocable. Many different cell types may be cultured by choosing the correct techniques (*see* Section 10.2.1 and Chapter 23). In general, trypsin is more severe than collagenase, but is sometimes more effective in creating a single-cell suspension. Collagenase does not dissociate epithelial cells readily, but this characteristic can be an advantage for separating the epithelial cells from stromal cells. Mechanical disaggregation is much quicker than the procedure using collagenase, but damages more cells. The best approach is to try out the techniques described in Protocols 12.4–12.9 and select the method that works best in your system. If none of those methods is successful, try using additional enzymes, such as pronase, dispase, Accutase, and DNase, and consult the literature for examples of previous work with the tissue in which you are interested.

12.3.11 Primary Records

Regardless of the technique used to produce a culture, it is important to keep proper records of the culture's origin and derivation, including the species, sex, and tissue from which it was derived, any relevant pathology, and the procedures used for disaggregation and primary culture (Table 12.2). If you are working under good laboratory practice (GLP; *www.oecd.org/department/0,2688,en_2649_34381_1_1_1_1_1,00.html*) conditions, then such records are not just desirable, but obligatory [Food and Drug Administration, 1992; Department of Health and Social Security, 1986; Organisation for Economic Co-operation and Development, 2004]. Records can be kept in a notebook or another hard copy file of record sheets, but it is best at this stage to initiate a record in a computer database; this record then becomes the first step in maintaining the *provenance* of the cell line. The database record may never proceed beyond the primary culture stage, but, looking at the opposite extreme, it could be the first stage in creating an accurate record of what will become a valuable cell line. If the record becomes irrelevant, it can always be deleted, but it cannot, with any accuracy, be created later if the cell line assumes some importance.

As it may be necessary to authenticate the origin of a cell line at a later stage in its life history, particularly if cross-contamination is suspected, it is important to save a sample of tissue, blood, or DNA from the same individual at the time of isolation of tissue for the primary culture. This sample can then be used as reference material for DNA fingerprinting (*see* Section 16.6.2) or DNA profiling (*see* Section 16.6.3).