Techniques of cell and tissue Culture-Primary culture

Primary Culture:

Tissue culture was first devised at the beginning of the twentieth century by Harrison, 1907; Carrel, 1912. Itwas used as a method for studying the behavior of animal cells free of systemic variations that might arise *in vivo* both during normal homeostasis and under the stress of an experiment.

Key Events in the Development of Cell and Tissue Culture:

1907	Frog embryo nerve fiber outgrowth in vitro	Harrison, 1907
1916	Trypsinization and subculture of explants	Rous & Jones, 1916
1920s/30s	Subculture of fibroblastic cell lines	Carrel & Ebeling, 1923
1925–1926	Differentiation in vitro in organ culture	Strangeways & Fell, 1925, 1926
1940s	Introduction of use of antibiotics in tissue culture	Keilova, 1948; Cruikshank & Lowbury, 1952
1952–1955	Establishment the first human cell line, HeLa, from a cervical carcinoma,	Gey et al., 1952
1952	Nuclear transplantation	Briggs & King, 1960
1973	DNA transfer, calcium phosphate	Graham & Van der Eb, 1973
1975	Fibroblast growth factor	Gospodarowicz et al., 1975

Tissue Culture:

Tissue Culture is commonly used as a generic term to include organ culture and cell culture.The term *organ culture* will always imply a three-dimensional culture of undisaggregated tissue retaining some or all of the histological features of the tissue *in vivo*. *Cell culture* refers to a culture derived from dispersed cells taken from original tissue, from a primary culture, or from a cell line or cell strain by enzymatic, mechanical, or chemical disaggregation. The term *histotypic culture* implies that cells have been reaggregated or grown to re-create a three-

dimensional structure with tissuelike cell density, e.g., by cultivation at high density in a filter well, perfusion and overgrowth of a monolayer in a flask or dish, reaggregation in suspension over agar or in real or simulated zero gravity, or infiltration of a three-dimensional matrix such as collagen gel. *Organotypic* implies the same procedures but recombining cells of different lineages, e.g., epidermal keratinocytes in combined culture with dermal fibroblasts, in an attempt to generate a *tissue equivalent*.

Harrison [1907] chose the frog as his source of tissue, because it was a cold-blooded animal, and consequently, incubation was not required. Furthermore, tissue regeneration is more common in lower vertebrates.

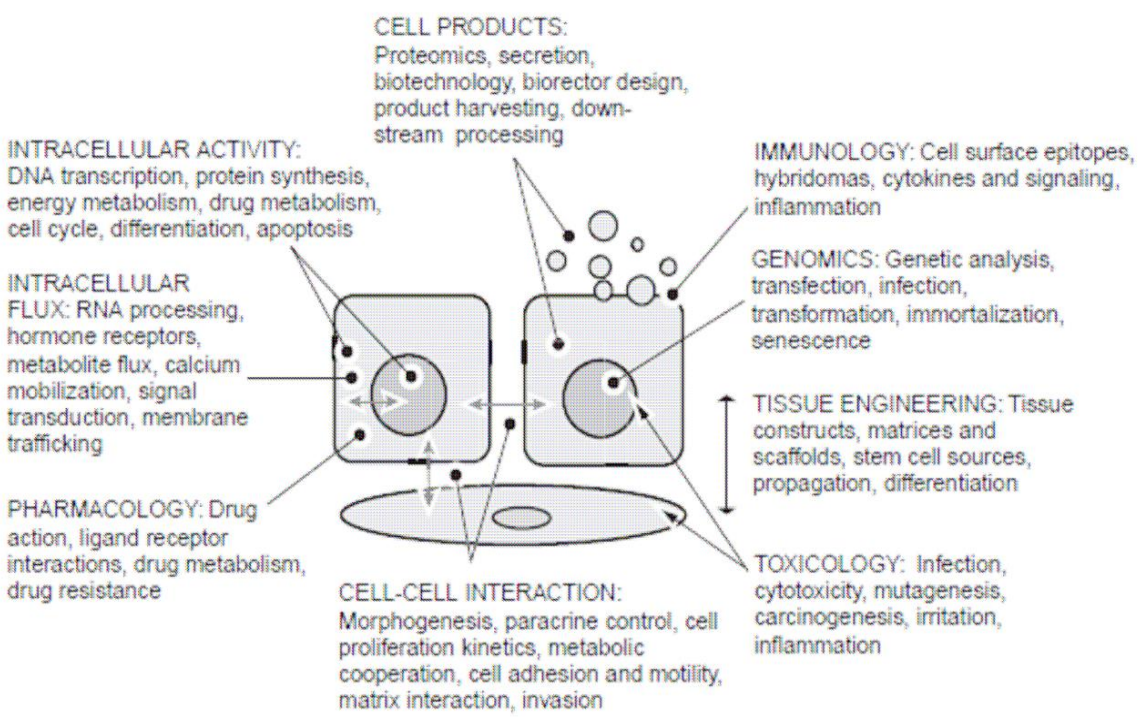


Fig. 1.2. Tissue Culture Applications.

The two major advantages of tissue culture are control of the physiochemical environment (pH, temperature, osmotic pressure, and O_2 and CO_2 tension) and the physiological conditions, which may be kept relatively constant. Most cell lines require supplementation of the

medium with serum or other poorly defined constituents. These supplements includes undefined elements such as hormones and other regulatory substances.

Characterization and Homogeneity of Sample

Tissue samples are invariably heterogeneous. Repli-cates—even from one tissue—vary in their constituent cell types. After one or two passages, cultured cell lines assume a homogeneous (or at least uniform) constitution, as the cells are randomly mixed at each transfer and the selective pressure of the culture conditions tends to produce a homo-geneous culture of the most vigorous cell type. Hence, at each subculture, replicate samples are identical to each other, and the characteristics of the line may be perpetuated over several generations, or even indefinitely if the cell line is stored in liquid nitrogen.

TABLE 1.2. Advantages of Tissue Culture

Category	Advantages
Physico-chemical environment	Control of pH, temperature, osmolality, dissolved gases
Physiological conditions	Control of hormone and nutrient concentrations
Microenvironment	Regulation of matrix, cell–cell interaction, gaseous diffusion
Cell line homogeneity	Availability of selective media, cloning
Characterization	Cytology and immunostaining are easily performed
Preservation	Can be stored in liquid nitrogen
Validation & accreditation	Origin, history, purity can be authenticated and recorded
Replicates and variability	Quantitation is easy
Reagent saving	Reduced volumes, direct access to cells, lower cost
Control of C × T	Ability to define dose, concentration (C), and time (T)
Mechanization	Available with microtitration and robotics
Reduction of animal use	Cytotoxicity and screening of pharmaceutics, cosmetics, etc.

TABLE 1.3. Limitations of Tissue Culture

Category	Examples
Necessary expertise	Sterile handling
	Chemical contamination
	Microbial contamination
	Cross-contamination
Environmental control	Workplace
	Incubation, pH control
	Containment and disposal of biohazards
Quantity and cost	Capital equipment for scale-up
	Medium, serum
	Disposable plastics
Genetic instability	Heterogeneity, variability
Phenotypic instability	Dedifferentiation
	Adaptation
	Selective overgrowth
Identification of cell type	Markers not always expressed
	Histology difficult to recreate and atypical
	Geometry and microenvironment change cytology

Major Differences *In Vitro* :

Many of the differences in cell behavior between cultured cells and their counterparts *in vivo* stem from the dissociation of cells from a three-dimensional geometry and their propagation on a two-dimensional substrate. Specific cell interactions characteristic of the histology of the tissue are lost, and, as the cells spread out, become mobile, and, in many cases, start to proliferate, so the growth fraction of the cell population increases. When a cell line forms, it may represent only one or two cell types, and many heterotypic cell–cell interactions are lost.

The culture environment also lacks the several systemic components involved in homeostatic regulation *in vivo*, principally those of the nervous and endocrine systems. Without this control, cellular metabolism may be more constant *in vitro* than *in vivo*, but may not be truly representative of the tissue from which the cells were derived.

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 disag-gregation 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 2002], collagenase with Dispase], and col-lagenase with hyaluronidase [.There are other, nonmammalian enzymes, such as Trypzean ,a recombinant, maize-derived, trypsin, TrypLE (Invitrogen), recombinant microbial, and Accutase and Accumax (Innovative Cell Technologies), also avail-able 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 disag-gregation, 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 .

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

(6) Embryonic tissue disaggregates more readily, yields more viable cells, and proliferates more rapidly in primary culture than does adult tissue.

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

For example, in the United Kingdom, the use of embryos or fetuses beyond 50% gestation or incubation is regulated under the Animal Experiments (Scientific Proce-dures) Act of 1986. Work with human biopsies or fetal mate-rial usually requires the consent of the local ethical committee and the patient and/or his or her relatives .

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 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.

PRIMARY CULTURE :

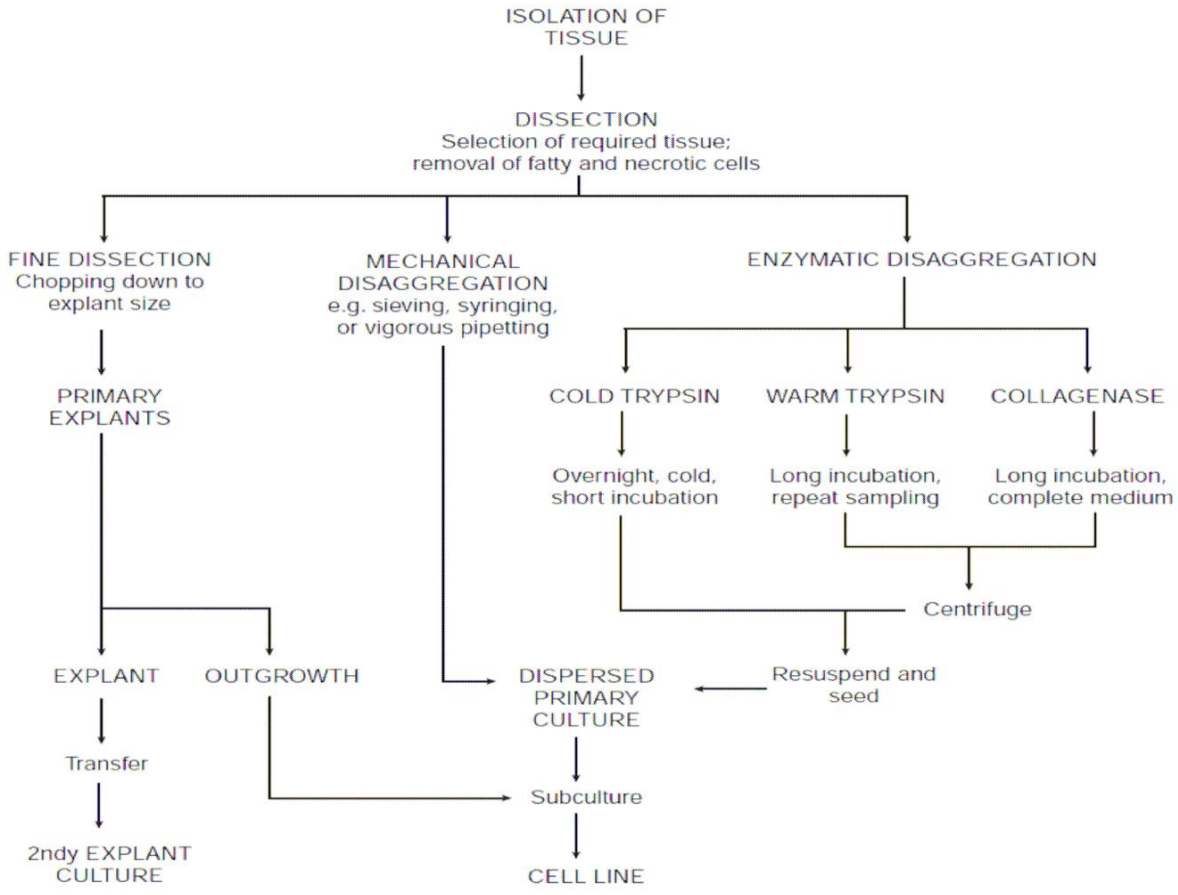
Several techniques have been devised for the disaggregation of tissue isolated for primary culture. These techniques can be divided into

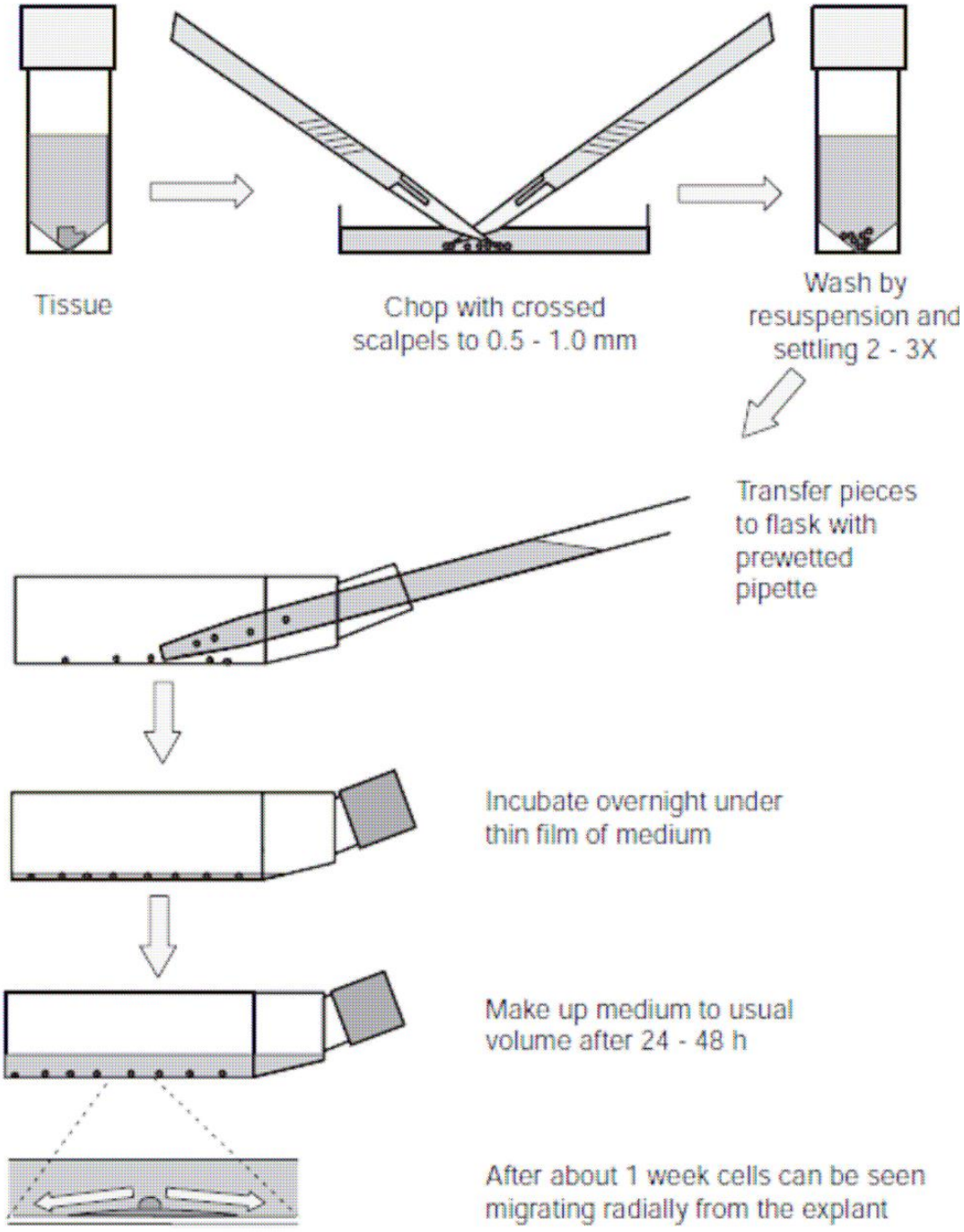
(1) purely mechanical techniques, involving dissection with or without some form of maceration, and

(2) techniques utilizing enzymatic disaggregation (Fig. 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.

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.





Enzymatic Disaggregation :

Cell–cell adhesion in tissues is mediated by a variety of homotypic interacting glycopeptides (cell adhesion molecules, or CAMs), 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 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. 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.

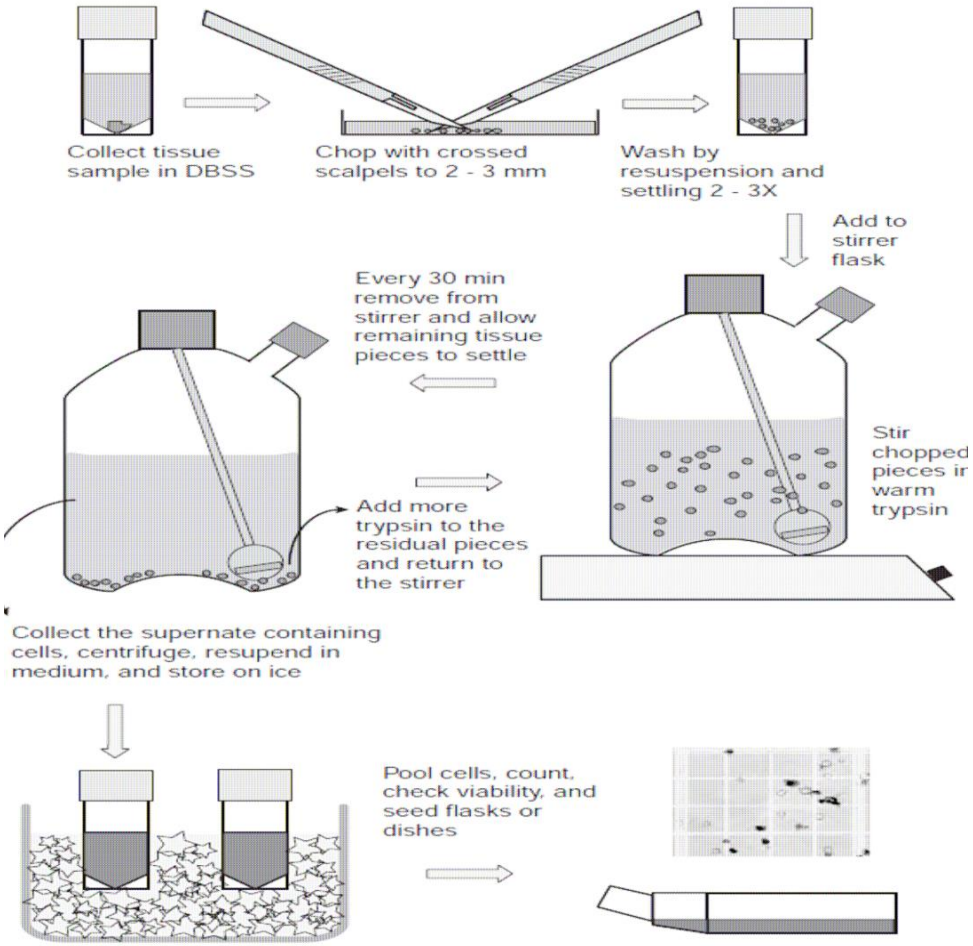
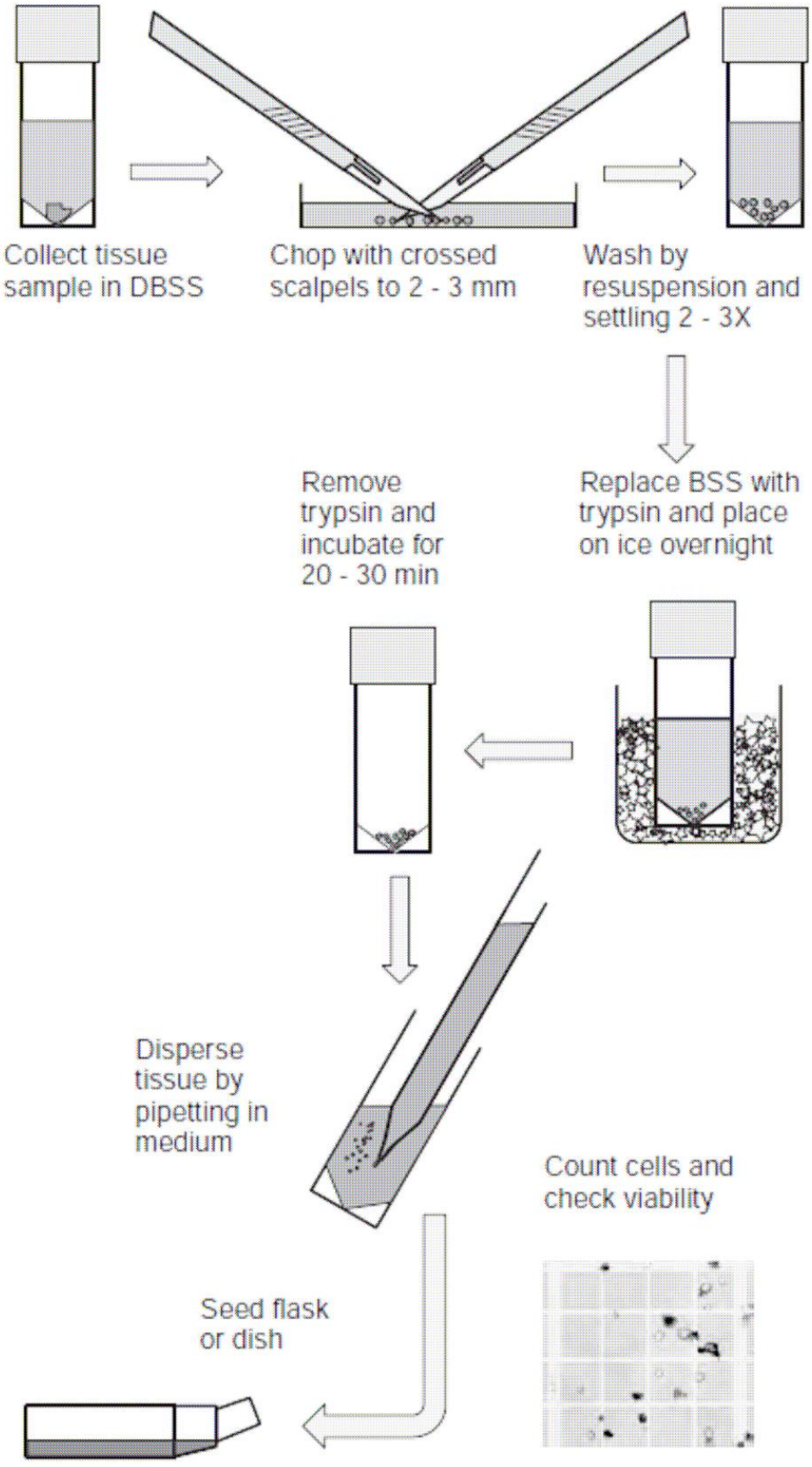
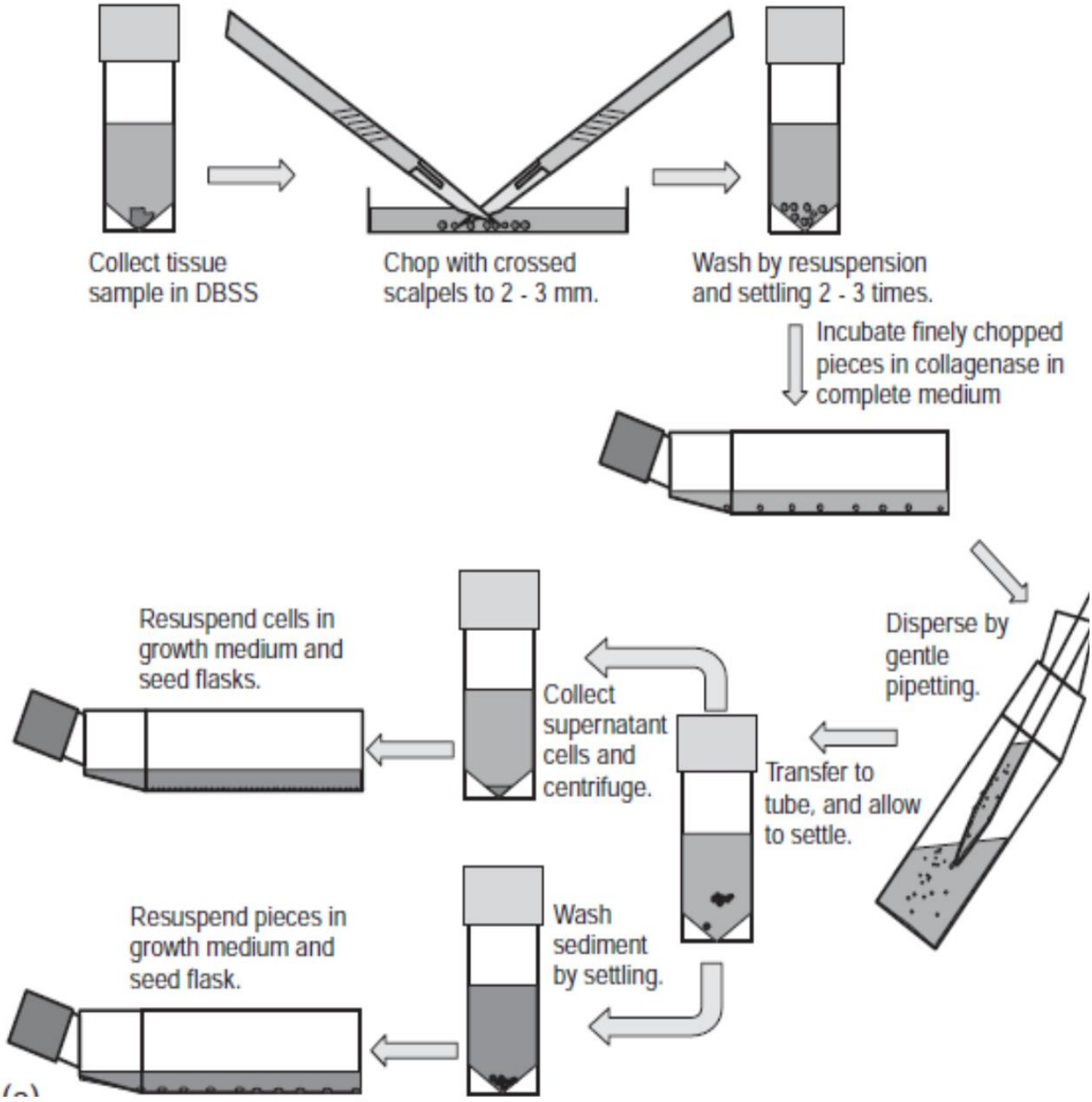


Fig. 12.7. Warm Trypsin Disaggregation.

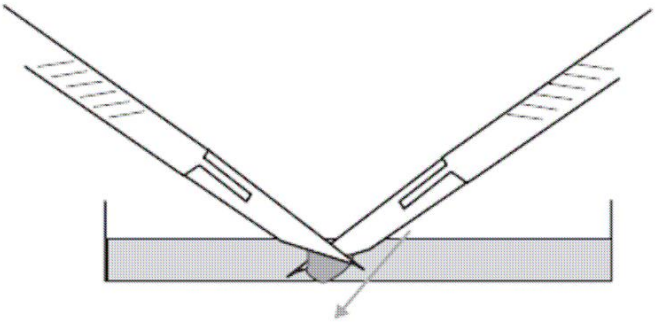


COLD TRYPSIN DISAGGREGATION

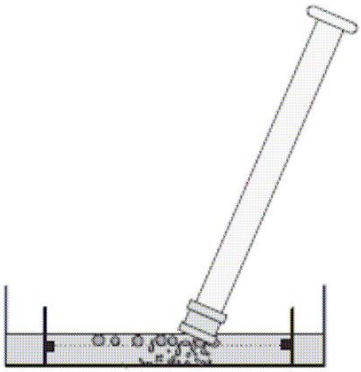


Tissue Disaggregation by Collagenase.

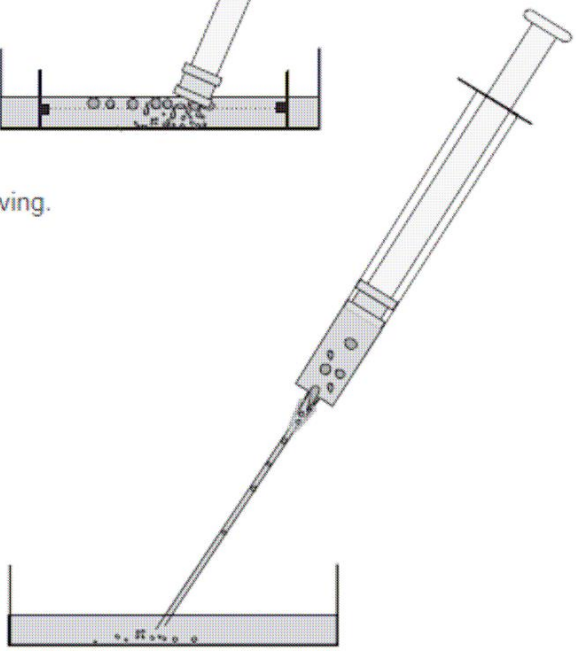
Mechanical Disaggregation. (a) Scraping or ‘‘spillage’’. Cutting action, or abrasion of cut surface, releases cells. (b) Sieving. Forcing tissue through sieve with syringe piston. (Falcon Cell Strainer can be used; (c) Syringing. Drawing tissue into syringe through wide bore needle or canula and expressing.(d)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



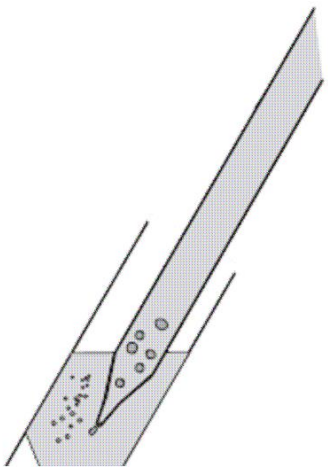
(a) Scraping or "spillage".



(b) Sieving.



(c) Syringing.



SUBCULTURE AND PROPAGATION

The first *subculture* represents an important transition for a culture. The need to subculture implies that the primary culture has increased to occupy all of the available substrate. Hence, cell proliferation has become an important feature. Although the primary culture may have a variable growth fraction,, depending on the type of cells present in the culture, after the first subculture, the growth fraction is usually high (80% or more). From a very heterogeneous primary culture, containing many of the cell types present in the original tissue, a more homogeneous cell line emerges. In addition to its biological significance, this process has considerable practical importance, as the culture can now be propagated, characterized, and stored, and the potential increase in cell number and the uniformity of the cells open up a much wider range of experimental possibilities.

Once a primary culture is subcultured (or *passaged*), it becomes known as a *cell line*. This term implies the presence of several cell lineages of either similar or distinct phenotypes. If one cell lineage is selected, by cloning , by physical cell, or by any other selection technique, to have certain specific properties that have been identified in the bulk of the cells in the culture, this cell line becomes known as a *cell strain.*

If a cell line transforms *in vitro*, it gives rise to a *continuous cell line* , and if selected or cloned and characterized, it is known as a *continuous cell strain*. The first subculture gives rise to a *secondary* culture, the secondary to a *tertiary*, and so on, although in practice, this nomenclature is seldom used beyond the tertiary culture. Each subculture divided the culture in half (i.e., the *split ratio* was 1:2), so passage number was the same as generation number. However, they need not be the same. The *passage number* is the number of times that the culture has been subcultured, whereas the *generation number* is the number of doublings that the cell population has undergone, given that the number of doublings in the primary culture is very approximate. When the split ratio is 1:2, the passage number is approximately equal to the generation number.

TABLE 13.1. Commonly Used Cell Lines

Cell line	Morphology	Origin	Species	Age	Ploidy	Characteristics	Reference
Finite, from Normal Tissue							
IMR-90	Fibroblast	Lung	Human	Embryonic	Diploid	Susceptible to human viral infection; contact inhibited	Nichols et al., 1977
MRC-5	Fibroblast	Lung	Human	Embryonic	Diploid	Susceptible to human viral infection; contact inhibited	Jacobs, 1970
MRC-9	Fibroblast	Lung	Human	Embryonic	Diploid	Susceptible to human viral infection; contact inhibited	Jacobs, et al., 1979
WI-38	Fibroblast	Lung	Human	Embryonic	Diploid	Susceptible to human viral infection	Hayflick & Moorhead, 1961
Continuous, from Normal Tissue							
293	Epithelial	Kidney	Human	Embryonic	Aneuploid	Readily transfected.	Graham et al., 197
3T3-A31	Fibroblast		Mouse BALB/c	Embryonic	Aneuploid	Contact inhibited; readily transformed	Aaronson & Todaro 1968
3T3-L1	Fibroblast		Mouse Swiss	Embryonic	Aneuploid	Adipose differentiation	Green & Kehinde, 1974
BEAS-2B	Epithelial	Lung	Human	Adult			Reddel et al., 1988
BHK21-C13	Fibroblast	Kidney	Syrian hamster	Newborn	Aneuploid	Transformable by polyoma	Macpherson & Stoker, 1962
BRL 3A	Epithelial	Liver	Rat	Newborn		Produce IGF-2	Coon, 1968
C2	Fibroblastoid	Skeletal muscle	Mouse	Embryonic		Myotubes	Morgan et al., 1992
C7	Epithelioid	Hypothalamus	Mouse			Neurophysin; vasopressin	De Vitry et al., 197
CHO-K1	Fibroblast	Ovary	Chinese hamster	Adult	Diploid	Simple karyotype	Puck et al., 1958
COS-1, COS-7	Epithelioid	Kidney	Pig	Adult		Good hosts for DNA transfection	Gluzman, 1981
CPAE	Endothelial	Pulmonary-artery endothelium	Cow	Adult	Diploid	Factor VIII, Angiotensin II converting enzyme	Del Vecchio & Smith, 1981
HaCaT	Epithelial	Keratinocytes	Human	Adult	Diploid	Cornification	Boukamp et al., 1988
L6	Fibroblastoid	Skeletal muscle	Rat	Embryonic		Myotubes	Richler & Yaffe, 1970
LLC-PKI	Epithelial	Kidney	Pig	Adult	Diploid	Na^+-dependent glucose uptake	Hull et al., 1976; Saier, 1984
MDCK	Epithelial	Kidney	Dog	Adult	Diploid	Domes, transport	Gaush et al., 1966; Rindler et al., 1979
NRK49F	Fibroblast	Kidney	Rat	Adult	Aneuploid	Induction of suspension growth by TGF-α,β	De Larco & Todaro 1978
STO	Fibroblast		Mouse	Embryonic	Aneuploid	Used as feeder layer for embryonal stem cells	Bernstein, 1975
Vero	Fibroblast	Kidney	Monkey	Adult	Aneuploid	Viral substrate and assay	Hopps et al., 1963
Continuous, from Neoplastic Tissue							
A2780	Epithelial	Ovary	Human	Adult	Aneuploid	Chemosensitive with resistant variants	Tsuruo et al., 1986
A549	Epithelial	Lung	Human	Adult	Aneuploid	Synthesizes surfactant	Giard et al., 1972
A9	Fibroblast	Subcutaneous	Mouse	Adult	Aneuploid	Derived from L929; Lacks HGPRT.	Littlefield, 1964b
B16	Fibroblastoid	Melanoma	Mouse	Adult	Aneuploid	Melanin	Nilos & Makarski, 1978
C1300	Neuronal	Neuroblastoma	Rat	Adult	Aneuploid	Neurites	Liebermann & Sachs, 1978
C6	Fibroblastoid	Glioma	Rat	Newborn	Aneuploid	Glial fibrillary acidic protein, GPDH	Benda et al., 1968

CULTURE AGE

Cell lines with limited culture life spans are known as *finite* cell lines and behave in a fairly reproducible fashion . They grow through a limited number of cell generations, usually between 20 and 80 cell population doublings, before extinction. The actual number of doublings depends on species and cell lineage differences, clonal variation, and culture conditions, but it is consistent for one cell line grown under the same conditions. It is therefore important that reference to a cell line should express the approximate generation number or number of doublings since explantation;

Continuous cell lines have escaped from senescence control, so the generation number becomes less important and the number of passages since last thawed from storage becomes more important .In addition, because of the increased cell proliferation rate and saturation density ,split ratios become much greater (1:20–1:100) and cell concentration at subculture becomes much more critical .

CELL LINE DESIGNATIONS

New cell lines should be given a code or designation [e.g., normal human brain (NHB)]; a cell strain or cell line number (if several cell lines were derived from the same source; e.g., NHB1, NHB2, etc.); and, if cloned, a clone number (e.g., NHB2-1, NHB2-2, etc.). It is useful to keep a log book or computer database file where the receipt of biopsies or specimens is recorded before initiation of a culture. The accession number in the log book or database file, perhaps linked to an identifier letter code, can then be used to establish the cell line designation; for example, LT156 would be lung tumor biopsy number 156. This method is less likely to generate ambiguities, such as the same letter code being used for two different cell lines, and gives automatic reference to the record of accession of the line. Rules of confidentiality preclude the use of a donor's initials in naming a cell line.

For finite cell lines, the number of population doublings should be estimated and indicated after a forward slash, e.g., NHB2/2, and increases by one for a split ratio of 1:2 (e.g., NHB2/2, NHB2/3, etc.), by two for a split ratio of 1:4 (e.g., NHB2/2, NHB2/4, etc.), and so on. When dealing with a continuous cell line a ''p'' number at the end is often used to indicate the number the number of passages since the last thaw from the freezer , e.g., HeLa-S3/p4.

CHOOSING A CELL LINE :

The general parameters to consider in selecting a cell line are :

(1) **Finite vs. Continuous.** Is there a continuous cell line that expresses the right functions?

A continuous cell line generally is easier to maintain, grows faster, clones more easily, produces a higher cell yield per flask, and is more readily adapted to serum-free medium (*see* Table 13.3).

(2) **Normal or Transformed.** Is it important whether the line is malignantly transformed or not? If it is, then it might be possible to obtain an immortal line that is not tumorigenic, e.g., 3T3 cells or BKK21-C13.

(3) **Species.** Is species important? Nonhuman cell lines have fewer biohazard restrictions and have the advantage that the original tissue may be more accessible.

(4) **Growth Characteristics.** What do you require in

terms of growth rate, yield, plating efficiency, and ease of harvesting? You will need to consider the following parameters:

a) Population-doubling time (*see* Section 21.9.7)

b) Saturation density (yield per flask; *see* Section 21.9.5)

c) Plating efficiency (*see* Section 21.10)

d) Growth fraction (*see* Section 21.11.1)

e) Ability to grow in suspension (*see* Section 18.5.1, Table 13.5)

(5) **Availability.** If you have to use a finite cell line, are there sufficient stocks available, or will you have to generate your own line(s)? If you choose a continuous cell line, are authenticated stocks available?

(6) **Validation.** How well characterized is the line (*see* Section 7.10), if it exists already, or, if not, can you do the necessary characterization (*see* Chapter 16)? Is the line authentic (*see* Section 16.3)? It is vital to eliminate the possibility of cross-contamination before embarking on a program of work with a cell line, as so many cross-contaminations have

been reported (*see* Table 13.2).

(7) **Phenotypic Expression.** Can the line be made to express the right characteristics (*see* Section 17.7)?

(8) **Control Cell Line.** If you are using a mutant, transfected, transformed, or abnormal cell line, is there a normal equivalent available, should it be required?

(9) **Stability.** How stable is the cell line .

Significance of Cell Morphology :

Whatever procedure is undertaken, it is vital that the culture be examined carefully to confirm the absence of contamination . The cells should also be checked for any signs of deterioration, such as granularity around the nucleus, cytoplasmic vacuolation, and rounding up of the cells with detachment from the substrate . Such signs may imply that the culture requires a medium change, or may indicate a more serious problem, e.g., inadequate or toxic medium or serum, microbial contamination, or senescence of the cell line. Medium deficiencies can also initiate apoptosis.

Replacement of Medium

Four factors indicate the need for the replacement of culture medium:

A Drop in pH. The rate of fall and absolute level should be considered. Most cells stop growing as the pH falls from pH 7.0 to pH 6.5 and start to lose viability between pH 6.5 and pH 6.0, so if the medium goes from red through orange to yellow, the medium should be changed. Try to estimate the rate of fall; a culture at pH 7.0 that falls 0.1 pH units in one day will not come to harm if left a day or two longer before feeding, but a culture that falls 0.4 pH units in one day will need to be fed within 24–48 h and cannot be left over a weekend without feeding.

Cell Concentration. Cultures at a high cell con-centration exhaust the medium faster than those at a low concentration. This factor is usually evident in the rate of change of pH, but not always.

Cell Type. Normal cells (e.g., diploid fibroblasts) usually stop dividing at a high cell density , because of cell crowding, growth factor depletion, and other reasons. The cells block in the G_1

phase of the cell cycle and deteriorate very little, even if left for two to three weeks or longer. Transformed cells, continuous cell lines, and some embryonic cells, however, deteriorate rapidly at high cell densities unless the medium is changed daily or they are subcultured.

Morphological Deterioration. This factor must be anticipated by regular examination and familiarity with the cell line .If deterioration is allowed to progress too far, it will be irreversible, as the cells will tend to enter apoptosis .

CRITERIA FOR SUBCULTURE :

The need to subculture a monolayer is determined by the following criteria:

(1) **Density of Culture.** Normal cells should be subcultured as soon as they reach confluence. If left more than 24 h, they will withdraw from the cycle and take longer to recover when reseeded. Transformed cells should also be subcultured on reaching confluence; although they will continue to proliferate beyond confluence, they will start to deteriorate after about two doublings, and reseeding efficiency will decline.

(2) **Exhaustion of Medium.** Exhaustion of the medium usually indicates that the medium requires replacement, but if a fall in pH occurs so rapidly that the medium must be changed more frequently, then subculture may be required. Usually, a drop in pH is accompanied by an increase in cell density, which is the prime indicator of the need to subculture. Note that a sudden drop in pH can also result from contamination, so be sure to check .

(3) **Time Since Last Subculture.** Routine subculture is best performed according to a strict schedule, so that reproducible behavior is achieved and monitored. If cells have not reached a high-enough density (i.e., they are not confluent) by the appropriate time, then increase the seeding density, or if they reach confluence too soon, then reduce the seeding density. Once this routine is established, the recurrent growth should be consistent in duration and cell yield from a given seeding density. Deviations from this pattern then signify a departure from normal conditions or indicate deterioration of the cells. Ideally, a cell concentration should be found that allows for the cells to be subcultured after 7 days, with the medium being changed after 3–4 days.

Requirements for Other Procedures. When cells are required for purposes other than routine propagation, they also have to be subcultured, in order to increase the stock or to change the type of culture vessel or medium. Ideally, this procedure should be done at the regular subculture time, when it will be known that the culture is performing routinely, what the reseeding conditions should be, and what outcome can be expected. However, demands for cells do not always fit the established routine for maintenance, and compromises have to be made, but (1) cells should not be subcultured while still within the lag period, and (2) cells should always be taken between the middle of the log phase and the time before which they have entered the plateau phase of a previous subculture (unless there is a specific requirement for plateau-phase cells, in which case they will need frequent feeding or continuous perfusion).

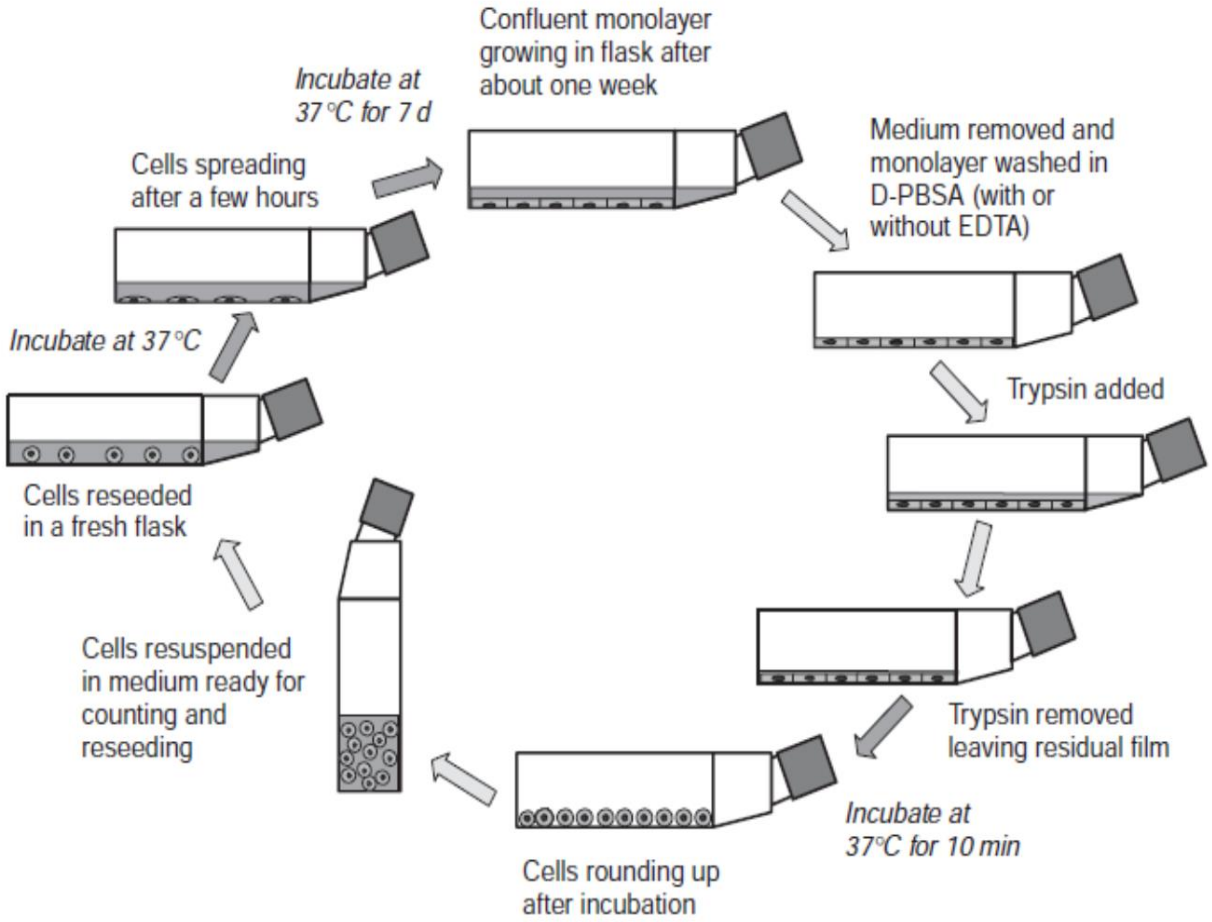


Fig. 13.3. Subculture of Monolayer. Stages in the subculture and growth cycle of monolayer cells after trypsinization

PROPAGATION IN SUSPENSION :

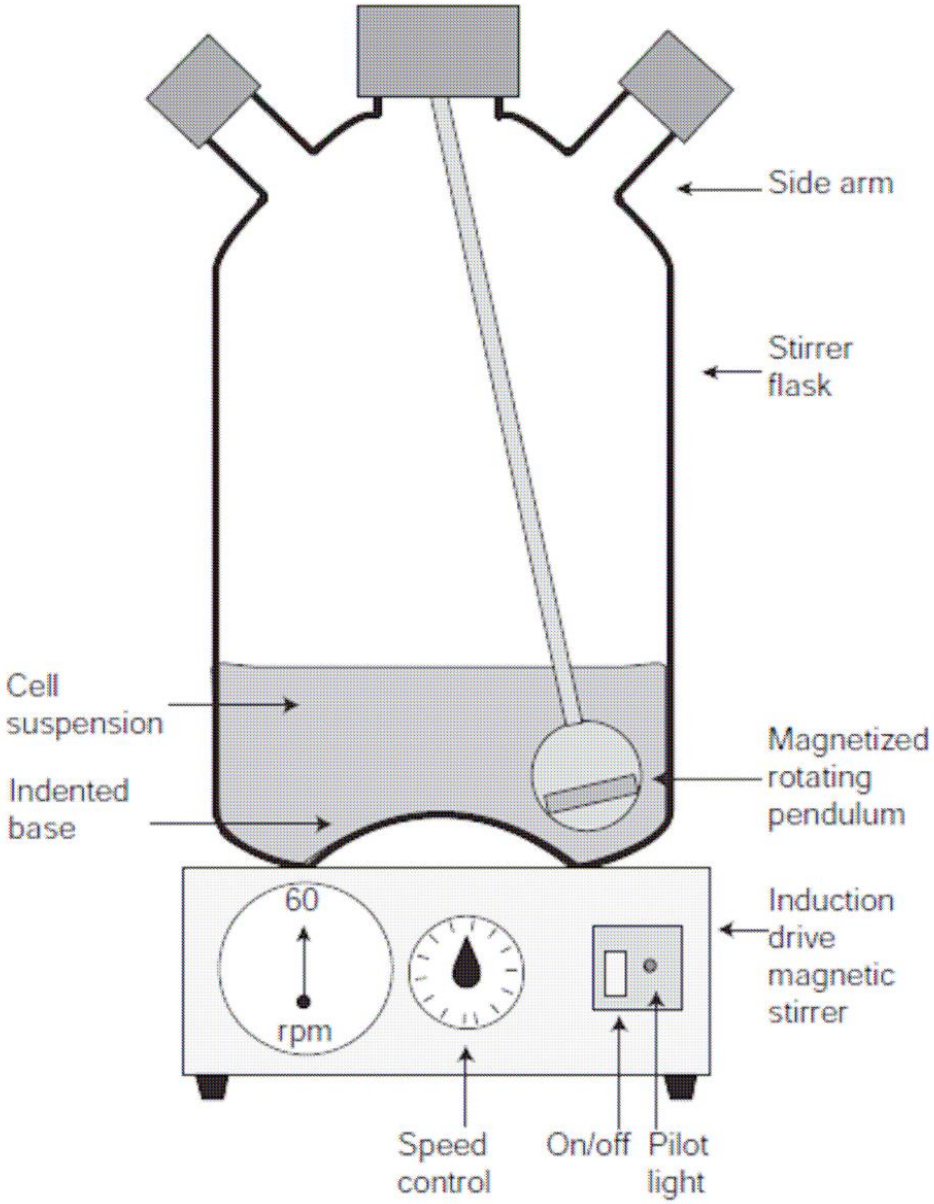


Fig. 13.5. Stirrer Culture. A small stirrer flask, based on the Techne design, with a capacity of 250–1000 mL. The cell suspension is stirred by a pendulum, which rotates in an annular depression in the base of the flask.

As a general rule, most continuous cell lines subculture satisfactorily at a seeding concentration of between 1×10^4 and 5×10^4 cells/mL.

The criteria for subculture are similar to those for monolayers:

(1) **Cell Concentration,** which should not exceed 1×10^6 cells/mL for most suspension-growing cells.

(2) **pH,** which is linked to cell concentration, and declines as the cell concentration rises.

(3) **Time Since Last Subculture,** which, as for monolayers, should fit a regular schedule.

(4) **Cell Production Requirements** for experimental or production purposes.

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