# CHAPTER 13

# Subculture and Cell Lines

## 13.1 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 (*see* Section 21.11.1), 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 (*see* Table 1.5).

## 13.2 TERMINOLOGY

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 (*see* Protocol 14.1), by physical cell separation (*see* Chapter 15), 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* (*see* Appendix IV). Some commonly used cell lines and cell strains are listed in Table 13.1 (*see also* Table 3.1). If a cell line transforms *in vitro*, it gives rise to a *continuous cell line* (*see* Sections 3.8, 18.4), and if selected or cloned and characterized, it is known as a *continuous cell strain*. It is vital at this stage to confirm the identity of the cell lines and exclude the possibility of cross-contamination; many cell lines in common use are not, in fact, what they are claimed to be, but have been cross-contaminated with HeLa or some other vigorously-growing cell line (Table 13.2). However, continuous cell lines have a number of advantages; the relative advantages and disadvantages of finite cell lines and continuous cell lines are listed in Table 13.3.

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. In Hayflick's work and others with human diploid fibroblasts [Hayflick & Moorhead, 1961], 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, as in Hayflick's experiments, the passage number is approximately equal to the generation number. However, if subculture is performed at split ratios greater than 1:2 the generation number, which is the significant indicator of culture age, will increase faster than the passage number based on the number of doublings that the cell population has undergone since the previous

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**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., 1977 |
| 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., 1974 |
| 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-$\alpha$,$\beta$ | 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 |

**TABLE 13.1.** Commonly Used Cell Lines (*Continued*)

| Cell line | Morphology | Origin | Species | Age | Ploidy | Characteristics | Reference |
|---|---|---|---|---|---|---|---|
| Caco-2 | Epithelial | Colon | Human | Adult | Aneuploid | Transports ions and amino acids | Fogh, 1977 |
| EB-3 | Lymphocytic | Peripheral blood | Human | Juvenile | Diploid | EB virus +ve | Epstein & Barr, 1964 |
| Friend | Suspension | Spleen | Mouse | Adult | Aneuploid | Hemoglobin | Scher et al., 1971 |
| GH1, GH2, GH3 | Epithelioid | Pituitary tumor | Rat | Adult | | Growth hormone | Buonassisi et al., 1962; Yasamura et al., 1966 |
| H4-11-E-C3 | Epithelial | Hepatoma | Rat | Adult | Aneuploid | Tyrosine aminotransferase | Pitot et al., 1964 |
| HeLa | Epithelial | Cervix | Human | Adult | Aneuploid | G6PD Type A | Gey et al., 1952 |
| HeLa-S3 | Epithelial | Cervix | Human | Adult | Aneuploid | High plating efficiency; will grow well in suspension | Puck & Marcus, 1955 |
| HEP-G2 | Epithelioid | Hepatoma | Human | Adult | Aneuploid | Retains some microsomal metabolizing enzymes | Knowles et al., 1980 |
| HL-60 | Suspension | Myeloid leukemia | Human | Adult | Aneuploid | Phagocytosis<br>Neotetrazolium Blue reduction | Olsson & Ologsson, 1981 |
| HT-29 | Epithelial | Colon | Human | Adult | Aneuploid | Differentiation inducible with NaBt | Fogh & Trempe, 1975 |
| K-562 | Suspension | Myeloid leukemia | Human | Adult | Aneuploid | Hemoglobin | Andersson et al., 1979a,b |
| L1210 | Lymphocytic | | Mouse | Adult | Aneuploid | Rapidly growing; suspension | Law et al., 1949 |
| L929 | Fibroblast | | Mouse | Adult | Aneuploid | Clone of L-cell | Sanford et al., 1948 |
| LS | Fibroblast | | Mouse | Adult | Aneuploid | Grow in suspension; derived from L929 | Paul & Struthers, personal communication |
| MCF-7 | Epithelial | Pleural effusion from breast tumor | Human | Adult | Aneuploid | Estrogen receptor +ve, domes, $\alpha$-lactalbumin | Soule et al., 1973 |
| MCF-10 | Epithelial | Fibrocystic mammary tissue | Human | Adult | Near diploid | Dome formation | Soule et al., 1990 |
| MOG-G-CCM | Epithelioid | Glioma | Human | Adult | Aneuploid | Glutamyl synthetase | Balmforth et al., 1986 |
| P388D1 | Lymphocytic | | Mouse | Adult | Aneuploid | Grow in suspension | Dawe & Potter, 1957; Koren et al., 1975 |
| S180 | Fibroblast | | Mouse | Adult | Aneuploid | Cancer chemotherapy screening | Dunham & Stewart, 1953 |
| SK/HEP-1 | Endothelial | Hepatoma, endothelium | Human | Adult | Aneuploid | Factor VIII | Heffelfinger et al., 1992 |
| WEHI-3B D+ | Suspension | Marrow | Mouse | Adult | Aneuploid | IL-3 production | Nicola, 1987 |
| ZR-75-1 | Epithelial | Ascites fluid from breast tumor | Human | Adult | Aneuploid | ER-ve, EGFr+ve | Engel et al., 1978 |

subculture (*see* Section 13.7.2). None of these approximations takes account of cell loss through necrosis, apoptosis, or differentiation or premature aging and withdrawal from cycle, which probably take place at every growth cycle between each subculture.

## 13.3 CULTURE AGE

Cell lines with limited culture life spans are known as *finite* cell lines and behave in a fairly reproducible fashion (*see* Section 3.8.1). They grow through a limited number of

**TABLE 13.2.** Cross-Contaminated Cell Lines

| Cell line | Species | Cell type | Contaminant | Species | Cell type | Source of Data |
|---|---|---|---|---|---|---|
| 207 | Human | Pre-B leukemia | REH | Human | pre-B leukemia | DSMZ |
| 2474/90 | Human | Gastric carcinoma | HT-29 | Human | Colorectal carcinoma | DSMZ |
| 2957/90 | Human | Gastric carcinoma | HT-29 | Human | Colorectal carcinoma | DSMZ |
| 3051/80 | Human | Gastric carcinoma | HT-29 | Human | Colorectal carcinoma | DSMZ |
| ADLC-5M2 | Human | Lung carcinoma | HELA/-S3 | Human | Cervical adenocarcinoma | DSMZ |
| AV3 | Human | Amnion | HeLa | Human | Cervical adenocarcinoma | ATCC |
| BCC-1/KMC | Human | Basal cell carcinoma | HELA/-S3 | Human | Cervical adenocarcinoma | DSMZ |
| BM-1604 | Human | Prostate carcinoma | DU-145 | Human | Prostate carcinoma | DSMZ |
| C16 | Human | Fetal lung fibroblast (MRC-5 clone) | HeLa | Human | Cervical adenocarcinoma | ECACC |
| CHANG liver | Human | Embryonic liver epithelium | HeLa | Human | Cervical adenocarcinoma | ATCC; JCRB |
| COLO-818 | Human | Melanoma | COLO-800 | Human | Melanoma | DSMZ |
| DAMI | Human | Megakaryocytic | HEL | Human | Erythroleukemia | DSMZ |
| ECV304 | Human | Endothelium | T24 | Human | Bladder carcinoma | ATCC |
| ECV304 | Human | Normal endothelial | T24 | Human | Bladder carcinoma | DSMZ |
| EJ | Human | Bladder carcinoma | T24 | Human | Bladder carcinoma | ATCC; JCRB |
| EPLC3-2M1 | Human | Lung carcinoma | HELA/-S3 | Human | Cervical adenocarcinoma | DSMZ; JCRB |
| EPLC-65 | Human | Lung carcinoma | HELA/-S3 | Human | Cervical adenocarcinoma | DSMZ |
| F2-4E5 | Human | Thymic epithelium | SK-HEP-1 | Human | Hepatoma | DSMZ |
| F2-5B6 | Human | Thymic epithelium | SK-HEP-1 | Human | Hepatoma | DSMZ |
| FL | Human | Amnion | HeLa | Human | Cervical adenocarcinoma | ATCC |
| GHE | Human | Astrocytoma | T-24 | Human | Bladder carcinoma | DSMZ |
| Girardi Heart | Pig | Adult heart | HeLa | Human | Cervical adenocarcinoma | ATCC |
| HAG | Human | Adenomatous goitre | T-24 | Human | Bladder carcinoma | DSMZ |
| HEp-2 | Human | Adult laryngeal epithelium | HeLa | Human | Cervical adenocarcinoma | ATCC |
| HMV-1 | Human | Melanoma | HeLa-S3 | Human | Cervical adenocarcinoma | DSMZ |
| HuL-1 | | | HeLa | Human | Cervical adenocarcinoma | JCRB |
| IMC-2 | Human | Maxillary carcinoma | HeLa-S3 | Human | Cervical adenocarcinoma | DSMZ |
| Intestine 407 | Human | Intestine epithelium | HeLa | Human | Cervical adenocarcinoma | ATCC |
| J-111 | | | HeLa | Human | Cervical adenocarcinoma | JCRB |
| JOSK-I | Human | Monocytic leukemia | U-937 | Human | Histiocytic lymphoma | DSMZ |
| JOSK-K | Human | Monocytic leukemia | U-937 | Human | Histiocytic lymphoma | DSMZ |
| JOSK-M | Human | Monocytic leukemia | U-937 | Human | Histiocytic lymphoma | DSMZ |
| JOSK-S | Human | Monocytic leukemia | U-937 | Human | histolytic lymphoma | DSMZ |
| JTC-17 | | | HeLa | Human | Cervical adenocarcinoma | Yamakage (JCRB) |
| KB | Human | Adult oral cavity epithelium | HeLa | Human | Cervical adenocarcinoma | ATCC; JCRB |
| KO51 | | | K562 | Human | Myeloid leukemia | DSMZ; JCRB |
| KOSC-3 | | | Ca9-22 | | | JCRB |

**TABLE 13.2.** Cross-Contaminated Cell Lines (*Continued*)

| Cell line | Species | Cell type | Contaminant | Species | Cell type | Source of Data |
|---|---|---|---|---|---|---|
| L132 | Human | Embryonic lung epithelium | HeLa | Human | Cervical adenocarcinoma | ATCC |
| LR10.6 | Human | Pre-B cell leukemia | NALM-6 | Human | Pre-B cell leukemia | DSMZ |
| MaTu | Human | Breast carcinoma | HELA/-S3 | Human | Cervical adenocarcinoma | DSMZ |
| MC-4000 | Human | Breast carcinoma | HELA/-S3 | Human | Cervical adenocarcinoma | DSMZ |
| MKB-1 | Human | T-cell leukemia | CCRF-CEM | Human | T-cell leukemia | DSMZ |
| MKN28 | | | MKN74 | | | JCRB |
| MOLT-15 | Human | T-cell leukemia | CTV-1 | Human | Monocytic leukemia | DSMZ |
| MT-1 | Human | Breast carcinoma | HELA/-S3 | Human | Cervical adenocarcinoma | DSMZ |
| NCC16 | | | PHK16-0b | | | JCRB |
| P1-1A3 | Human | Thymic epithelium | SK-HEP-1 | Human | Hepatoma | DSMZ |
| P1-4D6 | Human | Thymic epithelium | SK-HEP-1 | Human | Hepatoma | DSMZ |
| P39 TSU | Human | | HL60 | Human | Myeloid leukemia | JCRB |
| PBEI | Human | Pre-B cell leukemia | NALM-6 | Human | Pre-B cell leukemia | DSMZ |
| PSV811 | Human | Fibroblast | WI38 | Human | Fibroblast | JCRB |
| RAMAK-1 | Human | Muscle synovium | T-24 | Human | Bladder carcinoma | DSMZ |
| SBC-2 | Human | Bladder carcinoma | HELA/-S3 | Human | Cervical adenocarcinoma | DSMZ |
| SBC-7 | Human | Bladder carcinoma | HELA/-S3 | Human | Cervical adenocarcinoma | DSMZ |
| SCLC-16H | Human | SCLC | SCLC-21/22H | Human | SCLC | DSMZ |
| SCLC-24H | Human | SCLC | SCLC-21/22H | Human | SCLC | DSMZ |
| SNB-19 | | | U251MG | Human | Glioma | ATCC |
| SPI-801 | Human | T-cell leukemia | K-562 | Human | Myeloid leukemia | DSMZ |
| SPI-802 | Human | T-cell leukemia | K-562 | Human | Myeloid leukemia | DSMZ |
| TK-1 | Human? | | U251MG | Human | Glioma | JCRB |
| TMH-1 | | | IHH-4 | | | JCRB |
| WISH | Human | Newborn amnion epithelium | HeLa | Human | Cervical adenocarcinoma | ATCC |

**TABLE 13.3.** Properties of Finite and Continuous Cell Lines

| Properties | Finite | Continuous (transformed) |
|---|---|---|
| **Ploidy** | Euploid, diploid | Aneuploid, heteroploid |
| **Transformation** | Normal | Immortal, growth control altered, and tumorigenic |
| **Anchorage dependence** | Yes | No |
| **Contact inhibition** | Yes | No |
| **Density limitation of cell proliferation** | Yes | Reduced or lost |
| **Mode of growth** | Monolayer | Monolayer or suspension |
| **Maintenance** | Cyclic | Steady state possible |
| **Serum requirement** | High | Low |
| **Cloning efficiency** | Low | High |
| **Markers** | Tissue specific | Chromosomal, enzymic, antigenic |
| **Special functions (e.g., virus susceptibility, differentiation)** | May be retained | Often lost |
| **Growth rate** | Slow ($T_D$ of 24–96 h) | Rapid ($T_D$ of 12–24 h) |
| **Yield** | Low | High |
| **Control parameters** | Generation no.; tissue-specific markers | Stain characteristics |

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; I say "approximate" because the number of generations that have elapsed in the primary culture is difficult to assess.

Continuous cell lines (*see* Table 13.1) 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 (*see* Section 13.7.2). In addition, because of the increased cell proliferation rate and saturation density (*see* Section 18.5), split ratios become much greater (1:20–1:100) and cell concentration at subculture becomes much more critical (*see* Section 13.7.3).

## 13.4 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 (*see* Section 20.4.2), e.g., HeLa-S3/p4.

When referenced in publications or reports, it is helpful to prefix the cell line designation with a code indicating the laboratory in which it was derived (e.g., WI for Wistar Institute, NCI for National Cancer Institute, SK for Sloan-Kettering) [Federoff, 1975]. In publications or reports, the cell line should be given its full designation the first time it is mentioned and in the Materials and Methods section, and the abbreviated version can then be used thereafter.

It is essential that the cell line designation is unique, or else confusion will arise in subsequent reports in the literature. Cell banks deal with this problem by giving each cell line an accession number; when reporting on cell lines acquired from a cell bank, you should give this accession number in the Materials and Methods section. Punctuation can also give rise to problems when one is searching for a cell line in a database, so always adhere to a standard syntax, and do not use apostrophes or spaces.

## 13.5 CHOOSING A CELL LINE

Apart from specific functional requirements, there are a number of general parameters to consider in selecting a cell line:

(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 (*see* Section 18.3 and Plate 7)? Has it been cloned? If not, can you clone it,

and how long would this cloning process take to generate sufficient frozen and usable stocks?

## 13.6 ROUTINE MAINTENANCE

Once a culture is initiated, whether it is a primary culture or a subculture of a cell line, it will need a periodic medium change, or "feeding," followed eventually by subculture if the cells are proliferating. In nonproliferating cultures, the medium will still need to be changed periodically, as the cells will still metabolize and some constituents of the medium will become exhausted or will degrade spontaneously. Intervals between medium changes and between subcultures vary from one cell line to another, depending on the rate of growth and metabolism; rapidly growing transformed cell lines, such as HeLa, are usually subcultured once per week, and the medium should be changed four days later. More slowly growing, particularly nontransformed, cell lines may need to be subcultured only every two, three, or even four weeks, and the medium should be changed weekly between subcultures (*see also* Sections 13.6.2, 13.7.1, 21.9.2).

### 13.6.1 Significance of Cell Morphology

Whatever procedure is undertaken, it is vital that the culture be examined carefully to confirm the absence of contamination (*see* Section 19.3.1 and Fig. 19.1). 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 (Fig. 13.1). 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 (*see* Section 3.3.1 and Plate 17c,d). During routine maintenance, the medium change or subculture frequency should aim to prevent such deterioration, as it is often difficult to reverse.

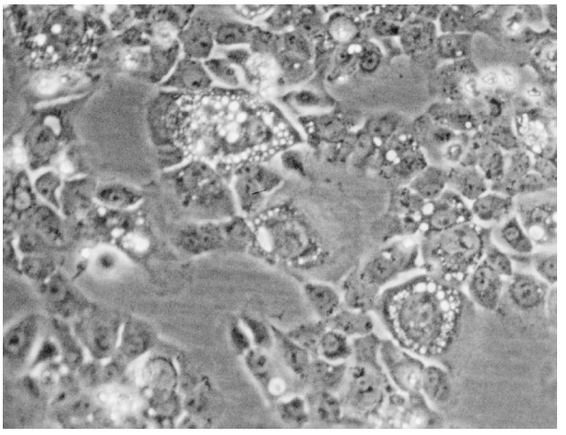

***Fig. 13.1. Unhealthy Cells.*** Vacuolation and granulation in bronchial epithelial cells (BEAS-2B) due, in this case, to medium inadequacy. The cytoplasm of the cells becomes granular, particularly around the nucleus, and vacuolation occurs. The cells may become more refractile at the edge if cell spreading is impaired.

Familiarity with the cell's morphology may also give the first indication of cross-contamination or misidentification. It is useful to have a series of photographs of cell types in regular use (*see* Fig. 16.2 and Plates 9 and 10), taken at different cell densities (preferably known cell densities, e.g., by counting the number of cells/cm$^2$) to refer to when handling cultures, particularly when a new member of staff is being introduced to culture work.

### 13.6.2 Replacement of Medium

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

(1) **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.

(2) **Cell Concentration.** Cultures at a high cell concentration 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.

(3) **Cell Type.** Normal cells (e.g., diploid fibroblasts) usually stop dividing at a high cell density (*see* Section 18.5.2), 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.

(4) **Morphological Deterioration.** This factor must be anticipated by regular examination and familiarity with the cell line (*see* Section 13.6.1). If deterioration is allowed to progress too far, it will be irreversible, as the cells will tend to enter apoptosis (*see* Section 3.3.1).

***Volume, depth, and surface area.*** The usual ratio of medium volume to surface area is 0.2–0.5 mL/cm$^2$ (*see also* Section 21.9.3). The upper limit is set by gaseous diffusion through the liquid layer, and the optimum ratio depends on the oxygen requirement of the cells. Cells with a high $O_2$ requirement do better in shallow medium (e.g., 2 mm), and those with a low requirement may do better in deep medium (e.g., 5 mm). If the depth of the medium is greater than

5 mm, then gaseous diffusion may become limiting. With monolayer cultures, this problem can be overcome by rolling the bottle (*see* Section 26.2.3) or perfusing the culture with medium and arranging for gas exchange in an intermediate reservoir (*see* Section 25.3.2, 26.2.5).

***Holding medium.*** A *holding medium* may be used when stimulation of mitosis, which usually accompanies a medium change, even at high cell densities, is undesirable. Holding media are usually regular media with the serum concentration reduced to 0.5% or 2% or eliminated completely. For serum-free media, growth factors and other mitogens are omitted. This omission inhibits mitosis in most untransformed cells. Transformed cell lines are unsuitable for this procedure, as either they may continue to divide successfully or the culture may deteriorate, because transformed cells do not block in a regulated fashion in $G_1$ of the cell cycle (*see* Section 3.3.1).

Holding media are used to maintain cell lines with a finite life span without using up the limited number of cell generations available to them (*see* Section 3.8.1). Reduction of serum and cessation of cell proliferation also promote expression of the differentiated phenotype in some cells [Maltese & Volpe, 1979; Schousboe et al., 1979]. Media used for the collection of biopsy samples can also be referred to as holding media.

***Standard feeding protocol.*** Protocol 13.1 is designed to accompany Exercise 4, using medium prepared from Exercise 3 (*see* Chapter 2). The cells and media are specified, but can easily be changed to suit individual requirements.

**PROTOCOL 13.1. FEEDING A MONOLAYER CULTURE**

***Outline***

Examine the culture by eye and on an inverted microscope. If indicated, e.g., by a fall in pH, remove the old medium and add fresh medium. Return the culture to the incubator.

***Materials***

*Sterile:*

- ❑ Cell cultures: A549 cells 4 days after seeding at $2 \times 10^4$ cells/mL, 25 $cm^2$ flasks ..... 4
- ❑ Growth medium ..... 100 mL
  e.g., Eagle's 1×MEM with Hanks's salts and 4 mM $HCO_3$, without antibiotics.
  If the training program is being followed, use the two media prepared in Exercise 3, one of which has been stored at 4°C and one at 37°C, for one week.
- ❑ Pipettes, graduated, and plugged. If glass, an assortment of sizes, 1 mL, 5 mL, 10 mL, 25 mL, in a square pipette can, or, if plastic, individually wrapped and sorted by size on a rack
- ❑ Unplugged pipettes for aspirating medium if pump or vacuum line is available

*Nonsterile:*

- ❑ Pipetting aid or bulb (*see* Figs. 5.5, 6.6)
- ❑ Tubing to receiver connected to vacuum line or to receiver via peristaltic pump (*see* Figs. 5.1–5.3)
- ❑ Alcohol, 70%, in spray bottle
- ❑ Lint-free swabs or wipes
- ❑ Absorbent paper tissues
- ❑ Pipette cylinder containing water and disinfectant (*see* Sections 5.8.8, 7.8.5)
- ❑ Marker pen with alcohol-insoluble ink
- ❑ Notebook, pen, protocols, etc.

***Protocol***

1. Prepare the hood by ensuring that it is clear and swabbing it with 70% alcohol.
2. Bring the reagents and materials necessary for the procedure, swab bottles with 70% alcohol and place items required immediately in the hood (*see* Protocol 6.1)
3. Examine the culture carefully for signs of contamination or deterioration (*see* Figs. 13.1, 19.1).
4. Check the previously described criteria—pH and cell density or concentration—and, based on your knowledge of the behavior of the culture, decide whether or not to replace the medium. If feeding is required, proceed as follows.
5. Take the culture to the sterile work area.
6. Uncap the flask.
7. Take sterile pipette and insert into bulb or pipetting aid, or, selecting an unplugged pipette, connect to vacuum line or pump.
8. Withdraw the medium, and discard into waste beaker (*see* Fig. 6.8). Or, preferably, aspirate medium via a suction line in the hood connected to an external pump (*see* Figs. 5.2, 5.3).
9. Discard pipette.
10. Uncap the medium bottle.
11. Take a fresh pipette and add the same volume of fresh medium as was removed, prewarmed to 37°C if it is important that there be no check in cell growth, and recap the bottle.
12. Discard the pipette.
13. Recap the flask and the medium bottle.
14. Return the culture to the incubator.
15. Complete record of observations and feeding on record sheet or lab book.
16. Clear away all pipettes, glassware, etc., and swab down the work surface.

***Note.*** When a culture is at a low density and growing slowly, it may be preferable to half-feed it—i.e., to remove only half of the medium at Step 8 and replace it in Step 11 with the same volume as was removed.

## 13.7 SUBCULTURE

When a cell line is subcultured the regrowth of the cells to a point ready for the next subculture usually follows a standard pattern (Fig. 13.2). A *lag period* after seeding is followed by a period of exponential growth, called the *log phase*. When the *cell density* (cells/cm$^2$ substrate) reaches a level such that all of the available substrate is occupied, or when the *cell concentration* (cells/mL medium) exceeds the capacity of the medium, growth ceases or is greatly reduced (*see* Fig. 16.2b,d,f,h,j,l and Plate 4d). Then either the medium must be changed more frequently or the culture must be divided. For an adherent cell line, dividing a culture, or *subculture* as it is called, usually involves removal of the medium and dissociation of the cells in the monolayer with trypsin, although some loosely adherent cells (e.g., HeLa-$S_3$) may be subcultured by shaking the bottle, collecting the cells in the medium, and diluting as appropriate in fresh medium in new bottles. Exceptionally, some cell monolayers cannot be dissociated in trypsin and require the action of alternative proteases, such as pronase, dispase, and collagenase (Table 13.4). Of these proteases, pronase is the most effective but can be toxic to some cells. Dispase and collagenase are generally less toxic than trypsin but may not give complete dissociation of epithelial cells. Other proteases, such as Accutase, Accumax (invertebrate proteases), and Trypzean or TrypLE (recombinant trypsins), are available, and their efficacy should be tested where either there is a problem with standard disaggregation protocols or there is a need to avoid mammalian (e.g., porcine trypsin) or bacterial (e.g., Pronase) proteases. The severity of the treatment required depends on the cell type, as does the sensitivity of the cells to proteolysis, and a protocol should be selected with the least severity that is compatible with the generation of a single-cell suspension of high viability.

The attachment of cells to each other and to the culture substrate is mediated by cell surface glycoproteins and $Ca^{2+}$ (*see* Section 3.2). Other proteins, and proteoglycans, derived from the cells and from the serum, become associated with the cell surface and the surface of the substrate and facilitate cell adhesion. Subculture usually requires chelation of $Ca^{2+}$ and degradation of extracellular matrix and, potentially, the extracellular domains of some cell adhesion molecules.

### 13.7.1 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

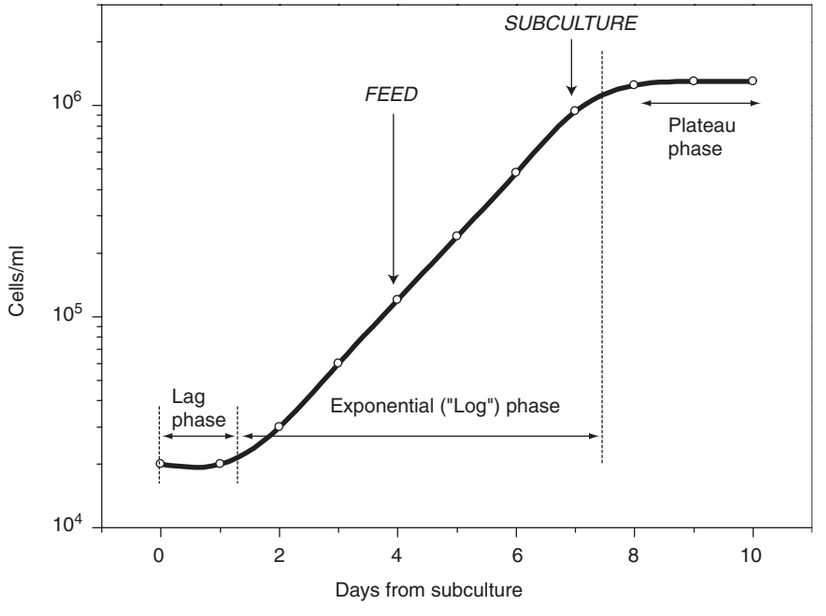


***Fig. 13.2. Growth Curve and Maintenance.*** Semilog plot of cell concentration versus time from subculture, showing the lag phase, exponential phase, and plateau, and indicating times at which subculture and feeding should be performed (*see also* Section 21.9.2 and Fig. 21.6).

**TABLE 13.4.** Cell Dissociation Procedures

| Procedure | Pretreatment | Dissociation agent | Medium | Applicable to |
|---|---|---|---|---|
| Shake-off | None | Gentle mechanical shaking, rocking, or vigorous pipetting | Culture medium | Mitotic or other loosely adherent cells |
| Scraping | None | Cell scraper | Culture medium | Cell lines for which proteases are to be avoided (e.g., receptor or cell surface protein analysis); can damage some cells and rarely gives a single-cell suspension |
| Trypsin* alone | Remove medium completely | 0.01–0.5% Crude trypsin; usually 0.25% | D-PBSA, CMF, or saline citrate | Most continuous cell lines |
| Prewash + trypsin | D-PBSA | 0.25% Crude trypsin | D-PBSA | Some strongly adherent continuous cell lines and many early-passage cells |
| Prewash + trypsin | 1 mM EDTA in D-PBSA | 0.25% Crude trypsin | D-PBSA | Strongly adherent early-passage cell lines |
| Prewash + trypsin | 1 mM EDTA in D-PBSA | 0.25% Crude trypsin | D-PBSA + 1 mM EDTA | Many epithelial cells, but some can be sensitive to EDTA; EGTA can be used |
| Trypsin + collagenase | 1 mM EDTA in D-PBSA | 0.25% Crude trypsin; 200 U/mL crude collagenase | D-PBSA + 1 mM EDTA | Dense cultures and multilayers, particularly with fibroblasts |
| Dispase | None | 0.1–1.0 mg/mL Dispase | Culture medium | Removal of epithelium in sheets (does not dissociate epithelium) |
| Pronase | None | 0.1–1.0 mg/mL Pronase | Culture medium | Provision of good single-cell suspensions, but may be harmful to some cells |
| DNase | D-PBSA or 1 mM EDTA in D-PBSA | 2–10 μg/mL crystalline DNase | Culture medium | Use of other dissociation agents which damage cells and release DNA |

*Digestive enzymes are available (Difco, Worthington, Roche, Sigma) in varying degrees of purity. Crude preparations—e.g., Difco trypsin, 1:250, or Worthington CLS-grade collagenase—contain other proteases that may be helpful in dissociating some cells, but may be toxic to other cells. Start with a crude preparation, and progress to purer grades if necessary. Purer grades are often used at a lower concentration (μg/mL), as their specific activities (enzyme units/g) are higher. Purified trypsin at 4°C has been recommended for cells grown in low-serum concentrations or in the absence of serum [McKeehan, 1977] and is generally found to be more consistent. Batch testing and reservation, as for serum, may be necessary for some applications.

to deteriorate after about two doublings, and reseeding efficiency will decline.

(2) **Exhaustion of Medium.** Exhaustion of the medium (*see* Section 13.6.2) 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 (*see* Section 19.3).

(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.

(4) **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).

***Handling different cell lines.*** Different cell lines should be handled separately, with a separate set of media and reagents. If they are all handled at the same time, there is a significant risk of cross-contamination, particularly if a rapidly growing line, such as HeLa, is maintained alongside a slower-growing line (*see* Section 19.5).

***Typical subculture protocol for cells grown as a monolayer.*** Protocol 13.2 describes trypsinization of a monolayer (Fig. 13.3; Plates 7–12) after an EDTA prewash to remove traces of medium, divalent cations, and serum (if used). This procedure can be carried out without the prewash, or with only D-PBSA as a prewash, and with 1 mM EDTA in the trypsin if required, depending on the type of cell (*see* Table 13.4).

The amounts of materials specified are designed for use with Exercise 13 in Chapter 2, but can be varied as required.

## PROTOCOL 13.2. SUBCULTURE OF MONOLAYER CELLS

### Outline

Remove the medium. Expose the cells briefly to trypsin. Incubate the cells. Disperse the cells in medium. Count the cells. Dilute and reseed the subculture.

### Materials

*Sterile:*

- ❑ A549 cells, 7 days and 14 days after seeding at $2 \times 10^4$ cells/mL, 25-$cm^2$ flasks ..... 1 each
- ❑ WI-38, MRC-5, or an equivalent normal diploid fibroblast culture, 7 days and 14 days after seeding at $2 \times 10^4$ cells/mL, 25-$cm^2$ flasks ..... 1 each
- ❑ Growth medium, e.g., 1×MEM with Earle's salts, 23 mM $HCO_3$, without antibiotics .. 100 mL
- ❑ Trypsin, 0.25% in D-PBSA (*see* Table 13.4) ..... 10 mL
- ❑ D-PBSA with 1 mM EDTA ..... 20 mL
- ❑ Pipettes, graduated, and plugged. If glass, an assortment of sizes, 1 mL, 5 mL, 10 mL, 25 mL, in a square pipette can, or, if plastic, individually wrapped and sorted by size on a rack
- ❑ Unplugged pipettes for aspirating medium if pump or vacuum line is available
- ❑ Universal containers or 50-mL centrifuge tubes ..... 4
- ❑ Culture flasks, 25 $cm^2$ ..... 8

*Nonsterile:*

- ❑ Pipetting aid or bulb (*see* Figs. 5.5, 6.6)
- ❑ Tubing to receiver connected to vacuum line or to receiver via peristaltic pump (*see* Figs. 5.1–5.3)
- ❑ Alcohol, 70%, in spray bottle
- ❑ Lint-free swabs or wipes
- ❑ Absorbent paper tissues
- ❑ Pipette cylinder containing water and disinfectant (*see* Sections 5.8.8, 7.8.5)
- ❑ Marker pen with alcohol-insoluble ink
- ❑ Notebook, pen, protocols, etc.
- ❑ Hemocytometer or electronic cell counter

### Protocol

1. Prepare the hood, and bring the reagents and materials to the hood to begin the procedure (*see* Section 6.5).
2. Examine the cultures carefully for signs of deterioration or contamination (*see* Figs. 13.1, 19.1).
3. Check the criteria (*see* Section 13.7.1), and, based on your knowledge of the behavior of the culture, decide whether or not to subculture. (Those following Exercise 13 should make particular note of the density and condition of the cells, e.g., evidence of mitoses, multilayering, cellular deterioration.) If subculture is required, proceed as follows.
4. Take the culture flasks to a sterile work area, and remove and discard the medium (*see* Protocol 13.1, Steps 7–9). Handle each cell line separately, repeating this procedure from this step for each cell line handled.
5. Add D-PBSA/EDTA prewash (0.2 mL/$cm^2$) to the side of the flasks opposite the cells so as to avoid dislodging cells, rinse the prewash over the cells, and discard. This step is designed to remove traces of serum that would inhibit the action of the trypsin and deplete the divalent cations, necessary for cell adhesion.
6. Add trypsin (0.1 mL/$cm^2$) to the side of the flasks opposite the cells. Turn the flasks over and lay them down. Ensure that the monolayer is completely covered. Leave the flasks stationary for 15–30 s.
7. Raise the flasks to remove the trypsin from the monolayer and quickly check that the monolayer is not detaching. Using trypsin at 4°C helps to prevent premature detachment, if this turns out to be a problem.

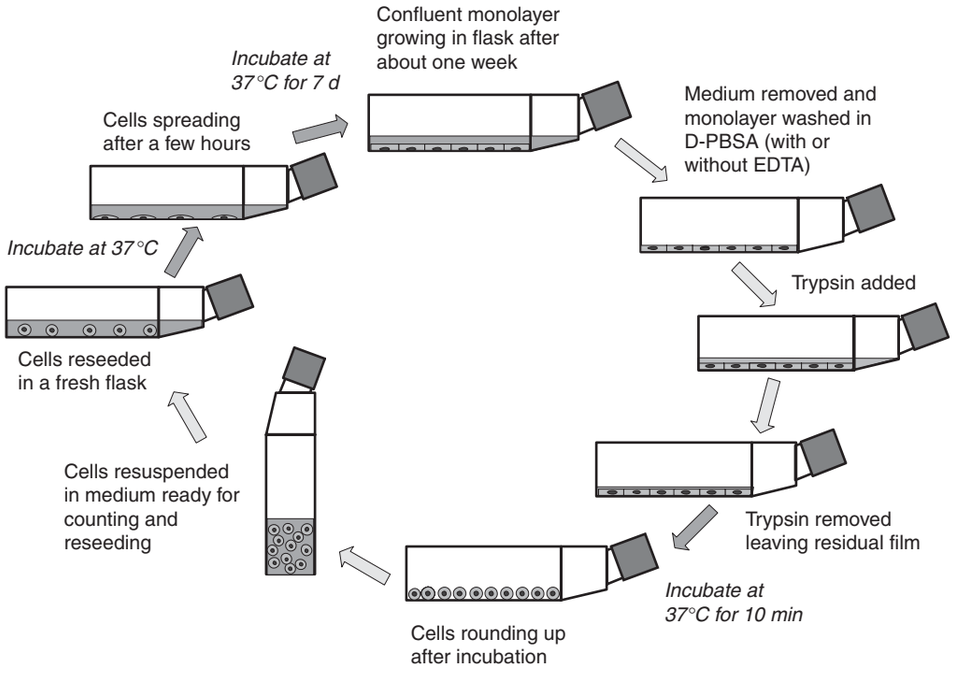


***Fig. 13.3. Subculture of Monolayer.*** Stages in the subculture and growth cycle of monolayer cells after trypsinization (*see also* Plates 4, 5).

**8.** Withdraw all but a few drops of the trypsin.

**9.** Incubate, with the flasks lying flat, until the cells round up (Figs. 13.3, 13.4, Plate 5); when the bottle is tilted, the monolayer should slide down the surface. (This usually occurs after 5–15 min.) Do not leave the flasks longer than necessary, but on the other hand, do not force the cells to detach before they are ready to do so, or else clumping may result.

***Note.*** In each case, the main dissociating agent, be it trypsin or EDTA, is present only briefly, and the incubation is performed in the residue after most of the dissociating agent has been removed. If you encounter difficulty in getting cells to detach and, subsequently, in preparing a single-cell suspension, you may employ alternative procedures (*see* Table 13.4).

**10.** Add medium (0.1–0.2 mL/cm$^2$), and disperse the cells by repeated pipetting over the surface bearing the monolayer.

**11.** Finally, pipette the cell suspension up and down a few times, with the tip of the pipette resting on the bottom corner of bottle, taking care not to create a foam. The degree of pipetting required will vary from one cell line to another; some cell lines disperse easily, whereas others require vigorous pipetting in order to disperse them. Almost all cells incur mechanical damage from shearing forces if pipetted too vigorously. Primary suspensions and early-passage cultures are particularly prone to damage, partly because of their greater fragility and partly because of their larger size, but continuous cell lines are usually more resilient and require vigorous pipetting for complete disaggregation. Pipette the suspension up and down sufficiently to disperse the cells into a single-cell suspension. If this step is difficult, apply a more aggressive dissociating agent (*see* Table 13.4).

A single-cell suspension is desirable at subculture to ensure an accurate cell count and uniform growth on reseeding. It is essential if quantitative estimates of cell proliferation or of plating efficiency are being made and if cells are to be isolated as clones.

**12.** Count the cells with a hemocytometer or an electronic particle counter (*see* Section 21.1), and record the cell counts.

**13.** Dilute the cell suspensions to the appropriate seeding concentration:

**(a)** By adding the appropriate volume of cell suspension to a premeasured volume of medium in a culture flask

or

**(b)** By diluting the cells to the total volume required and distributing that volume among several flasks.

Procedure (a) is useful for routine subculture when only a few flasks are used and precise cell counts and reproducibility are not critical, but procedure (b) is preferable when setting up several replicates, because the total number of manipulations is reduced and the concentrations of cells in each flask will be identical.

For Exercise 13, use procedure (b): dilute the cells from each flask to $2 \times 10^4$ cells per mL in 20 mL medium, and seed 5 mL from each suspension into two flasks.

**14.** If the cells are grown in elevated $CO_2$ (as indicated by the use of Eagle's MEM with Earle's salts and 23 mM $NaHCO_3$, as listed in Materials above), gas the flask by blowing the correct gas mixture (in this case 5% $CO_2$) from a premixed cylinder, or a gas blender, through a filtered line into the flask above the medium (*see* Fig. 6.11). Do not bubble gas through the medium, as doing so will generate bubbles, which can denature some constituents of the medium and increase the risk of contamination. If the normal gas phase is air, as with Eagle's MEM with Hanks' salts (*see* Protocol 13.1), this step may be omitted.

**15.** Cap the flasks, and return them to the incubator. Check the pH after about 1 h. If the pH rises in a medium with a gas phase of air, then return the flasks to the aseptic area and gas the culture briefly (1–2 s) with 5% $CO_2$. As each culture will behave predictably in the same medium, you eventually will know which cells to gas when they are reseeded, without having to incubate them first. If the pH rises in medium that already has a 5% $CO_2$ gas phase (as in this case), either increase the $CO_2$ to 7% or 10% or add sterile 0.1 N of HCl.

**16.** Repeat this procedure from Step 4 for the second cell line.

***Note.*** The procedure in Step 15 should not become a long-term solution to the problem of high pH after subculture. If the problem persists, then reduce the pH of the medium at the time it is made up, and check the pH of the medium in the incubator or in a gassed flask.

As the expansion of air inside plastic flasks causes larger flasks to swell and prevents them from lying flat, the pressure should be released by briefly slackening the cap 30 min after placing the flask in the incubator. Alternatively, this problem may be prevented by compressing the top and bottom of large flasks before sealing them (care must be taken not to exert too much pressure and crack the flasks). Incubation restores the correct shape as the gas phase expands.

### 13.7.2 Growth Cycle and Split Ratios

Routine passage leads to the repetition of a standard growth cycle (Fig. 13.4a; *see also* Fig. 13.2). It is essential to become familiar with this cycle for each cell line that is handled, as it controls the seeding concentration, the duration of growth before subculture, the duration of experiments, and the appropriate times for sampling to give greatest consistency. Cells at different phases of the growth cycle behave differently with respect to cell proliferation, enzyme activity, glycolysis and respiration, synthesis of specialized products, and many other properties (*see* Sections 21.9.4, 25.1.1).

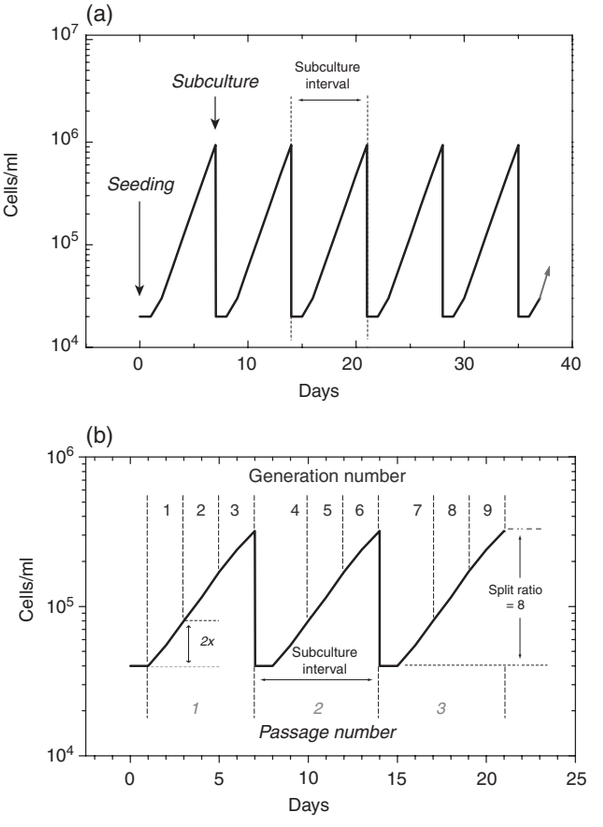


***Fig. 13.4. Serial Subculture.*** (a) Repetition of the standard growth cycle during propagation of a cell line: If the cells are growing correctly, then they should reach the same concentration (peaks) after the same time in each cycle, given that the seeding concentration (troughs) and subculture interval remain constant. (b) Generation number and passage: Each subculture represents one passage, but the generation number (in this case 3 per passage) depends on the split ratio (8 for 3 doublings per passage).

For finite cell lines, it is convenient to reduce the cell concentration at subculture by 2-, 4-, 8-, or 16-fold (i.e., a split ratio of 2, 4, 8, and 16, respectively), making the calculation of the number of population doublings easier (i.e., respectively, a split ratio of 2 corresponds to 1 population doubling, 4 to 2, 8 to 3, and 16 to 4); for example, a culture divided 8-fold requires three doublings to return to the same cell density (Fig. 13.4b). A fragile or slowly growing line should also be split 1:2, whereas a vigorous, rapidly growing normal cell line can be split 1:8 or 1:16 and some continuous cell lines may be split 1:50 or 1:100. Once a cell line becomes continuous (usually taken as beyond 150 or 200 generations), the cell concentration is the main parameter and the culture should be cut back to between $10^4$ and $10^5$ cells/mL. The split ratio, or dilution, is also chosen to establish a convenient subculture interval, perhaps 1 or 2 weeks, and to ensure that the cells (1) are not diluted below the concentration that permits them to reenter the growth cycle within a reasonable lag period (24 h or less) and (2) do not enter a plateau before the next subculture.

When handling a cell line for the first time, or when using an early-passage culture with which you have little experience, it is good practice to subculture the cell line to a split ratio of 2 or 4 at the first attempt, noting the cell concentrations as you do so. As you gain experience and the cell line seems established in the laboratory, it may be possible to increase the split ratio—i.e., to reduce the cell concentration after subculture—but always keep one flask at a low split ratio when attempting to increase the split ratio of the rest.

Even when a split ratio is used to determine the seeding concentration at subculture, the number of cells per flask should be recorded after trypsinization and at reseeding, so that the growth rate can be estimated at each subculture and the consistency can be monitored (*see* Protocols 21.7–21.9). Otherwise, minor alterations will not be detected for several passages.

### 13.7.3 Cell Concentration at Subculture

The ideal method of determining the correct seeding density is to perform a growth curve at different seeding concentrations (*see* Protocols 21.7–21.9) and thereby determine the minimum concentration that will give a short lag period and early entry into rapid logarithmic growth (i.e., a short population-doubling time) but will reach the top of the exponential phase at a time that is convenient for the next subculture.

As a general rule, most continuous cell lines subculture satisfactorily at a seeding concentration of between $1 \times 10^4$ and $5 \times 10^4$ cells/mL, finite fibroblast cell lines subculture at about the same concentration, and more fragile cultures, such as endothelium and some early-passage epithelial cells, subculture at around $1 \times 10^5$ cells/mL. For a new culture, start at a high seeding concentration and gradually reduce until a convenient growth cycle is achieved without any deterioration in the culture.

### 13.7.4 Propagation in Suspension

Protocol 13.2 refers to the subculture of monolayers, because the manner in which most primary cultures and cell lines grow. However, cells that grow continuously in suspension, either because they are nonadhesive (e.g., many leukemias and murine ascites tumors) or because they have been kept in suspension mechanically, or selected, may be subcultured like bacteria or yeast. Suspension cultures have a number of advantages (Table 13.5); for example, trypsin treatment is not required so subculture is quicker and less traumatic for the cells, and scale-up is easier (*see also* Section 26.1). Replacement of the medium (feeding) is not usually carried out with suspension cultures, and instead, the culture is either diluted and expanded, diluted and the excess discarded, or the bulk of the cell suspension is withdrawn and the residue is diluted back to an appropriate seeding concentration. In each case, a growth cycle will result, similar to that for monolayer cells, but usually with a shorter lag period.

Cells that grow spontaneously in suspension can be maintained in regular culture flasks, which need not be tissue culture treated (although they must be sterile, of course). The rules regarding the depth of medium in static cultures are as for monolayers—i.e., 2–5 mm to allow for gas exchange. When the depth of a suspension culture is increased—e.g., if it is expanded—the medium requires agitation, which is best achieved with a suspended rotating magnetic pendulum, with the culture flask placed on a magnetic stirrer (Fig. 13.5; *see also* Section 26.1). Roller bottles rotating on a rack can also be used to agitate suspension cultures (*see* Table 8.1 and Fig. 26.11).

### 13.7.5 Subculture of Cells Growing in Suspension

Protocol 13.3 describes routine subculture of a suspension culture into a fresh vessel. A continuous culture can be

**TABLE 13.5.** Monolayer vs. Suspension Culture

| Monolayer | Suspension |
|---|---|
| **Culture requirements** | |
| Cyclic maintenance | Steady state |
| Trypsin passage | Dilution |
| Limited by surface area | Volume (gas exchange) |
| **Growth properties** | |
| Contact inhibition | Homogeneous suspension |
| Cell interaction | |
| Diffusion boundary | |
| **Useful for** | |
| Cytology | Bulk production |
| Mitotic shake-off | Batch harvesting |
| *In situ* extractions | |
| Continuous product harvesting | |
| **Applicable to** | |
| Most cell types, including primaries | Only transformed cells |

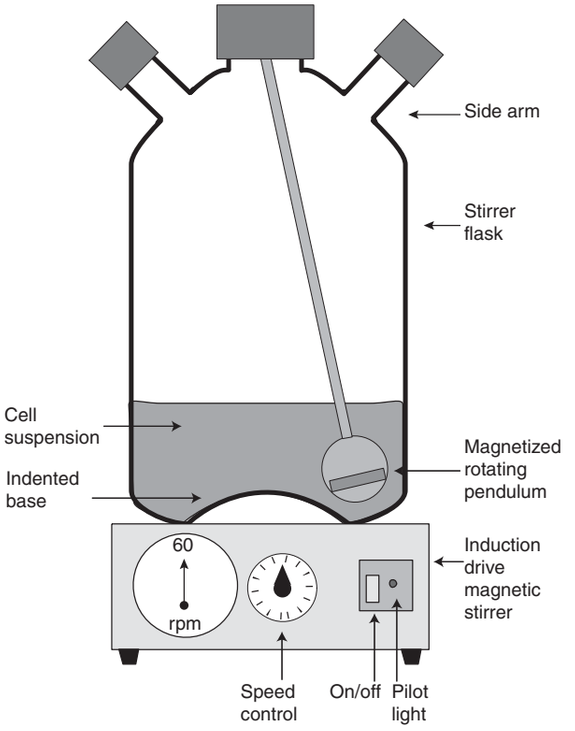


***Fig. 13.5. Stirrer Culture.*** A small stirrer flask, based on the Techne design, with a capacity of 250–1000 mL. The cell suspension is stirred by a pendulum, which rotates in an annular depression in the base of the flask.

maintained in the same vessel, but the probability of contamination gradually increases with any buildup of minor spillage on the neck of the flask during dilution. The criteria for subculture are similar to those for monolayers:

(1) **Cell Concentration,** which should not exceed $1 \times 10^6$ 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.

Protocol 13.3 is designed for use in conjunction with Exercise 12 in Chapter 2. The cell lines, culture vessels, and other parameters may be adjusted to suit other cell lines as required.

## PROTOCOL 13.3. SUBCULTURE IN SUSPENSION

### *Outline*

Withdraw a sample of the cell suspension, count the cells, and seed an appropriate volume of the cell suspension into fresh medium in a new flask, restoring the cell concentration to the starting level.

### *Materials*

*Sterile:*

- ❑ Starter culture: HL-60, L1210, or P388, 7 days and 10 days after seeding at $1 \times 10^4$ cells/mL, 25-$cm^2$ flasks ..... 1 each
- ❑ Growth medium, e.g., MEM with Spinner Salts (S-MEM) or RPMI 1640, with 23 mM $NaHCO_3$, 5% calf serum ..... 200 mL
- ❑ Pipettes, graduated, and plugged ..... 1 can of each
  If glass, an assortment of sizes, 1 mL, 5 mL, 10 mL, 25 mL, in a square pipette can, or, if plastic, individually wrapped and sorted by size on a rack
- ❑ Unplugged pipettes for aspirating medium if pump or vacuum line available ..... 1 can
- ❑ Universal containers or 50-mL centrifuge tubes ..... 4
- ❑ Stirrer flasks, 500 mL with magnetic pendulum stirrers (Techne, Bellco) ..... 2
- ❑ Culture flasks, 25 $cm^2$ ..... 4

*Nonsterile:*

- ❑ Pipetting aid or bulb (*see* Figs. 5.5, 6.6)
- ❑ Tubing to receiver connected to vacuum line or to receiver via peristaltic pump (*see* Figs. 5.1–5.3)
- ❑ Alcohol, 70%, in spray bottle
- ❑ Lint-free swabs or wipes
- ❑ Absorbent paper tissues
- ❑ Pipette cylinder containing water and disinfectant (*see* Sections 5.8.8, 7.8.5)
- ❑ Marker pen with alcohol-insoluble ink
- ❑ Notebook, pen, protocols, etc.
- ❑ Hemocytometer or electronic cell counter
- ❑ Magnetic stirrer platform

### *Protocol*

1. Prepare the hood, and bring the reagents and materials to the hood to begin the procedure (*see* Section 6.5).
2. Examine the culture carefully for signs of contamination or deterioration. This step is more difficult with suspension cultures than with monolayer cells, but cells that are in poor condition are indicated by shrinkage, an irregular outline, and/or granularity. Healthy cells should look clear and hyaline, with the nucleus visible on phase contrast, and are often found in small clumps in static culture.
3. Take the cultures to the sterile work area, remove a sample from them, and count the cells in the samples.

4. Based on the previously described criteria (*see* Section 13.7.1) and on your knowledge of the behavior of the culture, decide whether or not to subculture. If subculture is required (Exercise 12 will require subculture), proceed as follows.
5. Mix the cell suspension, and disperse any clumps by pipetting the cell suspension up and down.
6. Add 50 mL of medium to each of two stirrer flasks.
7. Add 5 mL of medium to each of four 25-$cm^2$ flasks.
8. Add a sufficient number of cells to give a final concentration of $1 \times 10^5$ cells/mL for slow-growing cells (36–48 h doubling time) or $2 \times 10^4$/mL for rapidly growing cells (12–24 h doubling time). For the cells cited in Materials, use a final concentration of $1 \times 10^4$ cells/mL.
9. Gas the cultures with 5% $CO_2$.
10. Cap the flasks, and take to incubator. Lay the flasks flat, as for monolayer cultures.
11. Cap the stirrer flasks and place on magnetic stirrers set at 60–100 rpm, in an incubator or hot room at 37°C. Take care that the stirrer motor does not overheat the culture. Insert a polystyrene foam mat under the bottle if necessary. Induction-driven stirrers generate less heat and have no moving parts.

Suspension cultures have a number of advantages (*see* Table 13.5). First, the production and harvesting of large quantities of cells may be achieved without increasing the surface area of the substrate (*see* Section 26.1). Furthermore, if dilution of the culture is continuous and the cell concentration is kept constant, then a steady state can be achieved; this steady state is not readily achieved in monolayer cultures. Maintenance of monolayer cultures is essentially cyclic, with the result that growth rate and metabolism vary, depending on the phase of the growth cycle.

### 13.7.6 Standardization of Culture Conditions

Standardization of culture conditions is essential for maintaining phenotypic stability. Although some conditions may alter because of the demands of experimentation, development, and production, routine maintenance should adhere to standard, defined conditions.

***Medium.*** The type of medium used will influence the selection of different cell types and regulate their phenotypic

**TABLE 13.6.** Data Record, Feeding

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

| | | | | | |
|---|---|---|---|---|---|
| | Date: | | | | |
| **Cell line** | Designation | | | | |
| | Primary or subculture | | | | |
| | Generation or pass no. | | | | |
| | | | | | |
| **Status** | Phase of growth cycle | | | | |
| | Appearance of cells | | | | |
| | Density of cells | | | | |
| | pH of medium (approx.) | | | | |
| | Clarity of medium | | | | |
| | | | | | |
| **Medium** | Type | | | | |
| | Batch no. | | | | |
| | Serum type and concentration | | | | |
| | Batch no. | | | | |
| | Other additives | | | | |
| | $CO_2$ concentration | | | | |
| | | | | | |
| **Other parameters** | | | | | |

**TABLE 13.7.** Data Record, Subculture

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

| | | | | | |
|---|---|---|---|---|---|
| | Date: | | | | |
| **Cell line** | Designation | | | | |
| | Generation or pass no. | | | | |
| **Status before subculture** | Phase of growth cycle | | | | |
| | Appearance of cells | | | | |
| | Density of cells | | | | |
| | pH of medium (approx.) | | | | |
| | Clarity of medium | | | | |
| **Dissociation agent** | Prewash | | | | |
| | Trypsin | | | | |
| | EDTA | | | | |
| | Other | | | | |
| | Mechanical | | | | |
| **Cell count** | Concentration after resuspension ($C_I$) | | | | |
| | Volume ($V_I$) | | | | |
| | Yield ($Y = C_I \times V_I$) | | | | |
| | Yield per flask | | | | |
| **Seeding** | Number ($N$) & type of vessel (flask, dish, or plate wells) | | | | |
| | Final concentration ($C_F$) | | | | |
| | Volume per flask, dish, or well ($V_F$) | | | | |
| | Split ratio ($Y \div C_F \times V_F \times N$), or number of flasks seeded ÷ number of flasks trypsinized, where the flasks are of same size | | | | |
| **Medium/serum** | Type | | | | |
| | Batch no. | | | | |
| | Serum type and concentration | | | | |
| | Batch no. | | | | |
| | Other additives | | | | |
| | $CO_2$ concentration | | | | |
| **Matrix coating** | e.g., fibronectin, Matrigel, collagen | | | | |
| **Other parameters** | | | | | |
| | | | | | |
| | | | | | |

expression (*see* Sections 9.6, 10.2.1, 10.2.2, 17.2, 17.7). Consequently, once a medium has been selected, standardize on that medium, and preferably on one supplier, if the medium is being purchased ready made.

***Serum.*** The best method of eliminating serum variation is to convert to a serum-free medium (*see* Section 10.2), although, unfortunately, serum-free formulations are not yet available for all cell types, and the conversion may be costly and time consuming. Serum substitutes (*see* Section 10.5.2) may offer greater consistency and are generally cheaper than serum or growth factor supplementation but do not offer the control over the physiological environment afforded by serum-free medium. If serum is required, select a batch (*see* Section 9.6.1), and use that batch throughout each stage of culture including cryopreservation (*see* Section 20.2).

***Plastics.*** Most of the leading brands of culture flasks and dishes will give similar results, but there may be minor variations due to the treatment of the plastic for tissue culture (*see* Section 8.1.2). Hence it is preferable to adhere to one type of flask or dish and supplier.

***Cell line maintenance.*** Cell lines may alter their characteristics if maintained differently from a standard regime (*see* Section 13.6). The maintenance regime should be optimized (*see* Section 13.7) and then remain consistent throughout the handling of the cell line (*see* Sections 13.7.1, 13.7.2, 13.7.3). Cultures should also be replaced from frozen stocks at regular intervals (*see* Section 20.4.2).

### 13.7.7 Use of Antibiotics

The continuous use of antibiotics encourages cryptic contaminations, particularly mycoplasma, and the development of antibiotic-resistant organisms (*see* Section 9.4.7). It may also interfere with cellular processes under investigation. However, there may be circumstances for which contamination is particularly prevalent or a particularly valuable cell line is being carried, and in these cases, antibiotics may be used. If they are used, then it is important to maintain some antibiotic-free stocks in order to reveal any cryptic contaminations; these stocks can be maintained in parallel, and stock may be alternated in and out of antibiotics (*see* Fig. 13.6) until antibiotic-free culture is possible. It is not advisable to adopt this procedure as a permanent regime, and, if a chronic contamination is suspected, the cells should be discarded or the contamination eradicated (*see* Sections 19.4.4, 28.6, 28.8.3), and then you may revert to antibiotic-free maintenance.

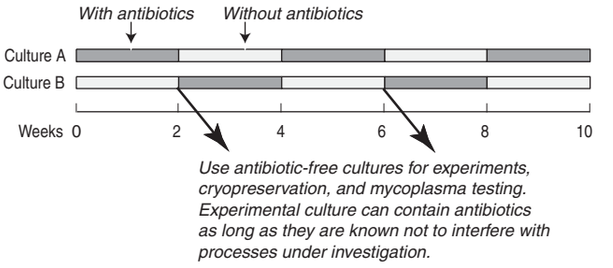


***Fig. 13.6. Parallel Cultures and Antibiotics.*** A suggested scheme for maintaining parallel cell cultures with and without antibiotics, such that each culture always spends part of the time out of antibiotics.

### 13.7.8 Maintenance Records

Keep details of routine maintenance, including feeding and subculture (Tables 13.6, 13.7), and deviations or changes should be added to the database record for that cell line. Such records are required for GLP [Food and Drug Administration, 1992; Department of Health and Social Security, 1986; Organisation for Economic Co-operation and Development, 2004], as for primary culture records (*see* 12.3.11), but are also good practice in any laboratory. If standard procedures are defined, then entries need say only ''Fed'' or ''Subcultured,'' with the date and a note of the cell numbers. Any comments on visual assessment and any deviation from the standard procedure should be recorded as well.

This set of records forms part of the continuing provenance of the cell line, and all data, or at least any major event—e.g., if the medium supplier is changed, the line is cloned, transfected, or changed to serum-free medium—should be entered in the database.