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Chapter 8

Animal Biotechnology

ANIMAL CELL SCIENCE AND TECHNOLOGY

Animal Cell Science focuses on the biological issues that subtend the productivity of animal cells in culture. Cell culture is no longer considered as a technique to create cell lines but as an indispensable science to understand the various cell-signaling mechanisms, the switches within the cells that control growth by the control of gene expression. In simple terms, it is the basic requisite for understanding of the intercellular and intracellular activities of a cell. Cultivation of animal cells is extremely important to the biotechnology industry. With recent advances in research in manipulating the environment to enhance cellular growth in vitro, novel compounds for therapeutic and prophylactic use have also been produced. Animal cell culture systems have been designed to yield the same therapeutic molecules in large scale. Prokaryotic cells have been genetically engineered to produce clinically important compounds. These recombinant molecules fail to give the desired results in eukaryotic systems owing to the differences in glycosylation. Eukaryotic cells are preferred for production and for studying the toxicity as well as the efficacy of the molecules. In many biomedical applications, tissue culture methods were developed which replaced the need for animal experimentation. In vitro pharmacotoxicology is now an established discipline for studying the drug activities and designing new forms of therapy. Currently cell culture methods are being used for histotypic cultures leading to formation of organs. Hybridoma technology helps in mass production of monoclonal antibodies from cell culture medium. Viral vaccines can be produced by mass culturing of host cells. Recombinant DNA technology uses cultured cells as a source of mRNA, gene sequence or as an expression vector for rDNA. Cell culture systems can now be used efficiently in conjunction with transfection methodology to analyze the function of genes. These systems help to evolve mechanisms to modify cell growth and differentiation which may have a major impact on our approach to understand human and animal diseases.

8.1 | Structure and Organization of Animal Cell

Cells are classified by the fundamental units of their structure and by the way they obtain energy. An animal cell is surrounded by a membrane called plasma membrane.

Plasma membrane is the lipid–protein–carbohydrate complex, providing a differentially permeable membrane through which transport of extracellular substances occurs. ***Nucleus*** has a double membrane surrounding the chromosomes and the nucleolus. Pores present in the nuclear membrane provide selective continuity between cytoplasmic and nuclear materials. The nucleolus disappears during cellular replication and is the site for synthesis of ribosome. ***Mitochondria*** are important organelles surrounded by a double membrane with the inner membrane thrown into a series of folds called cristae. Oxidation of food by Krebs cycle, production of energy by electron transport chain and beta-oxidation of fatty acids are important functions of these organelles. The mitochondrion contains its own DNA, and is believed to have originated as a captured bacterium. Although, ***rough endoplasmic reticulum*** (rER) is a network of interconnected membranes forming channels within the cell, covered with ribosomes (causing the 'rough' appearance), it provides surface for the biochemical reactions and also provides support to the colloidal complex of hyaloplasm. Ribosomes are sites for protein and RNA complexes responsible for protein synthesis. ***Smooth endoplasmic reticulum*** (sER) consists of a network of interconnected membranes forming channels within the cell. It is a site for synthesis and metabolism of lipids. It also contains enzymes for detoxifying chemicals including drugs and pesticides. ***Golgi apparatus*** stores the synthetic proteins and enzymes of cells. It has a series of stacked membranes. Vesicles (small membrane surrounded bags) carry materials from the rER to the golgi apparatus. Vesicles move between the stacks while the proteins are 'processed' to a mature form. Vesicles then carry newly formed membrane and secreted proteins to their final destinations including secretion or membrane localization. ***Lysosomes*** (only present in animal cells) are membrane-bound organelles that are responsible for degrading proteins and membranes in the cell, and also help degrade bacteria and foreign materials ingested by the cell. ***Vacuoles*** are storage depots for excess water, waste products and soluble pigments in plants. Peroxisomes or microbodies contain enzymes to degrade hydrogen peroxide, a toxic compound that can be produced during metabolism. Structure and organization of an animal cell have also been dealt with in Chapter 7.

8.2 | Primary Culture and Established Cell Line Cultures

8.2.1 | Historical Background

Cell lines have been cultured since 1830, and a number of them have been cited in literature. In 1907, Ross Harrison made the first attempt to culture animal cells wherein he cultivated embryonic nerve cells of frog by using hanging drop method. After supplementing with chick embryo plasma, cells proliferated better. Animal cell culture gained eminence after the use of viruses for producing vaccines in late 1940s, and became indispensable after the production of polio vaccine in cultured cells.

The cell lines can be from different animal groups (insect cell lines, human cell lines) and from various tissues. Cell lines are available with National Centre for Cell Science (NCCS), Pune, India; European Collection of Animal Cell Cultures (ECACC), UK; and American Type Cell Culture Collection (ATCC), USA. Although an array of cell lines exist in these centers, a scientist may consider isolating a primary cell line by himself.

8.2.2 | Primary Cultures from Various Sources

Primary culture refers to the original culture before passage or subculture. The 'passage number' is the number of times this procedure is performed after the original isolation of

Table 8-1 | Historical background of animal tissue culture

Name	*Date*	*Breakthrough*
Roux	1885	Medullary plate of chick embryo on warm saline
Jolly	1903	In vitro cell survival and cell division in salamander leucocytes
Ross Harrison	1907	Published experiments showing frog embryo nerve fiber growth in vitro
		Cultured connective tissue cells for extended periods and showed heart muscle tissue contractility over 2–3 months
Alexis Carrel	1912	Aseptic techniques to tissue culture. Use of trypsin Use of embryo extracts/animal serum
Rous and Jones	1913	Use of antibiotics: penicillin/streptomycin
	1916	Use of laminar air-flow cabinets
	1940	Trypsinization was used to produce homogenous cell strain; tissue culture media
	1950	Environment: Temperature, OP, pH, essential metabolites, inorganic ions, hormones, extracellular matrix
Katherine Sanford, et al.	1940s–50s	Were the first to clone – from L-cells. Human tumor cells could give rise to continuous cell lines. Nonmalignant rodent cell culture to study effects of carcinogens/viruses
Margaret Gey and George Gey	1948	Observed contact inhibition among fibroblasts – the beginning of quantitative cell culture experimentation
Abercrombie and Heaysmam	1952	*Polio virus* in human E-cells; production of polio vaccine
Enders, et al.	1954	Human cell lines for production of vaccines – human and veterinary
Harry Eagle	1962 1970	Developed defined media and described attachment factors and feeder layers
Hayflick and Moorhead	1955	Described the finite lifespan of normal human diploid cells.
	1961	Published methods for maintaining differentiated cells (of tumor origin)
Buonassisi, et al.	1962	Studied the differentiation of normal myoblasts in vitro
David Yaffe	1968	Human foetal lung fibroblasts

cells from a primary source. When divided in two flasks they are passaged or subcultured into secondary cultures. Theoretically primary cultures can be established from any tissue. For obtaining best results, the choice of starting material should be judicious. Choice with regard to species, whether to obtain from adult or embryo, and whether one wants to work with normal or tumor are extremely important points to be considered before starting a primary culture. Necessary permission from the ethical committee must be obtained before embarking on work with human tissues.

The first choice with scientists is to use embryonic tissue as it can undergo maximum number of cell doublings. The only limitation is that there are phenotypic differences in fetal tissue, for example liver, as compared to adult tissue.

It is important to choose a particular tumor tissue type to derive maximum benefits if the experiment requires a particular cell type present abundantly in tumors as compared to normal tissue. Immortal cell lines are all of tumor origin. It is relatively easier to collect a biopsy of tumor tissue in case of humans. Alternately, normal tissue has advantage over transformed cell lines as there are rarely any pathological differences in established cell lines. The disadvantage of normal tissue is that the cells have a finite life. Small animals such as mouse, rat, guinea pig and rabbit are used abundantly for scientific

research. Experiments like organ perfusion are possible in animals and not in humans. But the biochemical characteristics of the tissue may be different for different species, and ultimately all scientific achievements are targeted to improve life of humans, therefore, establishing a primary cell culture will be very appropriate.

8.2.2.1 | *Advantages/Disadvantages of Primary Cultures*

Tissues obtained are sufficient for short-term studies. The expense and inconvenience of maintaining stocks of established cell lines are obviated. They are suited for vaccine production since the probability of in vitro transformation of cells to malignancy is minimized. The survival in conditioned media is easy as they are hard. But there are some disadvantages. They are heterogenous – as different cell types are present. They also require frequent sacrifice of animals. They are easily contaminated by latent viruses. Long-term experiments cannot be carried out as they have a finite lifespan.

8.2.3 | *Established Cell Lines/Continuous Cell Lines*

Primary cell lines go on dividing for a long time at quite a high rate and can be passaged easily. Mostly, a primary cell stops proliferating after a large number of passages. Some occasional cell lines can be passaged indefinitely in vitro. These are then called established cell lines. The transition from primary cell line to established cell line is rather smooth and gradual in some cases, but in others cell transformation or cell alteration may cause this change. The transformed cell multiplies faster and may soon outnumber the other cell population, thus becoming the predominant cell type in the culture. Primary cell lines have normal number of chromosomes but established cell lines have unusual number of chromosomes.

8.2.3.1 | *Characteristics of Established Cell Lines*

They have short doubling times and are invariably aneuploid. Irrespective of their origin, their requirements are the same. There is no evidence of spatial orientation. They grow in higher densities than primary cultures. They can grow from a single cell or as dilute inocula. If required they can be established in suspensions, unlike primary cultures.

8.2.3.2 | *Establishment of Continuous Cell Lines*

Spontaneous: A large number of established cell lines have been obtained in the absence of any known exposure to a transforming agent. Such lines include the RMP promegakaryocyte line derived from rat bone that retains several differentiated functions including the ability to synthesize factor VIII, antigen and fibrinogen. The fibroblast cell line 3T3-L1 is derived from mouse embryo and is capable of lipid accumulation. However, in general it is unusual to isolate cell lines spontaneously, and those that have been obtained are predominantly from fetal rodent tissue.

Chemical transformation: Methyl cholanthrene has been widely used to establish a variety of cell lines including the mouse L-cell line and the rat muscle line L6. Another carcinogen, azoxy methane, has been used to transform normal human colon mucosal cells to give malignant lines with altered morphology, culture longevity, growth in soft agar, substrate adherence and peanut agglutinin binding. Human mammary epithelial cells exposed to benzopyrene develop an extended lifespan and apparently immortal cell lines can be isolated. These lines do not appear to be malignantly transformed as they do not form tumors in nude mice and they show little or no anchorage-independent growth. However, they resemble tumor-derived mammary epithelial cells more closely than their

normal progenitors. The mutagen EMS (ethyl methane sulphonate) has also been used in conjunction with simian virus 40 (SV40) infections to isolate the cell line HIT from Syrian hamster pancreatic islets.

Viral transformation*:* The provision of protocols for the propagation, titration and infection methods for different viruses is beyond the scope of this chapter. However, some examples of cell lines isolated as a result of viral infection are given below.

1. Simian virus 40: The monkey virus SV40 has been used to isolate transformed mouse and human fibroblast lines. In the case of the human cells, however, these lines are not truly immortal, but merely display a delay on the onset of senescence. This approach has led to the isolations of lines such as TPA30-1 human placental line and the RLA fetal rat hepatocyte line.
2. Epstein-Barr virus (EBV): The EBV has been widely used to isolate transformed cell lines from human B-lymphocytes. Such lines have important applications in the development of cell lines from individuals with chromosome translocations or inherited diseases and in the production of human monoclonal antibodies.
3. Other viruses: Cell lines have been isolated following infection with other viruses, for example murine lymphoid cell lines with the Abelson murine leukaemia virus (A MulV). Infection with the Rous rat sarcoma virus was used to isolate a cerebellar cell line, WC5, which (GFAP). expresses glial fibrillary acidic protein Animal cell culture has also Section 7.4.1. been dealt with briefly in Chapter 7,

8.3 | Equipments and Materials for Animal Cell Culture Technology

An ideal place for tissue culture should have: (a) a dust-free air conditioned room, (b) fitted with a temperature recorder, (c) small area for microscopic evaluation, (d) preparation room for media, (e) a marked area for sterilization of glassware, etc. The room should be free of through traffic, equipped with an air-flow cabinet which supplies filtered air in and around the work area. Air supply from a High Efficiency Particle Air Filter (HEPA) is a must. Clean laboratory must be specifically designated for clean culture work. Laboratory coats should be kept at the entrance and should not be worn outside the laboratory and brought back.

The storage areas should be such where the following can be kept properly: (a) liquids at 4–20 °C, (b) glassware shelves, (c) plastics shelves, (d) small item drawers, (e) chemical-sealed containers. All work surface, benches and shelves, and the base of airflow cabinets must be aseptic, that is cleaned by swabbing with 70% alcohol.

8.3.1 | Basic Aseptic Techniques

The following are the basic aseptic techniques:

1. While working on the bench, use a bunsen flame to heat the air surrounding the bunsen. This causes the movement of air and contaminants upwards and reduces the chance of contamination entering open vessels. Open all bottles and perform all actions in this area only.
2. Swab all bottles, tops, necks with 70% alcohol before opening them.
3. Flame all bottle-necks, caps, pipettes by passing them briefly through the hottest part of the flame. One must be cautious while using plasticware, as it can spoil the necks, caps, etc.
4. Avoid placing caps and pipettes down on the bench. While learning tissue culture techniques, care should be taken to practice holding bottle caps in the left little finger while holding the bottles for pouring or pipetting by the right hand.

5. Work systematically from left to right or vice versa so as to have all material on one side and finished part on the other.
6. Avoid touching the bottle-necks and flasks. Pouring from flasks and tubes should be avoided at all costs.
7. Take care not to spill media; however, if there is any spill, clear up the spill at the work area. Glassware and plasticware must be discarded in marked polybags. All
7. plasticware used for infectious work should be autoclaved before incineration. Reusable glassware should be immersed in disinfectant before autoclaving.

8.3.2 | Equipments

8.3.2.1 | Laminar Flow Hoods/Biosafety Cabinets

In the 1960s, due to the increased need for clean air in industry, laminar flow cabinets (also known as clean benches) were first developed to provide product protection for small-scale experimental procedures. Today laminar flow hoods are ubiquitous in the laboratory. A laminar flow cabinet provides a controlled environment in which levels of particulates, microbes and contamination of all kinds are regulated and kept to a minimum by constant air filtration with industrial-grade-filters. Laminar hoods work by drawing ambient air, under negative pressure, into the top of the unit. This air first passes through a prefilter, which traps the larger dust and dirt particles. The blower then directs this prefiltered air, now under positive pressure, through the 99.99% efficient HEPA filter engulfing the entire work area with sterile, unidirectional, ultraclean air. This air travels at a velocity calculated to prevent the intrusion of unfiltered room air into the work area. The laminar flow cabinet is usually enclosed on the sides and kept under constant positive pressure in order to prevent the infiltration of contaminated room air. Such cabinets operate by filter sterilizing the air taken in, thus excluding particles including bacterial and fungal organisms. Air thus sterilized passes vertically down on the work area – *vertical (downflow) flow hoods*. When the air is directed horizontally towards the operator then this is known as *horizontal (crossflow) flow hoods*. Horizontal flow hoods are not popular as these do not offer any protection to the operator from the potential hazards of some cultured cells. Sterile, particle-free air is essential in such diverse processing areas as electronic assembly, sterile packaging and hospital pharmacy I.V. preparation. These processes and products require isolation from the contaminants typically found in ambient air. This clean environment can be best achieved and maintained by utilizing filtered laminar flow air.

Hoods are classified on the basis of the degree of safety provided to the user. The air-borne microbial contamination is filtered by similar methods in both categories of laminar flow hoods. Greater protection to the user is offered by the following ways:

1. Providing a screen (front window) which gives minimum turbulence to the air drawn in from outside, while allowing adequate access for operator's arms. The air taken in at front acts as a safety curtain, preventing aerosols of potentially hazardous material from reaching the operator.
2. Providing an air-flow monitor and an alarm system which warns when the rate falls or rises above optimum safety level

BSC I, (Fig 8.1) has a fixed front window or a vertical sliding sash, a vertical downward airflow and HEPA [High efficiency Particulate Air]-filtered supply and exhaust air. HEPA filters are the backbone of the entire cell culture unit. They may be part of a building exhaust system or form part of a cabinet, will require frequent replacement depending on their use and their load that prevents sufficient airflow to be maintained. It is important to decontaminate the filters before removal. The formaldehyde gas that is regularly used for microbiological decontamination, can damage the filters as the exhaust systems containing HEPA filters should have airtight dampers on both the inlet and discharge side of the filter housing.

This safeguards restraint of the gas inside the filter casing during decontamination. A bag-in/bag-out filter assembly maybe used in situations involving bio-hazardous materials or toxic chemicals, when, it is not possible to gas or vapor decontaminate the HEPA filters, or when radionuclides have been used, and provide protection against exposure to the laboratory personnel. This defined prototype tests for airflow velocity profiles [75 lfm (linear feet per minute)], microbiological aerosol challenge and testing of leaks of HEPA filters. Such cabinets operate by filter sterilizing the air taken in, thus excluding particles including bacterial and fungal organisms.

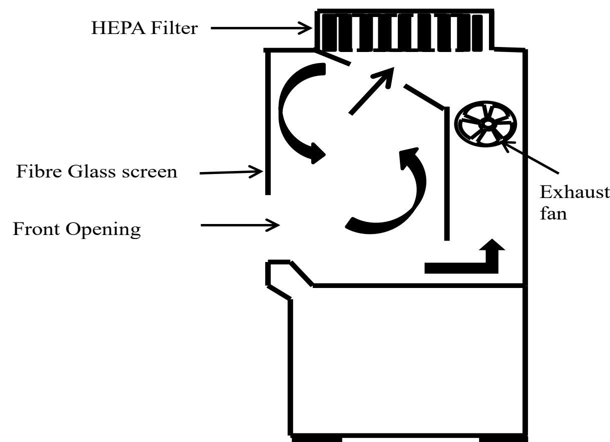

Fig 8.1 Class I Biological safety Cabinet

In BSC II, (Fig 8.2) cabinets offer more safety to the user and are apt for handling primates and human materials, virally infected cells, carcinogenic reagents, etc. The air is drawn from within the room or outside air in at the top of the cabinet, passes it through a HEPA filter and down into the work area of the cabinet. The supply air plus the additional volume of room air required to produce a minimum inflow face velocity of 100 lfm. The entire air entering this cabinet is exhausted, and passes through the HEPA filter prior to expulsion to the outside. This cabinet exhausts almost 1200 cubic feet per minute of conditioned room air making this cabinet quite expensive to operate. The movement of exhaust air must be monitored by a flow monitor that is a pressure-independent device.

Air thus sterilized passes vertically down on the work area – *vertical (downflow) flow hoods*. When the air is directed horizontally towards the operator then this is known as *horizontal (crossflow) flow hoods*. Horizontal flow hoods are not popular as these do not offer any protection to the operator from the potential hazards of some cultured cells. Sterile, particle-free air is essential in such diverse processing areas as electronic assembly, sterile packaging and hospital pharmacy I.V. preparation. The front sash of BSC II maybe modified, by the manufacturer to accommodate any equipment useful in the culture, like the eyepieces of the microscope, a vortex, or a centrifuge. These products require isolation from the contaminants typically found in ambient air. The workflow inside the sterile BSC should be from “clean to dirty”. Materials and supplies must be placed in the cabinet in such a way as to restrict the movement of “dirty” items over “sterile” ones.

Greater protection to the user is offered by the following ways.

1. Providing a screen (front window) which gives minimum turbulence to the air drawn in from outside, while allowing adequate access for operator’s arms. The air taken in from the front acts as a safety curtain, preventing aerosols of potentially hazardous material from reaching the operator.

2. Providing an air-flow monitor and an alarm system which warns when the rate falls or rises above optimum safety level.

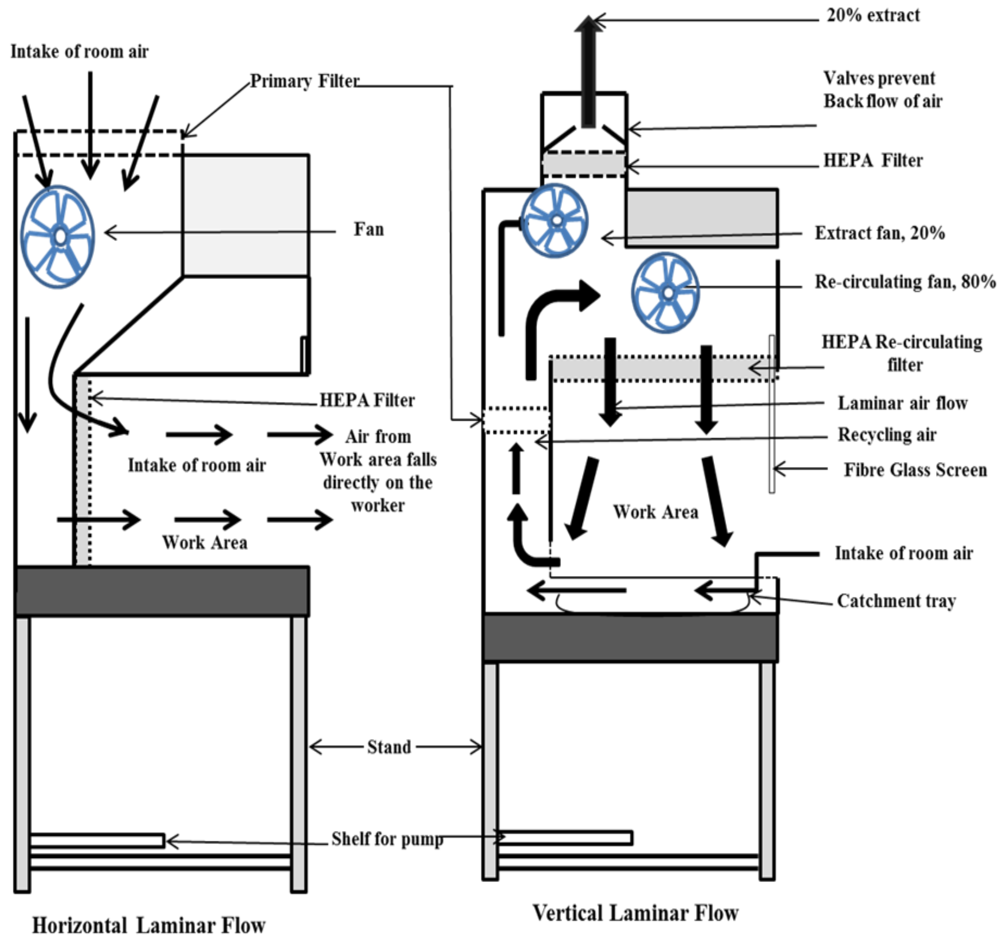

Fig 8.2 Class II Biological safety Cabinet

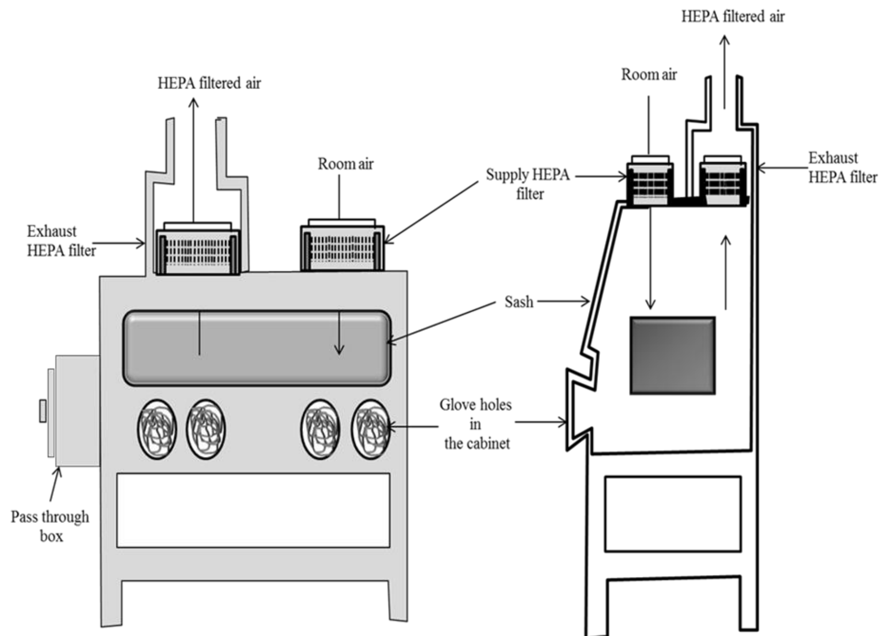

Fig 8.3 Class III Biological safety Cabinet

BSC III (Fig 8.3) is essential to work with highly infectious agents and for the conduct of hazardous operations. It should provide maximum protection for the environment and the laboratory personnel. It is a gas-tight (no leak with 1% test gas at 3 inches pressure Water Gauge14) enclosure with a view window that is non-opening. The material in the cabinet is passaed through a dunk tank, and can be accessible through double-door pass-through box that can be decontaminated intermittently, allowing the materials to be removed safely from the BSC III. Both supply and exhaust air are HEPA filtered. The exhaust air must pass through two HEPA filters, or a HEPA filter and an air incinerator, before discharge directly to the outdoors as these cabinets are not exhausted through the general laboratory exhaust system. Airflow is maintained by an exhaust system exterior to the cabinet, which keeps the cabinet under negative pressure (minimum of 0.5 inches of water gauge). Extended, thick and heavy rubber gloves are generally attached in a gas-tight manner to the holes in the cabinet to allow direct manipulation of the isolated materials kept inside. These gloves definitely restrict movement, but they also prevent the worker's direct contact with the hazardous materials. A little operating discomfort has to be tolerated for maximizing personal safety. The HEPA filter has to have 125 lfm vertical flow that provides particulate-free, although somewhat turbulent, airflow within the work environment.

8.3.2.2 | CO_2 Incubator

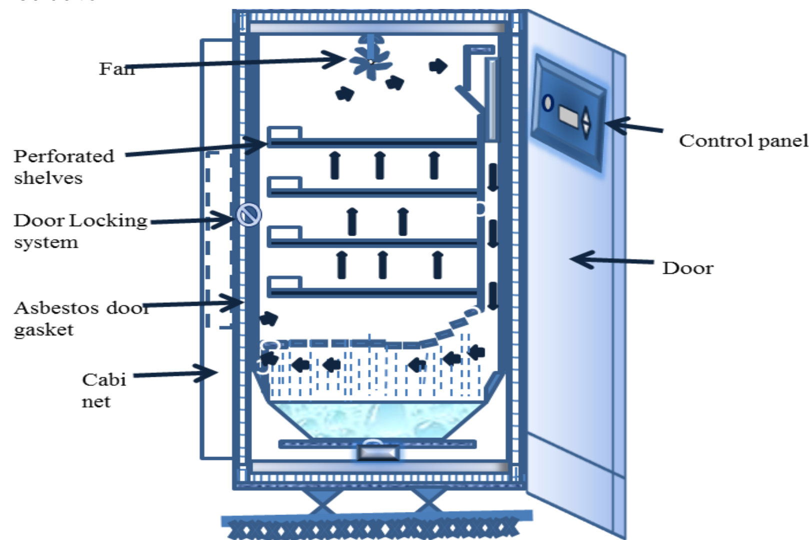

Fig 8.4 CO_2 Incubator

Incubators for cell culture work depend on an external supply of CO_2 so as to maintain a fixed level of CO_2 in the incubator. To maintain sterile handling area and cleanliness, the CO_2 cylinder should be kept outside the laboratory and the gas should be piped through. The gas is fed via a reduction valve on the cylinder head through pressure tubing to the incubator intake port. CO_2 monitors control the level gassed through incubators. As long as the incubator is closed, the usage of CO_2 is minimum. It is extremely useful to have extra CO_2 cylinders in case of unexpected running out of gas.

Leakage of CO_2 gas should be prevented at all costs. Constant precautions and routine checks can strike an effective balance. Correct pressure tubing, securing and testing all connections should avoid most problems. Also a wash bottle filled with soap solution (household washing liquid) squirted around connections, makes a cheap and effective detector. Dirt present on either face of the connection can cause significant leakage. Whenever a new cylinder is fixed, leak must be tested. CO_2 incubators should meet

requirements of an absolute constant and reproducible environment for growth of cell

cultures. A controlled atmosphere is achieved by blowing air over a humidifying tray and controlling the CO_2 tension by a CO_2 monitoring device. Air is circulated around the incubator by a fan to keep both CO_2 level and temperature uniform. Some incubators have a unique temperature management system enormously reducing evaporation and contamination risk; also there is no need for a fan. As the systems are based on air jacket technology, the units can be easily installed and maintained. Earlier the incubators were based on water jacket technology to distribute heat evenly around the cabinet. These take longer to cool in case of power failure. Care should be taken to empty out the water to move the incubator.

8.3.2.3 *Microscope*

An essential instrument for any culture laboratory is a good quality inverted microscope, preferably with phase-contrast optics and a photographic facility. Cell morphology, that is the degree of spreading, granularity, membrane blebbing, the proportion of multinucleates, vacuolation, and so on, should be monitored regularly for signs of stress in cells. Cell morphology is a sensitive indicator of problems with culture conditions. Early signs of a microbial contamination can also be detected with a good phase contrast microscope. Regular checking of cultures under the microscope can help to avoid catastrophic losses of irreplaceable material by allowing a problem to be noticed at an early stage. Also, experiments done on unhealthy cells may give variable or erroneous results. When choosing a microscope, select the long or extra-long working distance condenser so that flasks and even roller bottles can be viewed. Normally a 20× symbol objective is sufficient; their depth of field is often too low to obtain a sharp image of all but the very flattest cells. A good, low-power, wide-field objective, for example the Nikon 4X symbol, is very useful for scanning culture colonies.

8.3.2.4 *Centrifuge*

The main use of a centrifuge in a tissue culture laboratory is to spin-down cells during tissue disaggregation or in harvesting cell lines for subculture, freezing, analysis, etc. A very simple bench-top centrifuge which will reach 80–100 g, preferably with a variable braking system, is generally all that is needed. Various considerations may indicate the need for a more sophisticated piece of equipment. The volumes of cell suspensions to be handled in a single operation will dictate the bucket size and adaptability needed, for instance the capacity to take four swing-out buckets each holding five 50 ml tubes in one run, followed by a mixture of 15 and 50 ml tubes. If primary cultures are anticipated, it may be an advantage to have a cooled centrifuge with a timer for long runs with sensitive cells, which have been exposed to proteolytic enzymes. For some laboratories a bench-top microfuge is a useful addition for high-speed centrifugation of small volumes of reagents, which may generate a precipitate, for example after thawing from the freezer.

8.3.2.5 *Refrigerators and Freezers*

For most laboratories, domestic larder-type refrigerators (with no ice box) are adequate. Media storage requires considerable space and it may be more convenient to store unopened bottles in a nearby cold room, if available. The refrigerator in the tissue culture laboratory can then be reserved for media in current use, with each opened bottle designated for one individual's work with one cell line. Ideally, separate refrigerator should be used for sterile culture media and for non-sterile solutions, chemical stocks, etc. Freezers at $-20\ ^{\circ}C$ and, ideally, $-70\ ^{\circ}C$ will be needed for storage of sera solutions and reagents which are unstable at higher temperatures. At lower temperatures, sera and proteins such as collagenase, which is prone to degradation even at $20\ ^{\circ}C$, can be stored for extended periods. Some of these lower temperature freezers are available with liquid CO_2 or liquid nitrogen back-up facilities that permit temperature to be maintained even in the event of an electrical supply or compressor failure. Liquid nitrogen freezers for long-term cryopreservation of cells are also required.

8.3.2.6 *Pipetting Aids*

A number of micropipettes are available in the market to suit most budgets and needs. Some are autoclavable for additional safety of sterile procedures. For most laboratories, a range of micropipettes covering for example 1–20 l, 20–200 l, 100–1000 l, and 1–5 l will be adequate, perhaps with one or two fixed volume micropipettes, such as 50 or 100 ml for frequently used volumes. Depending on the number of regular users, sets may need to be replicated. In addition, it is useful to have a multichannel micropipette; this is essential if much work is done with 96-well plates. For many laboratories, an automated aid to pipetting larger volumes is also a high priority. Several companies make light weight, hand-held, battery- or mains operated units for this purpose with loading and dispensing push-button, controls and an air filter for sterility. For those on a tight budget, conventional manual pipette bulbs will be adequate; these are available in a range of volumes. Under no circumstances should mouth pipetting be considered for tissue culture work.

8.3.2.7 Miscellaneous Small Items of Equipments

The preparation area for reagents ideally should have a balance, a pH meter and a few magnetic stirrers. The balance should be sensitive enough to weigh milligram quantities. An osmometer is helpful but not essential. Spent medium should be drawn into an aspirator jar to be kept under the laminar flow hood. The aspirator jar should contain a small amount of hypochlorite solution (chloros), which will help to avoid both microbial contamination and cross-contamination between cell lines via the aspirator line.

Materials: The materials to be used are:

1. Plasticware: The following are the commonly used items in the laboratory.

   Flasks treated to produce electrically charged surface to enhance cell adhesion. Flat-bottomed flasks, petridishes, flat-bottomed/round-bottomed multi-well plates, roller bottles, tubes. Pasteur pipettes, micropipette tips, volumetric pipettes, centrifuge tubes (15 ml/50 ml) for handling cells in suspension and solutions. Cryovials, snap-cap tubes, Eppendorf tubes, sample tubes, screw-capped tubes for storage.
2. Glassware: Glassware has great propensity to adsorb substances such as alkaline detergent onto its surface than plastics such as polypropylene. A collection of volumetric cylinders, flasks and beakers for preparation of sterile solutions and bottles for storage is a must for every laboratory.

***Miscellaneous** items:* Pipette cans, pipette bulbs, tube racks, autoclave tapes, haemocytometers, oven tape, instrument tins, autoclave bags. If reusable pipettes are used, it is best to sterilize them in stainless steel cylindrical cans with a square cross-section. Shorter cans are required for Pasteur pipettes. Oven-sterilizable, tight-lidded flat tins are convenient for sterilizing instruments for dissection.

8.4 Cell Culture Contaminants

Cell culture is often done under artificial conditions. In short, natural conditions are simulated to provide all the nutrients at a suitable temperature and humidity for optimal growth. Owing to the rich nutrient medium provided opportunistic growth is unavoidable. Therefore, no cell culture problem is free from contamination. There is nothing as depressing as that of culture loss due to contamination. Any undesirable element in cell

culture that may cause an adverse effect on the growth of the cells or the experimental system is termed as a contaminant. Contaminants can be categorized under three sections:

1. Minor- Usually when the losses of flasks are occasional;
2. Major - When the frequency of contaminants increases turning into a serious problem.
3. Cataclysms - when the contaminants are discovered that put a doubt on the past and current work.

A contaminant can further be classified into:

i) Chemical contamination
ii) Biological contamination

8.4.1 ***Chemical contaminants*** may be the Media: from reagent and water, sera: batch to batch variation in hormone and growth factors, water borne endotoxins, and some other culture additives. Storage vessels may further add to the problems if not cleaned thoroughly. Excessive use of fluorescent lights and addition of sodium azide in the CO_2 incubators are generally a common cause of chemical contaminants.

Occasionally there may be white deposits on glassware, pipettes and on instruments that may have been left by disinfection or detergents. Metal ions, endotoxins, and other components of media, generally the sera form a thin coat on the plastic ware, in plastic tubing and storage bottles. Free radicals may be generated in the media, possibly by photo-activation of riboflavin or tryptophan. HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid), commonly used for buffering the media also gets reduced when exposed to fluorescent light. Residues from germicides used to disinfect equipment and incubators including the laboratories, also cause white deposits. The impurities in gases used in CO_2 incubators may also create unwanted deposits.

8.4.2 ***Biological contaminants*** include bacterial, fungal as in moulds, viruses, mycoplasmas, yeast, few protozoans and invertebrates. Cross-contamination by other cells in culture can also be a deterrent. There could be several possible sources to acquire these biological contaminants such as contact with non-sterile supplies, media, solution, or touch of your own personal belongings and clothes. Particulate or aerosol fallout during experimentation, manipulation of flasks, plates of culture vessel, transportation from one point to another, or negligence while putting for incubation, add to the troubles. Accidents and mistakes do happen, often by laxity on the part of the worker, and should be avoided at all costs.

The ubiquitous prevalence of Bacteria, Fungus and yeast coupled with their ability to rapidly colonize and flourish in the rich nutrient environment provided by cell culture is a constant source of contamination. In the absence of antibiotics, microbes can be detected in a culture within a few hours or days, either by direct microscopic examination or by the turbidity of the media. Occasionally, a pH shift followed by a floating cell population primarily due to cell death is visible too. But, the regular presence of antibiotics tends to make the microbes resistant, that forms a latent infection, that maybe very difficult to detect microscopically. Viruses are impossible to be visualized, due to their small size and hence are difficult to remove from media, solution, or any other biological fluids. Viral infection of cells is definitely not the prime concern, the potential health hazards posed for the laboratory personnel is worrisome. Therefore, utmost caution should always be maintained. While working with tissues or cells from human or other primates, it is absolutely necessary to avoid all possible transmission of viral infection (HIV, HBV, EBV, etc.).Protozoans include both parasitic and free living amoeba, are generally water borne.

Protozoans cause damage resembling a viral damage and may completely destroy the culture within two weeks. Owing to their slow growth coupled with morphological similarities to cultured cells, the amoebas are challenging to detect in culture. Although such contaminants are rare, still it is important for the worker to be aware of the possibility of their occurrence. Insects and arachnids are never directly the cause of any contaminant to the cell culture. However, their presence in and around the lab areas, especially cockroaches, ants, flies, and mites can cause contamination and may even cause microbial contamination. Mycoplasma was first detected in cell culture by Robinson and co-workers in 1956. At least 15% of cell cultures are generally infected by mycoplasmas. Mycoplasmas are not benign culture contaminants as they have the ability to alter cell function, growth, metabolism, morphology and cause chromosomal aberrations and damage, plaque formation. Mycoplasma appear to be the smallest self-replicating organism, they lack cell wall and very stringent and fastidious growth requirements allow them to grow at high densities in cell culture without any visible signs of contamination. Viruses are small in size too and have the ability to infect 100% of the cells in culture and impossible to remove from sera by membrane filtration. Hence, they have the ability to alter every parameter of the cell function making Viruses and Mycoplasma as the most serious, pervasive, and distressing culture contaminants. Some workers have even named Mycoplasmas as the "**crabgrass**" of the cell.

8.4.3 *How to avoid contamination?*

The most pertinent answer to the contaminant problem is the judicious use of proper aseptic techniques. Firstly, accidents can be reduced by keeping the laboratory area clean and free from dust. There should be a standard protocol for checking the contaminants routinely. The frozen cell repository should be used strategically. Antibiotics are best avoided but if necessary, should be used cautiously.

A clean and safe environment must be maintained at all costs. Routine cleaning of laboratories-work surfaces, floors and the equipment should be done diligently. Biosafety cabinets should be tested for the quality of purified air coming from the HEPA(High Efficiency Particulate Air) filters. The velocity of air coupled with the particulate count also determines the contaminant periodicity. The CO_2 incubator is a very good source of fungus as 90% humidity is maintained. The air jacketed incubators are safer but many a times they are supplemented with water trays that are good breeding grounds for fungus as well as bacteria. The microscope is important for regularly examining all the tissue culture flasks for any contamination (tiny dots of bacteria or stings of hyphae from fungi /mould). If any of the above is visible, immediately remove all infected flasks into an appropriate place in the laboratory away from the un-contaminated flasks, and discard the infected flasks after proper treatment with disinfectants.

The disinfectants can be categorized in 4 groups namely:

i) Hypo-chlorites[recommended for bacteria and viruses dose is 1000ppm for surface disinfection]

ii) Phenols [Not active against viruses]

iii) Alcohols [Mode of action is dehydration] 70% is sufficient to kill bacteria Generally used to clean equipment, work surfaces and hand sanitizer

iv) Aldehydes [a common irritant and best avoided. 4% glutaraldehyde is sufficient for sanitization purposes]

The contaminated flask should be topped with an equal amount of 10% sodium hypochlorite and left for two hours before spilling down the sink with running tap water. Wipe

the outside of all the non-infected flasks with 2.5% sodium hypochlorite and 70% isopropanol/alcohol/ethanol. Further, remember to discard all the aliquots of media, penicillin/streptomycin, foetal calf serum and any open bottles of water that could have been a source of contamination. It is always advisable to fumigate the Class I/II cabinet by formaldehyde and if possible the whole laboratory. Also, the incubator should be decontaminated by the usual cleaning procedure i.e. by spraying 70% ethanol and wiping it dry

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8.5 | Basic Techniques of Mammalian Cell Culture: Disaggregation of Tissue and Primary Culture, Maintenance of Cell Culture and Cell Separation

Before embarking on any new venture it is wise to get acquainted with its terminology.

1. Primary culture: As discussed earlier, cells derived from a tissue are called primary culture. A culture is considered primary till it is sub-cultured or passaged.
2. Subculture: When cells are transferred from an ongoing culture onto a new flask after trypsinization it is called subculture. In case of primary culture, the product of first culture is also known as secondary culture.
3. Cell line: A cell line is a cell population derived from primary cell line. Finite indicates a limited life span and continuous represents an unlimited life span.
4. Cell strain: It refers to a sub-cultured population selected on the basis of its expression of specific properties, functional characteristics or markers.
5. Clonal culture: Clonal selection refers to the establishment of a cultured cell popu-lation derived from a single cell. Cells derived from a single cell might not always be identical as expected. There is some degree of heterogeneity in cultures. A completely homogenous population is attained after long-term stability.

8.5.1 Material Source

Surgical samples are a convenient source and care should be taken to procure the sample in aseptic conditions. Once the tissue has been removed during operation, it should be placed in a sterile container having serum-free medium. Utmost care should be taken to avoid formalin fixation of the sample as maintaining a culture is different from doing cell culture. The media used to collect and culture tissue samples should be supplemented with antibiotics.

8.5.1.1 | *Isolation of Cell*

Blood is the most convenient source of cells, as it contains a variety of cell types. Therefore, protocols for separating different cell types should be intensely followed. But cells can be obtained in the form of organ or tissues, where it is important to disaggregate the tissue and purify the cell type of interest. Various disaggregation methodologies are followed where the tissue can be used freshly or even after storage at 4°C.

8.5.1.2 | *Enzyme Digestion of Tissues*

Proteolytic enzymes can digest the extracellular matrix. Below is a schematic representa-tion of the disaggregation process at both the temperatures.

Trypsin is the commonly used enzyme whereas collagenase is used specifically for

fibrous tissue. Elastase, hyaluronidase, pronase, dispase are used independently or in combinations. Collagenase and dispase digest the tissue slowly, but trypsin and pronase digest rather aggressively. Also, digestion is slower when the tissue is kept at 4 °C as compared to 37°C. Therefore, cell damage is less likely to occur at low temperatures.

8.5.1.3 | Mechanical Disaggregation

Some tissues can be subjected to mechanical means of cell separation – spleen cells – by simply squeezing through a wire mesh or gently mincing it between two sterile frosted slides. Mechanical disaggregation method is faster than enzymatic digestion. However, it can also damage the cells and one may have a low recovery of cells. The possible advantage of this method is that it is simple and inexpensive, and free from batch-to-batch variations caused due to enzymatic digestion.

8.5.1.4 | Explant Cultures

When punch biopsies are taken from human samples, the cultures established from these are known as explant cultures. These are chopped into small pieces in a petridish containing sterile media where they are kept for a few hours for allowing attachment of cells to the substrate. It may take days or even weeks to establish a culture. It is the slowest method known.

The replicative capacity of cultured cells varies depending on cell type and the species. Many cells can be passaged a number of times, however others die at an early stage. In rodent tissue, it is unusual for cells to divide indefinitely. Cells from human tissue never give rise to continuous cell lines unless treated with certain agents. Chicken cells are difficult to maintain beyond a few doublings.

Hayflick and Moorhead in 1961 studied the potential of human fetal lung fibroblasts to divide in culture by counting the cells at each passage starting with the explant from human tissue. They found a slow increase in the growth rate (phase I). During this phase, some cells die while some grow. If the media is continuously supplied they grow at a constant rate for an average of 50 generations (phase II) after which there is slowing down of the growth rate. The next phase of increased cell death (phase III) results in complete death of culture. Human cells can generally undergo between 20–80 passages but could be shorter. The limited replicative capacity of human cells in culture is sometimes called the Hayflick effect, named after its discoverer. Experiments conducted in 1970 on adult lung fibroblasts clearly indicated that the number of population doublings achieved in the culture is a function of the donor's age. A linear increase in the lifespan of the cells was found with increasing age of the donor.

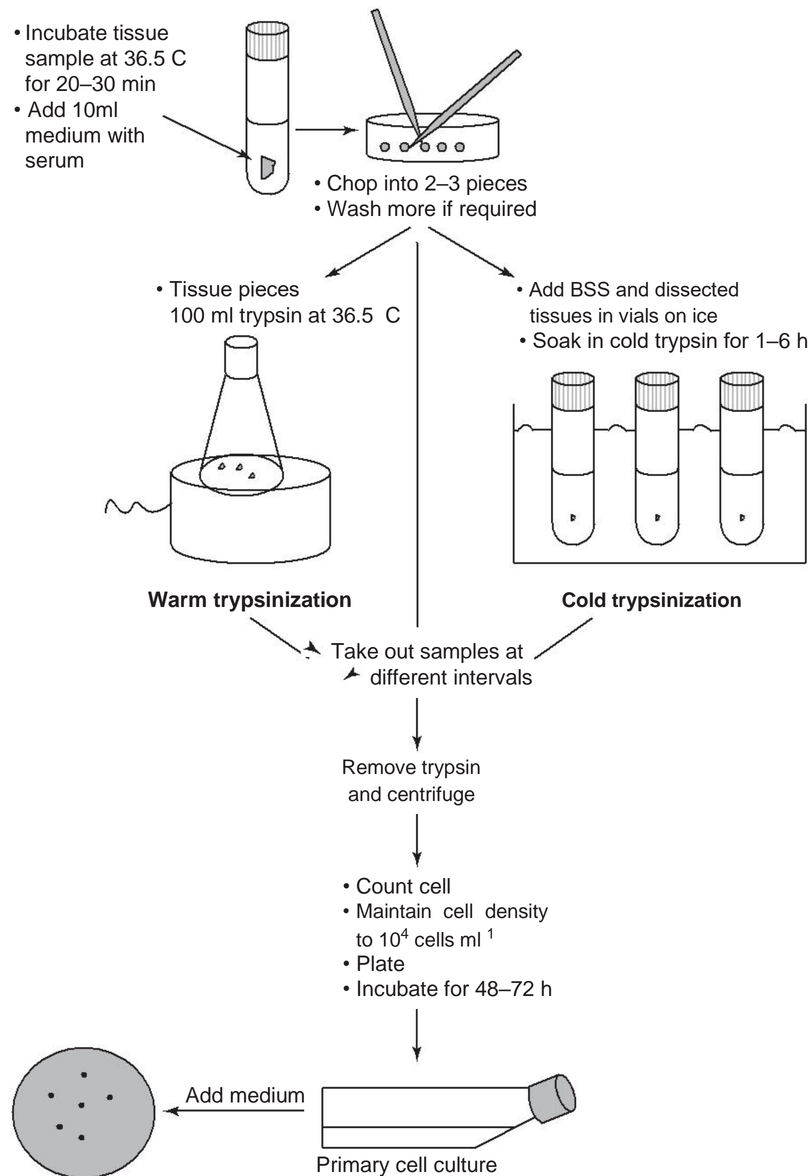

Figure 8-5 | Establishing a primary culture by cold and warm trypsinization.

8.5.2 | *Characteristics of Normal and Transformed Cell Lines*

8.5.2.1 | *Normal Cells*

They are anchorage dependent. This means that they need a substratum to adhere and proliferate. They have density-dependent inhibition of proliferation. Also their lifespan is finite. The characteristics are altered with increased in vitro age.

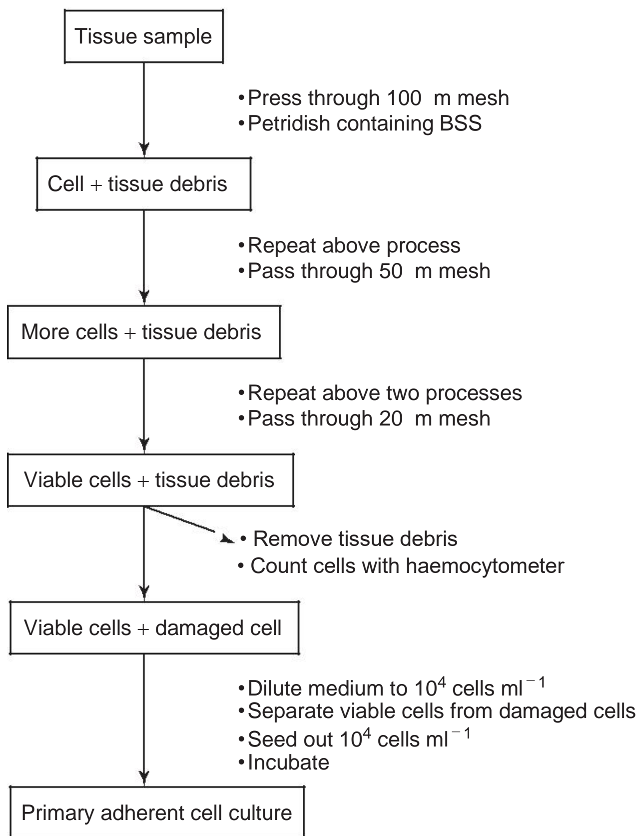

Figure 8-6 Flow chart of isolation of primary cells from a tissue by mechanical disaggregation.

8.5.2.2. Transformed Cells

These are immortal, that is ***they have*** no ***finite lifespan.*** They have a reduced requirement for serum or growth factors for optimal growth. Their population doubling time is short. They have reduced substrate adhesion as they are anchorage independent. They show heteroploidy or aneuploidy – genetic instability. They have altered growth control. Also there is loss of contact inhibition and loss of density-dependent inhibition of prolifer-ation. Increased colony formation in soft agar is a characteristic feature of transformed cells.

8.5.3 Maintenance of Stock Cultures

Consistency and reproducibility within successive experiments require that stock cultures be maintained by a routine protocol. Attention to several points as mentioned below will help improve culture performance.

1. Establish and maintain culture stocks for a given experimental series under identical seeding density, media and incubation conditions.
2. Replenish the medium on a routine schedule and before nutrients are severely depleted or pH drifts outside the acceptable range. Frequency of feeding is dependent on

population density. Carry cultures at a density that will permit replacement of the medium at practical intervals. For example, it is common to feed cultures on a set schedule three times weekly.

3. Passage stock cultures on a routine schedule and when they are at their 'healthiest' (i.e. during logarithm growth phase).

8.5.4 Antibiotic-free Stock Cultures

The uninterrupted use of antibiotics in the medium can mask low-level contamination by resistant microorganisms. Thus, some laboratories carry two sets of stocks for each cell line, one in complete medium and one in antibiotic-antimycotic-free medium. The antibiotic-free culture condition is a necessity.

If the laboratory works exclusively with antibiotic-treated cultures, it is wise to test periodically for the presence of low-level contamination. A simple test for the presence of bacterial or fungal contamination is to plate replicate cultures out of which one set is in antibiotic-free medium. These cultures are then followed (e.g. for 1–2 weeks) to see if frank contamination develops. Moreover, definitive testing to detect and identify contaminants should also be performed. Detailed protocols are included in a very useful volume published by the American Type Culture Collection (ATCC).

Mycoplasma contamination can be more difficult to detect. Mycoplasma may originate from a variety of sources such as serum added to the medium or the tissue used to establish primary cultures, and can be passed from the individuals who handle the cultures. These organisms can cause numerous problems such as acute cell lysis and can also lead to alter-ations in cell structure, function, karyotype, metabolism and growth characteristics that may not be overtly apparent for many cell generations. Mycoplasma are extremely small (300–800 nm diameter) and many strains can readily pass through 0.2 m filter steriliza-tion membranes. In addition, some strains are unaffected by routine antibiotics. For these reasons periodic mycoplasma screening is recommended. The ATCC presents detailed protocols for several mycoplasma testing methods including a direct colony growth assay, fluorescence DNA staining using a bisbenzimide dye, and use of the Gen-Probe Mycoplasma T.C. Rapid Detection System, a DNA:RNA hybridization assay (Gen-Probe). For laboratories that find in-house testing to be problematic, a number of companies offer a mycoplasma screening service (e.g. ATCC, Bionique, Q-One Biotech, Microbiological Associates).

8.5.5 Cell Separation

The alternative approach to cell cloning is to separate cells using physical methods. There are two popular methods by which cells may be selected on the basis of size, density, charge, surface area or specific affinities. *Flow cytometry* and *flow cytofluorimetry* measure the light scattering properties of cells, which are proportional to surface area. In flow of cytofluorimetry, specific fluorochromes are attached to cells and the cells are separated on the basis of fluorescence emission. Both methods can be performed very quickly using a sophisticated piece of apparatus called the Fluorescence Activated Cell Sorter (FACS). A stream of single cells passes through the flow chamber of the FACS and each particle in suspension is analyzed for the appropriate parameter (such as fluorescence emission or light scatter). The cells can then be separated according to these parameters and collected in a sterile manner. Up to 5000 live cells can be sorted in 1 s using the FACS.

8.5.5.1 | Physical Methods of Cell Separation

Cloning procedures are time consuming, so by the time a clone has produced enough cells to use, the line may well be close to senescence, particularly if the clones are derived from nontumor tissues. Tumor tissues and continuous cell lines are cloned more rapidly but, because of the nature of these rapidly dividing cells, considerable heterogeneity can arise even as the clone is being grown up.

Where cells do not grow efficiently enough for cloning or if time is limited it may be easier to employ physical separation techniques to produce populations of similar cell types. Cells may be separated on the basis of size, density (specific gravity), surface charge or surface chemistry (e.g. by the ability to be bound by antibodies or lectins).

8.5.5.2 | Separation Based on Cell Size

The relationship between cell size and sedimentation rate under gravity may be expressed as $v = r^2/4$, where v is the sedimentation rate (mm h^{-1}) and r is the radius of the cell (m). Thus, in principle, cells may be separated on the basis of their size. Many factors other than the radius such as cell density may influence its sedimentation rate. Obviously the properties of the suspending medium (e.g. viscosity, density) will also influence the rate of sedimentation. These may be used to improve the separation of different cell types.

8.5.5.3 | Separation Based on Cell Density

For this, a density gradient is established in a centrifuge tube using a suitable density medium. Usually either Percoll, Metrazamide or Ficoll is used for this purpose. The cells are layered on the surface of the density gradient and centrifuge so that the cells sediment to a point where the density is equivalent to their own density. The cell layer can then be siphoned off with a Pasteur pipette. This technique is known as isopycnic sedimentation.

8.5.5.4 | Separation Based on Cell Surface Charge

With this technique the cells are separated by electrophoresis. The cell suspension is passed between two electrodes (polarized plates) and the cells migrate to either plate according to their net charge, that is cells bearing a net negative charge migrate toward the positive plate and vice versa.

Cells get damaged by using a high voltage current, as there is a tendency for localized overheating. Population of cells do not usually show qualitative differences in surface charge, and one has to separate cells which are all negatively charged. In these cases, we are dependent upon separating cells on the basis of their net charges:mass ratios. Despite these difficulties, electrophoresis is the basis of cell separation used in cytofluorometry.

8.5.5.5 | Separation Based on Affinity

Cells in suspension can be passed through affinity columns which contain a matrix coated with antibodies or lectins. As the cells pass through they bind to or are captured by the

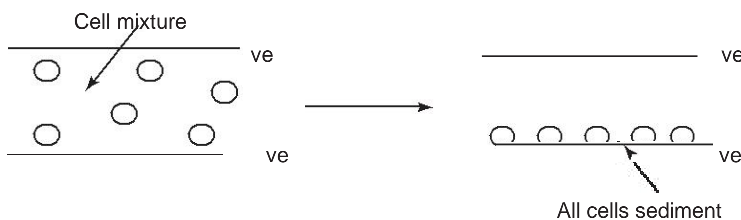

Figure 8-7 | Separation of cells on the basis of surface charge.

matrix and can be specifically eluted by washing the column with detergent or enzyme solutions. Care has to be taken not to damage the cells during this process.

8.5.5.6 Separation by Cytofluorometry

Fluorescent dyes called fluorochromes can be used to label cell surfaces or cytoplasmic molecules. The cells are separated by passing the cell mixture through the flow chamber of a machine such as the FACS (Becton-Dickinson) or a cytofluorograph (Ortho) or a Coulter Cell Sorter.

8.6 Growth Media

Cell culture medium is the single most important factor in promoting cell survival and proliferation. A nutrient is defined as a chemical substance that enters a cell and is used as a substrate for biosynthesis or energy metabolism. Any other requirement for cell growth is termed as a supplement, including serum, undefined media and biological fluids. The function of media is to provide an environment for survival and also to provide substances required for the growth of cells. Early tissue culture was done on biological fluids such as plasma, lymph, and serum or extracts of embryonic origin.

8.6.1 Basal Salt Solution

Balanced salt solution (BSS) is composed of inorganic salts that maintain the physiological pH and osmotic pressure. The physiological role played by the inorganic ions is to maintain the membrane potential. They also work as cofactors in enzyme reaction and in cell attachment. Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Cl^{-}, $S0^{2}{}_{4}{}^{-}$, $PO^{3}{}_{4}{}^{-}$ and $HCO^{-}{}_{3}$ are the inorganic molecules involved. To maintain the osmolality, concentration of NaCl is generally adjusted. Four main categories of BSS are EBSS (Earle's balanced salt solution); DPBS (Dulbecco's phosphate-buffered saline); HBSS (Hank's balanced salt solution); ESSS (Eagle's spinner salt solution). For maintaining the correct pH, HBSS and DPBS are equilibrated with air, while ESSS and EBSS require equilibration with gas phase containing 5% CO_2.

8.6.2 Other Constituents of Basal Media

8.6.2.1 Amino Acids/Vitamins

Carbohydrates are a major source of energy in cultured cells. Sugars such as glucose, maltose, sucrose, mannose, fructose and galactose are commonly used. Glutamine can also alternatively supply energy required by some cells. Animal cell culture requires essential amino acids as they cannot be synthesized from raw materials by heterotrophic organisms. The concentration of amino acids influences cell yield, survival and growth rates. Amino acid deficiency inhibits cell division, induces chromosomal damage and increases lysosomal activity and cell death. Imbalance of amino acid concentrations can also produce karyotype changes. During the lag phase of the growth cycle, there is an enhanced requirement of cysteine, glutamine, isoleucine and serine. Glutamine serves both as an energy source and carbon source in the synthesis of nucleic acids. It is the most unstable (labile) of the amino acids and needs to be replenished regularly.

Vitamins are required as cofactors in metabolism [e.g. Niacin, NAD(P), riboflavin, FMN, FAD]. The vitamins added to the basal media are *para*-amino benzoic acid, biotin, choline, folic acid, nicotinic acid, pantothenic acid, pyridoxal, riboflavin, thiamine and inositol. Retenoids help cells to adhere to the substrate. A sufficient supply of choline is

Table 8-2 | Balanced salt solutions used in cell culture

Substance	*Ringer* ($g\ l^{-1}$)	*Tyrode* ($g\ l^{-1}$)	*Simms* ($g\ l^{-1}$)	*Earle* ($g\ l^{-1}$)	*Hanks* ($g\ l^{-1}$)	*Dulbecco (PBS)* ($g\ l^{-1}$)
NaCl	9.00	8.00	8.00	6.80	8.00	8.00
KCl	0.42	0.20	0.20	0.40	0.40	0.20
$CaCl_2$	0.25	0.20	0.147	0.20	0.14	0.10
$MgSO_2.7H_2O$				0.10	0.10	
$MgCl_2.6H_2O$		0.10	0.20		0.10	0.10
$NaH_2PO_4.H_2O$		0.05		0.125		
$NaH_2PO_4.2H_2O$			0.21		0.06	1.15
KH_2PO_4					0.06	0.20
Glucose		1.00	1.00	1.00	1.00	
Phenol red			0.05	0.05	0.02	
$NaHCO_3$		1.00	1.00	2.20	0.35	
Gas phase	Air	Air	2% CO_2 in air	5% CO_2 in air	Air	Air

Note: PBS – phosphate buffered saline.

necessary for incorporation into membrane phospholipids. Vitamins A and E are added in Medium199.

Common examples of protein supplements are fetuin, -globulin, fibronectin, albumin and transferrin. Low molecular weight factors are carried into cells by binding onto transport proteins. *Albumin* is a transport protein, which carries vitamins, lipids and hormones into cells. *Transferrin* is involved in binding and transport of iron.

Phospholipids and cholesterol, Prostaglandins E and F_2, are involved in cell growth. Their action may possibly be in conjunction with epidermal growth factor (EGF) and other growth factors.

Insulin is essential for all media. It is very sensitive to inactivation by cysteine and hence large quantities need to be added. Glucocorticoid hormones such as hydrocortisone and dexamethasone can either stimulate cell growth or inhibit cell growth depending on cell type. Growth factors like nerve growth factor (NGF), EGF have been used to promote cell growth. Certain interleukins, colony stimulating factors and fibroblast growth factors (FGF) are also used. Copper, zinc, cobalt, manganese, molybdenum and selenium are considered to be activating enzymes and protecting against free radicals, which cause damage to DNA.

Nontransformed cells have to adhere to solid substrate in order to multiply. Only haemopoetic and transformed cells can multiply without attachment. Chondronectin is required for the adhesion of chondrocytes and laminin for adhesion of epithelial cells. Fibronectin is also important for cell adhesion.

8.6.1.2 | *Antibiotics*

Antibiotics are routinely used in laboratories although their use in biopharmaceutical production is not acceptable. Careful selection of the antibiotics is a necessity. Ideally, antibiotics should have no toxicity, preferably a broad spectrum type and be economical in cost. Commonly used antibiotics are penicillin (100 IU ml^{-1}) and streptomycin (50 g ml^{-1}). Gentamycin (50 g ml^{-1}) is expensive but is widely used as an antifungal agent. Nystatin (25 g ml^{-1}) also is an effective antifungal agent.

8.6.2.3 Role of Carbon Dioxide

Animal cell culture requires a gas phase consisting of O_2 and CO_2, although the oxygen requirement by cells and organs may differ. These gases influence the pH and the bicarbonate ion concentration of the culture. The main decision in selecting a BSS depends whether or not the cells are to be maintained under high CO_2 atmosphere or the nature of the buffer used. Media needs to be buffered to compensate for the evolution of CO_2 and the production of lactic acid from glucose metabolism. Bicarbonates form a cheap and most abundantly used buffering system. Other buffers available which keep the pKa values around pH 6.8–8.0 without the need for enhanced CO_2 levels are MOPS (pKa 7.2), HEPES (pKa 7.2–7.6), TES (pKa 7.4), DIPSO (pKa 7.6) and HEPPSO (pKa 7.8).

Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) is most abundantly used as it is more resistant to rapid pH changes than bicarbonates. Care should be taken to use the optimal concentration of Hepes (toxic to cells above 100 mM). The above organic buffers function in the absence of CO_2.

8.6.3 Serum as a Complex Supplement

Blood serum (plasma minus fibrinogen) may be used either as the entire culture media or as a supplement in basal medium. Serum provides a nutritive substrate and a supporting structure of many types of cell culture. It provides a cocktail of growth factors necessary for maintenance and proliferation of cells. It protects cells and tissues from excessive traumatic damage during subculture. It buffers the system against a variety of perturbations and toxic effects such as pH change, presence of heavy metal ions, proteolytic activity or endotoxin shock. Use of serum is crucial owing to its complexity and multiplicity of growth-promoting, cell-protection and nutritional advantages that it has. The important known constituents of serum are enlisted below.

8.6.3.1 Growth Factors

Proteins of molecular weight 5–30 kDa are the peptide growth factors which act via cell surface receptors and stimulate cells into proliferation or differentiation. Serum requirement varies depending on the cell type and the concentration of the cells. Different types and batches of serum may contain different levels of growth factors.

8.6.3.2 Albumin

Albumin, major protein component of serum, acts as a carrier for small molecules across cell membrane. Transport of fatty acids is an important function of albumin. Steroids and fat-soluble vitamins also bind to albumin. Lipids such as cholesterol, triglycerides and phospholipids are transported in serum in micellar form complexed with specific lipoproteins. Albumin has specific binding sites for thyroxine and some metal ions. Because of its absorbtive role it functions as a detoxifier by binding to metal ions such as Ni^{2+} and Cu^{2+}. It also acts as a pH buffer, protecting the cells against damage.

8.6.3.3 Transferrin

It is a major iron transport protein in vertebrates, representing 3–6% of total serum protein. The transferrin–Fe^{3+} complex is taken by cell surface receptors after the release of iron; apotransferrin is liberated from the cell and recycled. Copper may also be transferred by a protein called ceruloplasmin, and in chelated form by small peptides such as Gly-His-Lys (GHL, liver growth factor).

Table 8-3 | Some known constituents of serum

Factors	*Concentration*
Specific growth factors	
EGF, PDGF, IGF, FGF, IL-1, IL-6, insulin	1–100 mg ml^{-1}
Trace elements	
Iron	1–10 M
Zinc	0.1–1 M
Se, Co, Cu, I, Mn, Mo, Cr, Ni, As, Si, Sn, F	0.01 M
Lipids	
Cholesterol	10 M
Linoleic acid	
Steroids	
Polyamines	0.01–1 M
Attachment Factors	
Fibronectin	1–2 M
Laminin and fetuin	–
Mechanical protection	
Albumin	–
Buffering	
Albumin	–
Neutralization of toxic factors	
Albumin	–
Transport of metals	
Transferrin/Fe^{3+}	2–4 mg ml^{-1}
Protease inhibitors	
1 antitrypsin	1.5 –2.5 mg ml^{-1}
2 macroglobulin	0.7–2.0 mg ml^{-1}

8.6.3.4 | Attachment Factors

It is inevitable for anchorage-dependent cells to bind to the substratum for proliferation. Serum provides fibronectin, fetuin and laminin.

8.6.3.5 | Protease Inhibitors

Two classes of inhibitors are present in serum, each representing 2% of the total serum proteins. The antiproteolytic activity of serum helps and protects the cell from proteolytic damage.

8.6.4 | Other Complex Supplements

The other naturally occurring substances used as supplements are complex natural media: Different combinations of biological fluids have been used since a long time. A few examples are stated in Table 8-5.

8.6.4.1 Blood Plasma

Burrows in 1910 for the first time substituted a coagulum prepared from chick plasma. Plasma is still being used. Plasma from adult chicken is preferred over mammalian plasma as it forms a clear solid coagulum, even after dilution. Mammalian plasma is too opaque and it fails to form solid clots. Plasma is obtained after centrifugation from whole blood before coagulation takes place.

Table 8-4 | Constituents of serum with their respective functions

Component	***Function***
Proteins	
Albumin	Transports lipids, hormones, minerals and provides osmotic pressure
Fibronectin	Promotes cell attachment
Fetuin	Enhances cell attachment
Transferrin	Binds iron
¿-Macroglobulin	Inhibits trypsin
Hormones and growth factors	
Insulin	Uptake of glucose and amino acids
Platelet derived growth factor (PDGF)	Mitogen for fibroblasts, smooth muscle cell
Fibroblast growth factor (FGF)	Mitogen – growth factor
Endothelial growth factor (ECGF)	Mitogen – growth factor
Epidermal growth factor (EGF)	Mitogen – growth factor
Hydrocortisone	Promotes cell attachment
Steroid hormones	Mitogen – growth factor
Thyroid hormones (T_3, T_4)	Oxygen consumption, metabolic rate control, growth and differentiation of various cells
Lipids	
Cholesterol	Membrane synthesis
Linoleic acid	
Prostaglandins	
Metabolites	
Amino acids	Cell proliferation
Polyamines	

8.6.4.2 | *Tissue Extract*

Carrel in 1912 demonstrated that embryo extract could promote cell growth in connective tissue cultures originating from chick heart. Several experiments have since been worked out to determine the growth-enhancing properties of these tissue extracts. Growth-promoting activity is predominantly due to nucleoproteins of the liver and spleens have also been used but there is no organ specificity. Activity also depends on the total

Table 8-5 | Constituents of complex natural media

Complex natural media	***Composition***
1. Supplemented Hanks–Simms medium	3 parts Hank's balanced salt + 1 part Simm's ox serum ultrafiltrate, Hanks–Simms 85%, beef embryo extract 10%, inactivated horse serum 5–20%, pencillin 50 g ml^{-1}, streptomycin 50 g ml^{-1}
2. Supplemented bovine amniotic medium	Bovine amniotic fluid 37.5%, inactivated horse serum 20%, bovine embryo extract 5%, HBSS 37.5%, streptomycin 100 IU/ml, penicillin 100 g ml^{-1}, mycostatin 100 g ml^{-1}
3. Serum-supplemented yeast extract medium	Yeast extract medium 76 parts, 10 parts of 1% Difco's yeastolate 2.5 parts glucose solution, 87.5 parts HBSS, human serum 20 parts, 1.4% sodium bicarbonate (4 parts)
4. Serum-supplemented lactalbumin hydrolysate and yeast extract medium	Earle's saline containing lactalbumin 0.5%, yeast extract 0.1%, human or ox serum 10–20%

Table 8-6 | Serum-free medium for certain cell and cell lines

Serum	*Serum-free medium*	*Cell or cell lines*
CS	MCDB 202	Chick embryofibroblasts
	CMRL 1066	Continuous cell line
	MCDB 110, 202	Fibroblasts, human diploid fibroblasts
	MCDB 402	Fibroblasts, mouse embryofibroblasts, 3T3 cell
FB	MCDB 130	Endothelium
	F12	Skeletal muscles
	HoS	Mouse leukemia, mouse erythroleukemia, skeletal muscles

Note: CS – calf serum, FB – fetal bovine serum.

nucleic acid content and the age of the individual from which the fraction has been isolated.

8.6.5 | *Serum-free and Protein-free Media and Their Applications*

In early 1970s, Gordan Sato and his colleagues published papers on the specific requirements of different cell types for protein growth factors, attachment factors and hormones. The mixture of supplements required for the serum-free culture of neural cells, defined by Bottenstein and Sato in 1979, still forms the basis of many serum-free supplements for a wide variety of cell types. serum-free media is the only option for people looking for reproducibility, and not being dependent on the world cattle market. serum-free media has no growth factors, viruses or growth inhibitors. A great deal of effort with very limited success has gone into developing serum-free media. The requirements of cell line differ greatly and success of serum-free culture with one does not guarantee success with other. With the identification of growth factors and nutrients required by different cells,several effective serum-free media have been formulated, which are enlisted in Table 8-6.

This group includes special types of media for use without the addition of serum or with reduced serum concentrations. These are the serum-free, protein-free and reduced-serum media for a variety of cell types. In a defined serum-free media the components are formulated together to optimize performance of a single cell type. Each component is of known concentration and purity. The origin of the cell line, that is species and tissue; the compatibility of media components and their interactions; and the specific application for which the cell line is being cultured, that is production of biomass and generation of product are important factors to be considered. Two methods followed in designing a serum-free medium are the following.

1. Reduced serum: The concentration of serum is progressively reduced while the other components, that is growth factors and hormones, are added to identify the factors capable of restoring the level obtained in presence of serum.
2. Basal media: Add components individually or in combinations to the basal media in a stepwise manner so as to achieve an equivalent cell growth to the serum-supplemented growth.

8.6.5.1 | *Important Factors for Defined Serum-free Media*

Basal medium: It is extremely important as it is the source of energy, buffers and inorganic ions. The choice of the medium depends largely on the cell line being used.

Lipids: Fatty acids, sterols, phospholipids, ethanolamine and phosphoethanolamine are the lipids used. Totally synthetic hydrophilic carriers such as cyclodextrins are used for the transport of lipids. Bound fatty acids are used in the form of phospholipid-enclosed vesicles, that is liposomes.

Buffers: They are most important as they maintain a proper environment for the metabolism, growth and functioning of the cells. Na^+, K^+, HCO^-_3 and HPO^{2-}_4 are important in maintaining pH, along with H^+ and OH^-. Amino acids when present in high concentrations can also help in buffering the medium. Sodium glycerophosphate is an important buffer to be used in the presence of low or no bicarbonate.

Trace elements: Electrolyte balance also contributes to the osmotic equilibrium of the medium. Trace elements are included because of their beneficial effects. Fe^{2+}, Zn^{2+} and Cu^{2+} ions are required by some cells. Co^{2+} and SeO^{2-}_3 are known to be included in some serum-free media. Cells derived from heart and kidney tissue have a high requirement for K^+, while Ca^{2+} is required to control mitosis. Ca^{2+}/Mg^{2+} ratio is important in controlling cell proliferation and cell transformation. Other trace elements include Sn, V, Al and As. Iron is added as a transferring complex but can be supplemented as ferric citrate, ferrous nitrate or ferrous sulfate.

8.6.5.2 *Advantages/Disadvantages of Serum-free Media*

The use of serum in culture media is not so common because it has following disadvantages:

1. The quality of serum varies from batch to batch and deteriorates within one year. Therefore, every batch of serum needs fresh testing.
2. If more than one cell type is used, each may require different serum batch, therefore, many batches are to be maintained and co-culturing may be difficult.
3. The demand of serum usually exceeds the supply for a variety of reasons.
4. When cell culture is used for downstream processing to cover cell products, the presence of serum is an obstacle for purification.
5. Serum increases the cost of medium.
6. Serum may stimulate undesirable growth and may even inhibit growth in some cases.

Advantages of serum-free media:

1. It has the ability to make a medium selective for a particular cell type, since each cell type appears to require a different recipe.
2. It has high degree of purity of reagents and water.
3. It needs high degree of cleanliness of all apparatus.

8.6.5.3 *How to Develop Serum-free Media?*

This is a very important, interesting but time-consuming facet of cell culture. Initially, a known recipe for a related cell type may be used. If possible, individual constituents of the serum may be altered until the medium is optimized. The assessment of constituents is a time-consuming process. Sometimes it takes about three years for development of a new medium. In the second approach, an existing medium like RPMI 1640 or HAM's F12 or DMEM (Dulbecco's modified minimal essential medium) is taken and a shorter

Table 8-7 | Serum-free medium formulations for cell lines

Cell line	*Source*	*Basal medium*	*Supplements*	*Substratum modification*
G H3	Rat pituitary carcinoma	F12	INS, TRF, T3, TRH, SOM, FGF	None
HeLa	Human cervical carcinoma	F12	INS, TRF, FGF, EGF, HC, TEL	None
PCC.4az a-1	Mouse embryonal carcinoma	F12	INS, TRF, 2-ME, FET	None
M2R	mouse melanoma	DMEM/ F12	INS, TRF, TES, FSH, NGF, LR	None
TM4	Mouse testes	DMEM/F12	HC	None
RF-1	Rat ovarian follicle	DMEM/F12	INS, TRF, FSH, SOM, GH, RA	Fibronectin
M 1	Mouse myeloid leukaemia	F12	INS, TRF, HC	None
B104	Rat neuroblastoma	DMEM/F12	INS, TRF, TEL	Fibronectin
C62 BD	Rat glioma	DMEM/F12	INS, TRF, PRG, PUT, SEL	Polylysine
MCF-7	Human mammary carcinoma	DMEM/F12	INS, TRF, T3, HC, PGE, SEL	None
B H K-21	Hamster kidney	DMEM/F12	INS, TRF, EGF, PGF	Fibronectin
3T3	Mouse embryo fibroblasts	DMEM/Waymouth	INS, TRF, EGF, FGF, BSA	Fibronectin
116NS-19	Mouse hybridomia	MEM or RPMI 1640	INS, $FeSO_4$, EGF, INS, TRF	None
HL60	Human promyelocytic Leukaemia	DMEM/F12	INS, TRF, SEL	None
MPC-11	Mouse plasmacytoma	DMEM/F12	TRF, LH, SEL, LRH, PGE, EGF T3, GLU, NGF, PGF	None
Flow 2000	Human embryo fibroblasts	MCDB 108	INS, EGF, DEX	None
WI 38	Human embryo fibroblasts	MCDB 104	INS, TRF, EGF, DEX, PDG	Polylysine
K562	Human erythroleukaemia	RPMI 1640	TRF, SEL, BSA	None
U-251	Human glioma	DMEM	TRF, FGF, HC, SEL, BIO	None
MGNCI-H69	Human small cell lung carcinoma	RPMI 1640	INS, TRF, SEL, HC, OES	Fibronectin
LA-N-1	Human neuroblastoma	DMEM/F12	INS, TRF, PRG, PUT, SEL	None
MDCK	Dog renal epithelium	DMEM/F12	INS, TRF, T3, HC, PGE, SEL	Polylysine

Note: BIO, biotin; BSA, bovine serum albumin; DEX, dexamethasone; EGF, epidermal growth factor; FT, fetuin; FGF, fibroblast growth factor; FSH, follicle-stimulating hormone; GH, growth hormone; GLU, glucagons; HC, hydocortisone; HDL, high-density leptoprotein; INS, insulin; LDL, low-density lipoprotein; LH, luteinizing hormone; LRH, luteinizing releasing hormone; NGF, nerve growth factor; OES, oestradiol; PDGF, platelet-derived growth factor; PGE, prostaglandin E_1; PGF, prostaglandin F_{2c} ; PRG, progesterone; PTH, parathyroid hormone; PUT, putrescine; RA, retinoic acid; SEL, selenium; SOM, somatomedin C; T3, triiodothyronine; TEL, trace elements; TES, testosterone; TRF, transferring; TRH, thyrotropin releasing hormone; 2-ME, 2-mercaptoethanol.

list of constituents like selenium, transferrin, albumin, insulin, androgen, hydrocortisone, estrogen, etc. is used for manipulation.

8.6.5.4 | *Influence of Culture Conditions and Medium on Protein Expression*

Many factors in the culture environment affect the quantity and authenticity of proteins produced by cells. Apart from dependence on the basic capacity of the medium to supply cells with nutrients and oxygen and to maintain pH, osmolality, etc., protein expression may be affected by other more subtle factors.

Extracellular matrices (ECM) can affect the growth regulation and maintenance of normal cellular functions (e.g. normal mammary epithelial cells when cultured on collagen substrate produce 4- to 20-fold more caesin than when grown on polystyrene substrate).

Composition of medium can affect the glycosylation of the expressed protein. Glucose limitation results in incomplete and/or aberrant protein glycosylation. ***Ammonium ion accumulation*** in cell cultures can result in glycoproteins deficient in terminal sialylation. Treatment of cells with different hormones, vitamins and differentiation factors may result in altered glycosylation patterns.

8.7 Biology and Characterization of Cultured Cells, Measurement of Viability and Cytotoxicity, Measuring Parameters of Growth

Any scientific analysis depends solely on the methods of quantifying experimental data. Routine culturing of cell lines also requires quantification of the cell number/density to enable optimum cell culturing. Methods available for cell growth can be divided in two subgroups:

1. Direct method: Cell numbers are directly determined by either counting them on electronic particle counter or by using a counting chamber.
2. Indirect method: Measurement of DNA content or protein content related to cell number to determine the biomass.

8.7.1 *Direct Methods for Quantification*

Commonly, the improved Neubauer haemocytometer is used which was originally used for counting blood cells. It is the cheapest and simplest method for counting. The haemocytometer is a modified microscope slide with two polished surfaces/chamber of known depth, which displays a precisely ruled grid, etched out on a silver base. The grid consists of nine primary squares, 1 mm each side (area 1 mm^2) having two to three closely spaced lines (2.5 mm apart). These lines determine the limits of the cell to be counted – whether inside or outside. The primary square consists of more lines which are basically to keep sight and assessment of the cell counted. The plane of the grid lies 0.1mm below

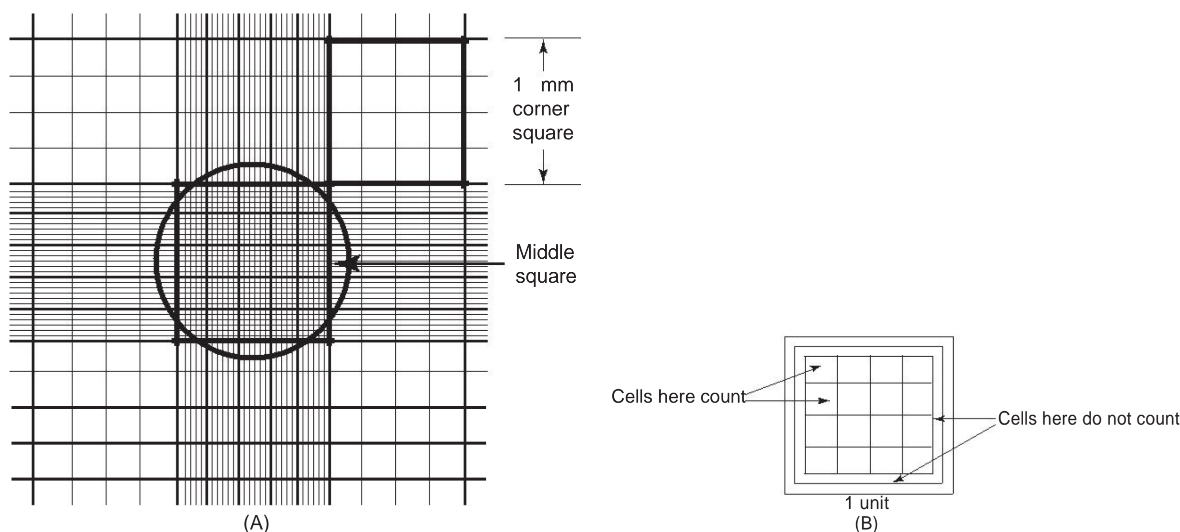

Figure 8-8 (A) Grid of a Neubauer haemocytometer; (B) side square of a haemocytometer.

two ridges, which support the coverslip. Also there is a depression on the outer edge of each polished surface where the cell suspension is added; this is to be drawn by capillary action.

Cells per ml = $100 \times 5 \times \text{dilution} \times 10^{-4}$.

The cell concentration per ml of suspension can be calculated simply by

$$C = n/v,$$

where C is the cell concentration, n the number of cells counted, v the volume (ml) represented by grid.

Going by the dimensions mentioned earlier, the grid size is $1 \times 1 \times 0.1\ \text{mm}^3 = 0.1\ \text{mm}^3 = 0.1 \times 10^{-3}$ ml.

Therefore, if we counted say 100 cells, the cell density would be

$$\frac{100}{0.1 \times 10^{-3}} = 10^6\ \text{ml}^{-1}.$$

Cell lines are unstable and change with time; hence it is important to keep track of its in vitro age. This is again done by two methods: (1) keeping a record of the passage number. Passage number is a documented record of the number of times it has been subcultured. (2) The other is to calculate the cumulative population doubling or the estimation of the cell generation.

8.7.1.1 *Measurement by Electronic Particle Counter*

Cells in suspension can be counted accurately and rapidly using a coulter counter. Electronic particle counter consists of two electrodes separated by a small orifice. If a potential is applied to the electrodes, current will pass between them through the buffer in the orifice. The amount of current will be dependent on the conductance (dielectric constant) of the buffer. Cells are suspended in an electrolyte and a metered volume of the suspension is pulled through a narrow aperture that carries a nominal current. As the cells pass through the orifice, it produces a fluctuation in the pulse. The pulse is amplified and the pulse within a prefixed threshold is counted and displayed on an oscilloscope.

Cell size can also be determined as the amplitude is directly proportional to cell volume. The size of the change in current flow depends on the size of the particle and the difference in the dielectric constant or conductivity. Cell clusters can also create a pulse. Such an error can be eliminated by adjusting the threshold of the pulse amplitude.

8.7.1.2 *Cell Viability by Dye Exclusion Method*

This method is used to count the number of cells by mixing an aliquot of cell suspension with a colored dye that is visible under the microscope. The dye should be membrane lipid insoluble, for example 0.4% erythrocin B or trypan blue. Only the cells with damaged plasma membranes will take up the dye. The viable cells will exclude the dye, hence the name. Hence we can correctly assess the number of cell that have not taken up the dye, and therefore percent viability can be easily calculated.

8.7.1.3 *Cytotoxicity Assay*

Four kinds of nonradioactive cell growth and cytotoxic assays are being routinely used. They are:

1. cell or colony counts;
2. macromolecular dye-binding assays;

3. metabolic impairment;
4. membrane integrity.

An ideal assay would be simple, rapid, sensitive, reproducible, quantitative, inexpensive, objective and reliable. Cell and colony counts are slow and time consuming, subject to individual discrepancies in methodology. Cell counts enumerate morphologically intact cells but fail to discriminate between live and dead cells.

Dye-binding assays are definitely more reliable and sensitive for growth and cytotoxicity. They are simple and rapid but require a spectrophotometer and a 96-well plate. They can indicate cell killing and growth inhibition.

Metabolic impairment assays measure the enzyme decay kinetics following toxic insults. They are more unreliable and complex than dye-binding assays. The success of these experiments relies heavily on reproducibility under identical conditions which are difficult to achieve in biological reactions. Deviations in experiments may cause serious errors. Their advantage is that they are capable of distinguishing normal cells from the cells having altered cellular metabolism.

Membrane integrity assays measure the ability of the cell to impermeat extracellular molecules. It can either be colorimetric or fluorescent, similar to dye-binding assays. They provide a surrogate index of the viability of the cells by their ability to exclude the dye, but cells can slowly accumulate the molecule. Hence extra caution should be taken while designing experimental protocols.

8.7.2 *Indirect Methods for Quantification*

8.7.2.1 *MTT Assay*

This can be put under membrane integrity assay as well as colorimetric assay. Tetrazolium salts have been extensively used to localize dehydrogenase enzyme activity. These salts do not react with dehydrogenase per se but with their products NADH and NADPH. These methods are developed to estimate cell number based on the cellular content of enzyme or substrate and subsequent extraction of the dye. A yellow-colored water-soluble salt 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide [MTT] reacts with the mitochondrial dehydrogenase enzymes of live cells, which reduces them to purple-colored insoluble formazan crystals which are precipitated in the immediate vicinity. Dehydrogenase content is consistent among cells of a specific type and the amount of formazan reduced is proportional to the cell number. Different cell types may have varied amount of dehydrogenase. Culture conditions may alter the enzyme content and activity. DNA content of cells can be determined to estimate changes in population densities. Amount of DNA per cell progresses as the cell grows. Presence of bi-nucleate and multinucleate cells in culture, hinder the accurate estimation of the cell number based on DNA content. DNA assays are unable to differentiate between viable and nonviable cells. Other methods such as radiolabeling and estimation of total protein content are used less frequently and are useful when cells are grown in microwell plates or hanging drop cultures. The cells can be mixed in situ, stained and counted manually. DNA and protein assays are extremely inaccurate.

Once a cell line has been established there is an urgent need to characterize it so as to confirm its species and tissue of origin, and also to determine the differentiated status of the cell within the lineage. To begin with, a complete record of the species and tissue of origin needs to be recorded. The species of origin can be confirmed by cytogenetics, isoenzymology, karyotyping and immunological methods.

8.7.3 Characterization of Cultured Cells

8.7.3.1 Cytogenetics

There is need to verify that the cells are derived from the particular species. Chromosome content and analysis is species specific and is a reliable method of species characterization. The correlation of chromosomal structure with heredity and variation is termed cytogenetics.

8.7.3.2 Karyotype

Karyotype is the collective term used to describe the chromosome number, size and shape. Primary cell lines retain diploid chromosomes and also the chromosomes of the donor. Lines from individuals with cytogenetic abnormalities have been extensively used for mapping genes, not merely to individual chromosomes but also to specific regions of chromosomes.

8.7.3.3 Isoenzymology

Enzymes that exist in multiple forms in animal tissue and in different molecular forms, catalyzing the same reaction, are called isoenzymes.

8.7.3.4 Immunological Test

This test depends on the specificity of antigen–antibody reaction. Species-specific antigens can be detected by immunofluorescence.

8.7.3.5 Intra-species Contamination

Once the species has been characterized, the next logical step is to characterize the tissue of origin. A number of techniques have been used for this:

Recombinant DNA methods: It is possible to directly determine an individual's genotype by typing the genes themselves. For many genes there are slight variations between individuals of a species, and such molecular differences in the DNA of a particular gene are called polymorphism. The presence of dissimilar arrangements of DNA bases means that each individual's DNA is cleaved at slightly different sites by restriction enzymes, generating distinct lengths of restriction fragments. DNA probes can be used to detect polymorphisms in a technique known as restriction fragment length polymorphism (RFLP). Cellular DNA is digested with restriction enzymes and the fragments of DNA are separated by agarose gel electrophoresis. The DNA is then transferred to a membrane by southern blotting and the DNA on the membrane is 'melted' to form single strands. The membrane is then exposed to radioactively labeled DNA probes. These probes are prepared using highly repetitive nucleotide sequences (so-called satellite DNA). These radioactive probes will react with the DNA fragments, which contain complementary nucleotide sequences, and they are detected by autoradiography. This process is called DNA fingerprinting.

High-resolution two-dimensional *electrophoresis:* The different proteins in the cell can be separated on the basis of isoelectric point and molecular weight using isoelectric focusing in the presence of urea in one dimension and electrophoresis in the presence of sodium dodecyl sulfate (SDS) in the second dimension. This process separates the proteins according to isoelectric point and also according to their size. By this method one can resolve a number of proteins simultaneously.

Allozyme analysis: Every individual belonging to the same species expresses different alleles for a given enzyme locus. Allelic isoenzymes are referred to as allozymes. This analysis is like a genetic signature for that cell line.

Blood group antigens and HL-A: This involves the determination of the antigens present on the surface of the cell. Blood group antigens are present on normal human epithelium in primary culture and on some continuous epithelial lines. The use of anti-human A, B or AB typing antiserum can be used to type these cell lines. The major histocompatibility antigens (or major histocompatibility complex – MHC) are highly variable antigens which are responsible for the immunological individuality of each person. These antigens are present on the plasma membrane of nucleated cells and are responsible for the fact that tissue transplanted from one individual to an unrelated individual will be recognized as foreign and will be rejected by the recepient's immune system. In man, the histocompatibility antigens are referred to as the human lymphocyte antigens (HLA) system. The genes coding for the HLA antigens are on chromosome 6 and occupy 4 loci (designated A, B, C and D) along the chromosome. The genes at any locus are not always the same, and the different forms of each gene are called alleles.

8.7.4 Morphology

Viewing the cells under the microscope can give us a clear picture. Epithelial cells are polygonal, regular with marked edges. Fibroblasts are bipolar and form definite parallel patterns, visible to the naked eye. Use of different substrates and culture media used may alter the morphology of the cells. A number of markers are currently available for more specific identification, viz., cell surface antigens, intermediate filament proteins, analysis of isoenzymes, expression of functional properties of the mature cell.

8.7.5 Parameters of Growth

Growth is the change in a quantity over a period of time. It is a process that requires two measurements, that is one at the onset of experiment and one at the end of the assay. For measuring growth kinetics, intermediate measurements are also recorded. A tissue can grow by: (i) increasing the number of cells, (ii) increasing the size of cells, (iii) increase in the amount of intercellular substances. There are static and dynamic ways of measuring growth and cell division. Counting the number of cells in a petridish tells us how much the cell population has grown, but it does not indicate the proliferation of the cells. Other methodologies are necessary to ascertain details of cell proliferation, for example autoradiography with ^{3}H thymidine, flow cytometry, immunohistochemistry, etc.

Indirect measurement of cell number: The number of cells present in a culture is by far the best parameter to use. But one can also measure the amount of DNA per flask or per gram of tissue, or the amount of RNA or the amount of protein, or count the number of mitoses.

8.7.6 Phases of Cell Growth

Conditioning and adaptation to culture is important for cells plated at clonal density. Growth of cells in culture generally shows a sigmoidal pattern which indicates adaptation to culture, conditioning to the environment and the availability of substrate and nutrient media to support proliferation.

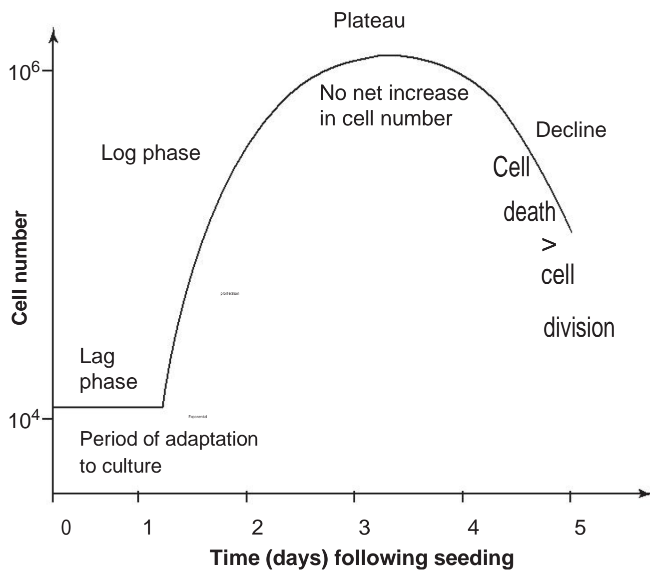

Figure 8-9 | Growth phases for normal cells in culture.

8.7.6.1 | *Lag Phase*

The time required for the freshly seeded cells to adapt, attach to the substrate and subsequently divide is termed as lag phase. The length of the lag phase depends on two factors: seeding density and the cell cycle phase in which the subculture is done. Cultures seeded at lower densities have longer lag phases.

8.7.6.2 | *Logarithmic (log) Phase*

This represents the most active phase in the entire growth pattern as it has 90–99.5% population growing exponentially. To study any functional assay, cells in the log phase are considered the best. Cell proliferation kinetics during log phase are characteristic for every cell line. Population doubling time is also calculated in this phase. Seeding density, rate of cell growth and saturation density of the cell line are crucial factors that influence this phase.

8.7.6.3 | *Plateau or Stationary Phase*

Cell proliferation tapers off after the population attains confluency. In this phase the number of cells dividing is equal to the number of cells dying; also the cells in cell cycle are as low as 1–10%. This is the plateau phase. It is not a stable period for many cell lines as this phase ismore susceptible to injury. Cells in suspension also reach saturation density.

8.7.6.4 | *Decline Phase*

Plateau phase leads to the next phase, that is the decline phase. Here the balance between cell division to cell death is disrupted. There is more cell death and there is decrease in the number of cells. This is not due to nutrient deficiency.

8.7.7 | *Population Doubling Level (PDL)*

It is assumed that cells in a culture undergo sequential symmetric divisions; hence there is exponential increase in cell population. At the end of n generations, each cell of the seeded inoculum produces 2^n cells. Therefore at the end of each generation the total

number of cells is given by $N_H = N_I 2^n$, where N_H is the number of cells harvested and N_I is the number of cells inoculated.

Number of population doublings or the number of generations represented by logarithms (base 10) is given by

$$2^n = N_H/N_I$$

$$n \log 2 = \log(N_H/N_I)$$
$$= \log N_H - \log N_I.$$

Since, log 2 = 0.301, therefore we have

$$0.301n = \log N_H - \log N_I,$$

which implies that

$$n = 3.32(\log N_H - \log N_I).$$

The number of generations between inoculum and harvest is added up to give the PDL. Previous PDL is added to the present PDL to give the cumulative PDL, which is documented on the flask after subculture.

8.7.8 *Multiplication Rate*

To know the exact in vitro age of the cells in culture, one must calculate another useful parameter on log phase – multiplication rate (r). It is the number of generations occurring per unit time and is expressed as population doublings per 24 h. Population doubling time (PDT) is the time, expressed in hours, taken for a known cell population to double. PDT is the reciprocal of the multiplication rate, that is

PDT = total time elapsed/number of generations,

PDT = t/r.

Population doubling level, multiplication rate and population time all indicate the growth characteristics of the cell population.

8.8 Manipulation of Cultured Cells and Tissues

8.8.1 *Scaling-up of Animal Cell Culture*

Routine cell culture for the purposes of studying cell morphology and functional toxicity and drug efficacy testing can be done on a small scale in the laboratory. But for the produc-tion of vaccines, monoclonal antibodies, etc., there is a need to scale-up cell culture. A new milestone has been reached in biotechnology that brings complete design flexibility and simplicity to the production of mammalian cells and their by-products. The large-scale culture of mammalian cells is far more costly than microbial culture. Various 'perfusion systems' have been developed where cells are retained in an all-purpose stirred-tank bioreactor. By increasing the culture volume and the concentration of cells in a reactor and by continuous perfusion of fresh medium, one can economically produce a product in large amounts. Continuous perfusion has a dual effect of supplying nutrients and simultaneously removing toxic metabolites. The cells are cultivated in two different phases as freely suspended cells in liquid phase and immobilized cells in solid phase. It is now possible to use a variety of cell culture methodologies for the successful growth of virtually all eukaryotic cell lines.

8.8.1.1 | *Homogenous Fermenter Systems*

Traditionally fermenters were used for upscaling of cell culture. These were essentially of two types: stirred tank and airlift types. Suspension cultures are grown in stirred tank type while microcarrier systems are used for adherent cell lines.

***Suspension** culture:* In suspension culture the cells are dispersed in liquid medium and grow freely but are not attached with any solid. The medium is agitated so that they do not form sediments. Therefore, it depends on choice of cells whether they are capable of growing freely in suspension or as immobilized cultures. A fully automated bioreactor is used that maintains the physiochemical and biological factors at optimum level and maintains the freely suspended cells in an agitated low viscous liquid medium. The most suitable bioreactor used is a compact-loop bioreactor that consists of marine impellers. It also relies on the integrated monitoring and control of physical, chemical, biological and biochemical parameters.

As compared to bacteria, animal cells grow slowly. This results in bringing the cells in unfavorable metabolic state due to very small changes in culture. The main carbon and

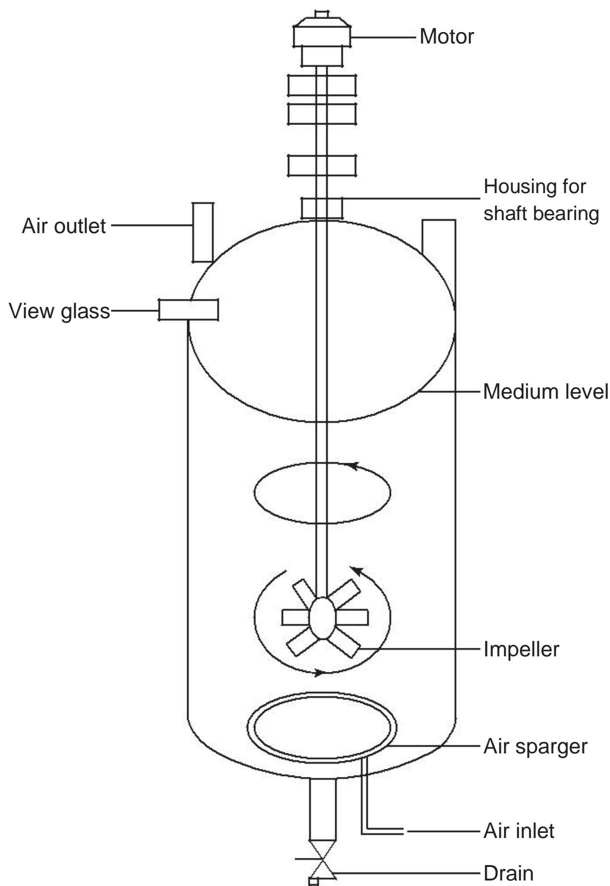

Figure 8-10 | Diagram of a stirred tank bioreactor (arrow indicates the direction of flow of liquid).

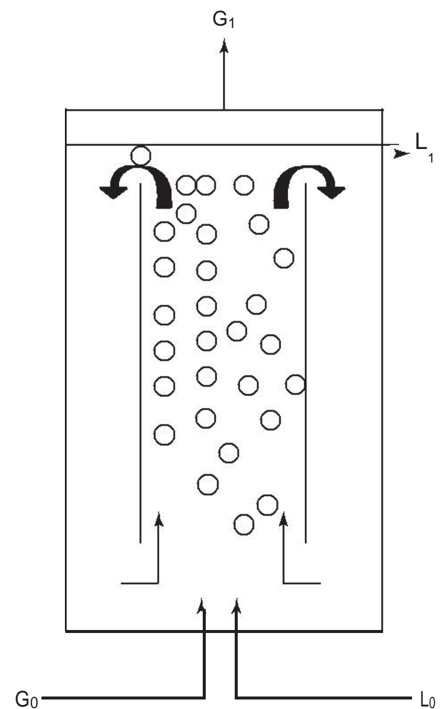

Figure 8- 1 1 | Airlift bioreactor.

energy sources are glucose and glutamine. Lactate and ammonia are their metabolic products that affect growth and productivity of cells. Therefore, on-line monitoring of glucose, glutamate and ammonia can be carried out by on-line flow injection analysis (FIA) by using gas chromatography (GC), high performance liquid chromatography (HPLC), etc.

Sometimes cell death also occurs; therefore, for measuring differences between viable and dead cells some enzyme activities are measured and correlated with cell density. Lactate dehydrogenase is used to distinguish cell density of viable cells. Cell lines derived from haemopoietic tissues, for example H9, Hut 78, Raji and hybridoma lines, normally grow in suspension either as single cells or in small clumps of cells derived from a single cell.

These studies included the use of serum-free medium, materials and agitation issues, and the correlation of specific cell populations with metabolic patterns. The next step in spinner culture development was scaling-up the suspension culture and adding pH and dissolved oxygen (DO) probes to maintain control over these parameters. Using a daily dilution feeding protocol, an excellent cell expansion (400-fold) in this configuration can be obtained.

Preliminary results have demonstrated that suspension culture holds great promise as an efficient system for culturing primary hematopoietic tissue, but the traditional spinner-flask or continuously stirred tank reactor (CSTR) system suffers from some drawbacks (such as the need for perfusion) that prevent their use as efficient and economical systems for routine clinical application. In general, current suspension systems are descended from systems designed to efficiently produce soluble proteins from a relatively homogenous cell population.

Microcarriers: Microbeads of different diameters ~ 150 m (i.e. 90–300 m) made of dextran, plastic, glass, gelatin or collagen can be effectively used to grow monolayer cells. Culturing monolayer cells on microbeads gives a maximum ratio of the surface area of the culture to volume of medium, almost up to 90,000 $cm^2\ l^{-1}$, depending on the size and density of the beads. It has the additional advantage that the cells may be treated as a suspension. Microcarrier uses a pendulum or paddle as in suspension culture to achieve efficient stirring without damaging the beads.

Anchorage-dependent cells: For anchorage-dependent cells growing on microcarriers, a double-screen cell-lift impeller can be employed. This low shear gas-exchange impeller increases oxygen transfer rates at very low stirring speeds. A marine blade or pitched blade impeller can also be utilized for these applications. Use of a double-screen impeller creates an inner cell-free aeration chamber containing a ring sparger. Cells and microcarriers are too large to pass through the screen and are kept outside, protected from shear forces. Only the culture medium can flow freely to and from the aeration chamber where high volumes of gas are absorbed by the medium and transferred to the culture.

8.8.1.2 *Non-homogenous Membrane Systems*

Hollow-fiber bioreactor (HFBR) systems: HFBR systems are perfusion systems based on the principle of compartmentalization, where the cells are retained in a low-volume compartment at a very high concentration and medium is perfused through hollow fibers within the cell compartment. They have been used extensively to grow many types of mammalian cells under entrapped conditions, with the primary objective of

obtaining large quantities of concentrated excreted proteins. HFBR systems offer a virtually shear-free environment for cell proliferation and simplicity of operation for the culture of cells to near-tissue-like densities. HFBR systems have also been explored for the production of cells for tumor infiltrating lymphocytes (TIL) cell therapy, lymphokine activated killer (LAK) cell therapy and other cytotoxic T-cell therapies. Recently, expansion of human stem cells from fetal liver has also been reported. While these are excellent systems for producing high concentrations of excreted proteins, they suffer from several disadvantages when considering tissue engineering applications, in which the cells are the desired product. First, the culture environment is spatially inhomogeneous, and this creates potentially large concentration gradients of critical nutrients, as well as oxygen and pH. Since hematopoietic differentiation is influenced by these culture variables, there would be less phenotypic control, leading to a loss of selectivity in the production of particular cell types. Second, when scaled-up, the space between fibers is not typically kept constant, which would significantly change the oxygen transfer characteristics of the system. Thus, while the culture performance of the HFBR systems might be adequate, the difficulties encountered in monitoring and controlling the culture environment suggest that a different type of reactor would be more appropriate for the expansion of primary hematopoietic cells.

Multiarray disks, spirals and tubes: Disks, spirals and tubes have all been used to increase the surface area for monolayer growth, but few of these systems are now available on a commercial basis. Most matrix or multisurface propagators have now gone toward perfusion with the emphasis on product recovery. Corning Costar markets a multisurface perfusion system, called cell cube, that is a hollow polystyrene cube with multiple inner lamellae, perfused with oxygenated, heated medium. The inner lamellae are capable of supporting monolayer growth on both surfaces.

Roller culture: If cells are seeded into a round bottle or tube that is then rolled around its long axis, the medium carrying the cell runs around the inside of the bottle. It is useful for adherent cells as cells adhere to curved surface area thereby increasing the growing area, and grow to form a monolayer. If the cells are nonadherent, they will be agitated by the rolling action, but will remain in the medium. Bottles can be used in specialized CO_2 incubators with attachment that turn the bottles along an axis. Volume of the medium used should be just enough to cover the monolayer. The roller platform consists of steady frame incorporating two or more moving belts on which the bottles rest. Bottles turn at approximately 2 revolutions per hour. Roller bottle systems have major advantages over static monolayer culture: (1) surface area is greatly increased; (2) the constant, but gentle, agitation of the medium and (3) the increased ratio of the medium's surface area to its volume, which allows gas exchange to take place at an increased rate through the thin film of medium over cells not actually submerged in the deep part of the medium. Care should be taken to keep the platform in the correct horizontal position. If kept incorrectly, it may lead to cell death.

Immobilized cell culture: Microcarrier beads are used for cell immobilization as these are capable of developing positive or negative surface charge. For example DEAE sephadex (diethylaminoethyl type of crosslinked dextran) developed positive charges, polycysteine negative charges and gelatin beads slightly positive or negative charges. The beads require surface coating for adhesion of sufficient number of cells, because energy is required for

cell adhesion. For adhesion, glass and ceramic materials are used as carriers as they have high surface energy.

In different types of bioreactors different materials such as glass spheres are packed for cell attachment. The materials increase surface area for cultivation of cells en masse. This type of bioreactor is called bed bioreactor. Fixed bed bioreactors are nonhomogenous. Ceramic matrix reactors are used for both adherent and suspension cultures. Plate heat exchangers and glass bead reactors are exclusively meant for adherent cultures. For a bioreactor of 100 l capacity, about 50 kg of 3 mm diameter spheres are used. This gives a total surface area of about 20 m^2.

Flat-bed perfusion systems: Flat-bed perfusion bioreactors were developed in an attempt to address some of the unique concerns of primary hematopoietic culture. The first system consisted of flat-bed chambers containing stromal (supportive cell layer) co-cultures that were perfused with media and demonstrated modest cell expansion. A modification of a flat-bed culture chamber has allowed the development of stroma-free hematopoietic perfusion culture. The chamber contains multiple microgrooves (perpendicular to medium flow) that allow the diffusion of nutrients but retain cells in the chamber. This reactor design, however, is not optimal for the tightly controlled production of large numbers of mature hematopoietic cell products. In the stromal co-culture system, cells are in intimate contact with a stromal layer that has a high metabolic rate. The hematopoietic cells in proximity to this stromal layer will therefore be exposed to gradients of pH and dissolved oxygen that will differ from those of the bulk medium. In the grooved system, cells are isolated from the main convective flow of medium, making it more difficult to monitor or control the environment near the cells. Thus for both types of flat-bed reactors we have a situation in which total cell capacity is limited by the monolayer nature of the cultures, and it is difficult to control the environmental conditions near the cell surface. Primary hematopoietic cell cultures are extremely sensitive to many materials in the culture system and perform poorly in the presence of substances (such as polysulfone and stainless steel) that have been shown to be biocompatible for other applications. These detrimental effects appear to be caused by leaching of plasticizers and metal ions, adsorption of proteins onto surfaces and autoclave-induced changes of surface characteristics. The observation that fresh polystyrene, whether tissue-culture treated or not, provides superior performance for primary hematopoietic tissue culture was consistent throughout these experiments. These observations demonstrate that for clinical applications of hematopoietic culture, as many reactor components as possible should be disposable and ideally composed of polystyrene.

Microencapsulation: The sol or gel transition phase of some biopolymers is exploited to encapsulate cells. Sodium alginate is commonly used as the transition phase and it depends on the concentrations of the divalent cations. It gels as a hollow sphere around the cells in suspension cultures. Macromolecules secreted by the cells are retained within the hollow sphere created by alginate. There is free movement of metabolites, nutrients and gas. Recovery of cells is done by reducing the concentrations of the divalent cations. These can be used in vivo as the biopolymer gels have low immunoreactivity.

8.8.2 Cell Synchronization

When all the cells of a culture divide at the same time it is called synchronous growth. A synchronously growing population of cells is essential for examining many aspects of cellular growth and metabolism. Analysis of cell cycle and proteins involved in cell cycle

control, expression of enzymes involved in DNA replication and repair, mitotic events, virus-cell interactions during lytic and latent infection, cell differentiation and cell death are the issues which can be solved by having a synchronized cell population. Two different methodologies are used to achieve cell synchronization. First, cells should be at a particular point in the cell cycle based on physical properties. Differences in adherence properties of the cells are exploited to obtain the selective detachment of mitotic cells. Second, force the cells to accumulate at a particular point in the cell cycle and subsequently release to allow continued synchronous growth by use of agents that inhibit specific events in cell cycle such as DNA replication or by use of techniques that prevent cell cycle progression such as contact inhibition of growth or deprivation of nutrients.

8.8.2.1 *Factors to be Considered before Synchronization*

Every cell line requires optimization of synchronization methods. Hence, choose a cell line on which methods of synchronization have already been standardized, that is, choose a stage in the cell cycle where the population will be synchronized and the number of cells that are necessary to complete the study.

It is important to calculate the degree of synchrony of a culture. There are several methods to assess the synchrony of cells. These methods depend on detection of the differences in DNA content throughout the cell cycle or on measuring the amount of DNA synthesis. Flow cytometry can be performed to determine the number of cells in culture having a particular DNA content. It can give an insight into the number of cells in G_1, S and G_2/M phases of the cell cycle. The other method is to measure the amount of labeled nucleotide incorporated that is [^{3}H] thymidine or analogs of nucleotide for example bromodeoxyuridine, into DNA. It is possible to measure the number of cells in S phase as well as to measure the amount of DNA being synthesized in cell culture.

8.8.2.2 *Methods for Synchronization of Cell Populations*

Protocols for achieving cell synchronization can be achieved by arresting cells in G_1 phase using isoleucine deprivation, in S phase using DNA synthesis inhibitors and in mitosis using selective detachment. Finally, centrifugal elutriation is done to separate the cells on the basis of size as they proceed through the cell cycle.

G_1 synchronization by isoleucine deprivation: Ley and Tobey observed in 1970 that CHO cells could be reversibly arrested in G_1 phase when grown in isoleucine deficient medium. On replenishing the medium with isoleucine the cells resume their growth and enter S phase within 4 h. Other hamster cell lines, some mouse transformed cell lines and some transformed human cell lines have been used with varying effectiveness. This procedure is applicable to cells in G_1 stage. It can also be used in combination with agents that block cells at the G_1/S border to provide an extremely efficient synchronization in early S phase.

G_1/S-phase synchronization: DNA synthesis inhibitors can be efficiently used in syn-chronizing the cell population in S phase. Synthesis of deoxyribonucleotide triphosphates can be inhibited by using excess thymidine and hydroxyurea. Some plant proteins, mimosine efficiently synchronizes cells in G_1/S phase, possibly inhibits the production of deoxyribonucleotide triphosphates through the interaction with intracellular proteins. Aphidicolin, inhibits entry into S phase as it inhibits DNA polymerase.

The difficulty in using these DNA synthesis inhibitors is that it will effectively arrest the cells in S phase and those cells which are not in S phase or are approaching S phase will not be affected till they reach the G_1/S stage.

***Double** thymidine synchronization:* The above problem can be addressed by using these agents in a double-arrest method. It can also be applied to a population that has been previously arrested or synchronized in G_1 phase by another method. After which a double block that is a thymidine block, can be added to cell preceding G_1 to block them at the G_1/S boundary. Therefore, to obtain a population with all the cells synchronized at the same point, it is important that the cells which have stopped growth within the S phase proceed completely through S phase. This method allows synchronization of a large number of cells, and is applicable to both monolayer and cells in suspension. The problem of this method is that it is difficult to cause complete inhibition of the DNA synthesis, hence it is called as 'leaky'. However, this problem can be addressed by checking the cultures periodically by flow cytometry to determine the exact cycle time schedule, the degree of synchrony of the culture after release from thymidine arrest.

Synchronization in mitosis using selective detachment: Cells growing in monolayers become loosely attached to their substrate because they tend to become spherical during the mitotic phase of their cell cycle. Cells are gently 'tapped out' by mechanical treatment, which selectively detaches them. 90–97% of cells isolated in this way are in the mitotic phase, hence the cells can be allowed to grow synchronously into G_1. The major drawback of this method is that only a small fraction of the entire population of the cells is

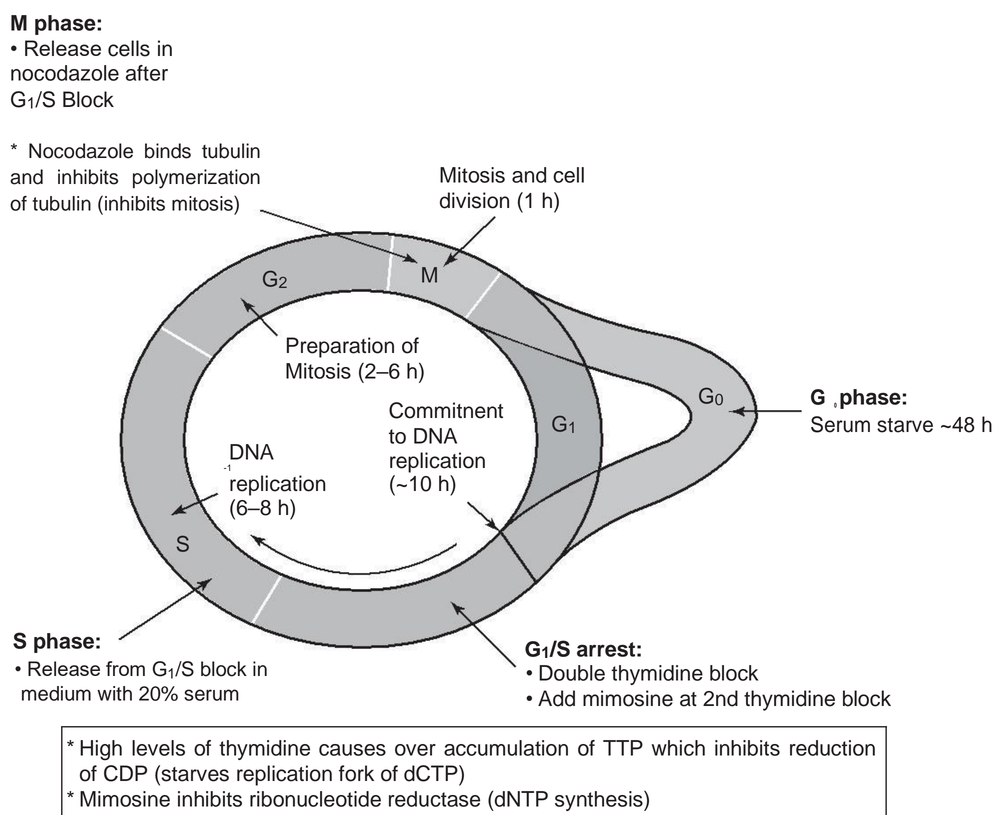

Figure 8-12 Cell cycle and cell synchronization.

undergoing mitosis at a given point of time. However, it is possible to collect cells periodically from the same culture flask a number of times. Cells thus collected should be kept in ice (for a maximum of 2 h) and subsequently transferred to 37 °C. Also, drugs that arrest cells in mitosis can also be used to increase the yield, for example nocodazole which causes selective loss of non-kinetochore microtubules. In 1992, Reider and Palazzo observed that colcemid disrupts tubulin assembly. The major advantages of using agents are that they increase the mitotic cell in cultures as well as arrest the cells in mitosis (metaphase) that allow easy assaying of the mitotic index or the degree of synchrony.

Limitations of this assay are that it can be applied to monolayer cells; hence the yield of synchronized cells is generally low. Also the assay needs to be modified for different cell lines – the difference in attachment, the force needed to detach the cells from the substrate.

Centrifugal elutriation: This exploits the fact that cells increase in size linearly as they proceed through the cell cycle as shown by Brown and co-workers in 1987. Based on this assumption, cells are isolated at the same point. A specialized centrifuge is used, where the cells are subjected to two opposing forces within a cell chamber, having a rotor and an opening near the center of rotation. The centrifugal force is exactly balanced by the flow of medium in the opposite direction. Inside the chamber, cells are in equilibrium in such a way that the smallest cells (G_1) are closest to the opening and the largest cells (G_1/M) are closest to the inlet. Cells of a particular size are periodically eluted by increasing the flow rate and there is a new equilibrium status attained by the cell population after each elution. Hence the population eluted is quite synchronous.

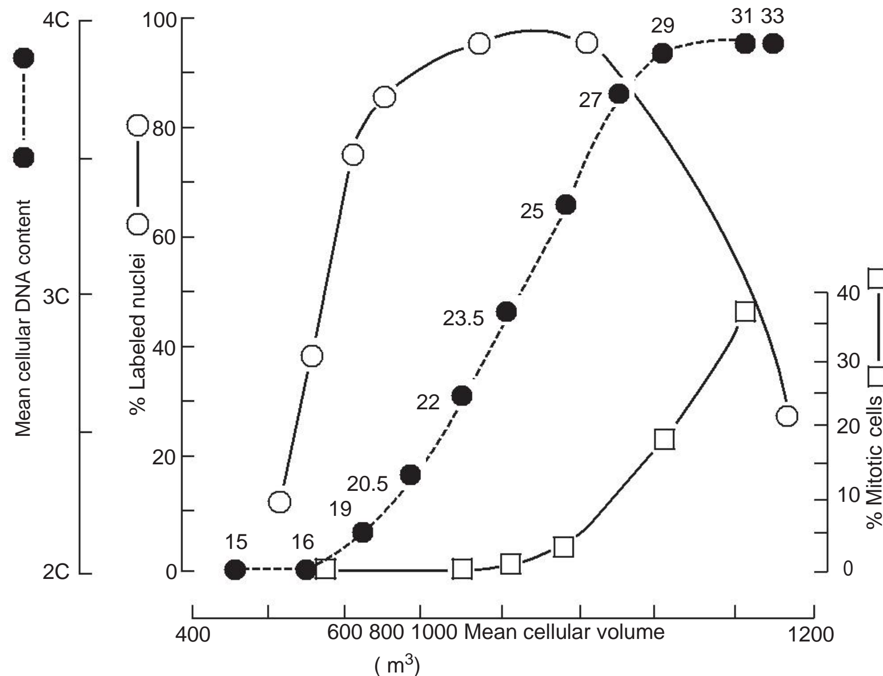

Figure 8-13 Analysis of cell fraction collected from a centrifugal elutriation.

The major advantage of this technique is that there is no use of chemicals for separation and the entire procedure is completed within a few hours from the same exponentially growing population. It is very effective for cells growing in suspension, but specialized elutriation rotors have to be used for monolayer cultures, which can only be used after trypsinization followed by resuspension.

All the methods enlisted above are meant for obtaining synchronized cell populations to define regulatory mechanisms that mediate competency of cell cycle. Parameters of control that relate to competency for initiation of proliferation, unquestionably associated with responsiveness to growth factors, may evolve additional and unique considerations. The initial entry of cells into S phase following induction of proliferative activity maybe somewhat different from those that traverse the G_1/S phase transition point in continuously dividing cells. The G_0–G_1 transition or exit from a 'deep' or 'prolonged' G_1 must be further defined at the biochemical, molecular and ultrastructural levels.

8.8.2.3 Cell Cloning and Micromanipulation

A clone is a population of cells that have descended from a single parental cell. Clones are made to minimize the degree of genetic and phenotypic variation within a cell population. During cloning, a population of cells is derived from a single-cell mitosis to produce a genetically homogenous clone, which can then be characterized and stored. The uniformity of the cells within the cell clone and the potential to increase cell number opens up a wide range of experimental possibilities.

1. In dilution cloning, the cells are seeded at low density and are incubated until colonies are formed after which they are isolated and propagated into cell strains.
2. By using selective media for example, fibroblasts. can be eliminated either by complement-mediated lysis using monoclonal antibodies against fibroblasts or by using chemicals which suppress fibroblasts overgrowth (such as cis-OH-proline, sodium ethylmercurithiosalicylate and phenobarbitone). In complement-mediated lysis, the monoclonal antibodies bind to the fibroblasts. This activates complement (a cascade system of blood proteins) that lyses those cells to which the antibody is attached.

Applications of cloned cells from continuous lines:

1. Many continuous lines are genetically unstable and their properties may alter during passage. Cloning can be used to isolate cultures with properties more closely resembling those of the original population. Examples of the latter may include karyological and biochemical variants and cells that exhibit different levels of product secretion or different susceptibilities to viruses. Treatment with mutagens can be used to increase the proportion of variant cells.
2. Variation within a continuous cell line may be studied by examining the properties of panels of clones established at different passage levels.
3. Cells transfected with DNA do not necessarily form populations with homogeneous genetic constituents, and cloning can enable cells to be selected and cultures developed with the required characteristics.
4. In applied biotechnology, it may be desirable from a regulatory standpoint that a cell line used to derive a product can be defined as originating from a single cell.

Cloning rings: In this technique, cells are grown at low cell density in conventional plastic or glass tissue culture vessels, but once discrete colonies have been formed cloning

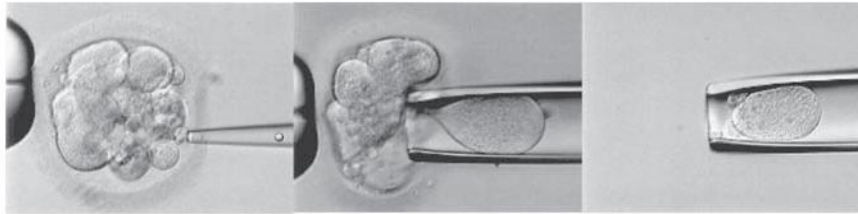

Figure 8-14 Micromanipulation of cells.

rings are used to isolate individual colonies and permit their trypsinization and removal of subculture. Cloning rings are small, hollow cylinders, generally made from glass, stainless steel or PTFE, and can be of any size which is convenient. They may be cut from suitable tubing and the ends smoothed with file or stone. Alternatively, they may be purchased from Bellco (cloning cylinders, code 2090) who supplies them in a variety of sizes in either stainless steel or borosilicate glass.

Stainless steel rings of 8 mm inside diameter, 2 mm wall thickness and ca.12 mm height have been found to be particularly satisfactory as their weight seats them firmly in the silicone grease, permitting a good seal to be maintained during the cell detachment procedure. However, the height of the cloning rings to be used must be chosen with reference to the dimensions of the vessel within which they will be used, as they must not be so tall that they prevent closure of the vessel.

Petriperm dishes: Another cloning technique applicable only to attached cells involves growing cells at low density on petriperm dishes (Bachofer GmbH). The bottom of these dishes is made of a thin fluorinated ethylene propylene (FEP) foil, which can be produced with either a hydrophobic or a hydrophilic surface. The latter (hydrophilic) would normally be used for cell cloning. If the cells are plated at a sufficiently low density such that the resulting colonies are well separated, individual colonies can be removed while still attached to the substrate simply by cutting the FEP foil with a scalpel.

***Spotting** technique:* This technique is simple and convenient, lending itself well to aseptic technique and giving low risk of adventitious contamination. An enhanced antibiotic regime is not usually required.

Micromanipulation: This technique permits the selection of cells with particular morphologies or other observable characteristics. It may be performed either manually or using a micromanipulator. In both cases, especially the former, some dexterity and practice are required to achieve consistent results. A sufficient uninterrupted period of time should be set aside to carry out the procedure.

8.8.3 Cell Transformation

Transformation is defined as the acquisition by cells of certain phenotypic properties associated with cancer. A number of assays have been developed to establish whether or not cells are transformed and they have generally proved reliable if used in the correct context. Transformed adherent cells can be identified by changes in the control of their growth and behavior. Some of these changes are described in more detail below.

8.8.3.1 *Phenotypic Properties of Transformed Cells*

The changes to cells when they become transformed can be treated under five headings:

1. Ability to form foci in in vitro culture.
2. Changes in morphology.
3. Changes in growth factor requirements.
4. Release from anchorage dependence for cell division.
5. Ability to form tumors in vivo.

These changes provide the basis for determining whether or not cells have been transformed.

Morphology: The morphology of cells changes upon transformation. Usually trans-formed cells tend to round up, probably due to the disruption and reorganization of cytoskeletal structures.

Growth factor requirement: Transformed cells proliferate under conditions in which normal cells do not. When such cells are cultivated in vitro they will grow in media containing low serum concentrations whereas nontransformed cells will not do so. This reduced requirement for exogenous mitogenic stimuli is now known to result from abnormal expression of growth factors or growth factor receptors or by subversion of the signal transduction pathways involved in normal growth control.

Transformed cells become anchorage independent: Cells are known to grow as adherent monolayers with the exception of haemopoietic cells. Adherent cells are said to be anchorage dependent which means that they need to attach to a substrate in order to proliferate. When cells are transformed, they acquire anchorage independence that is the ability to grow without attachment to a substrate. In practice, when cells are suspended

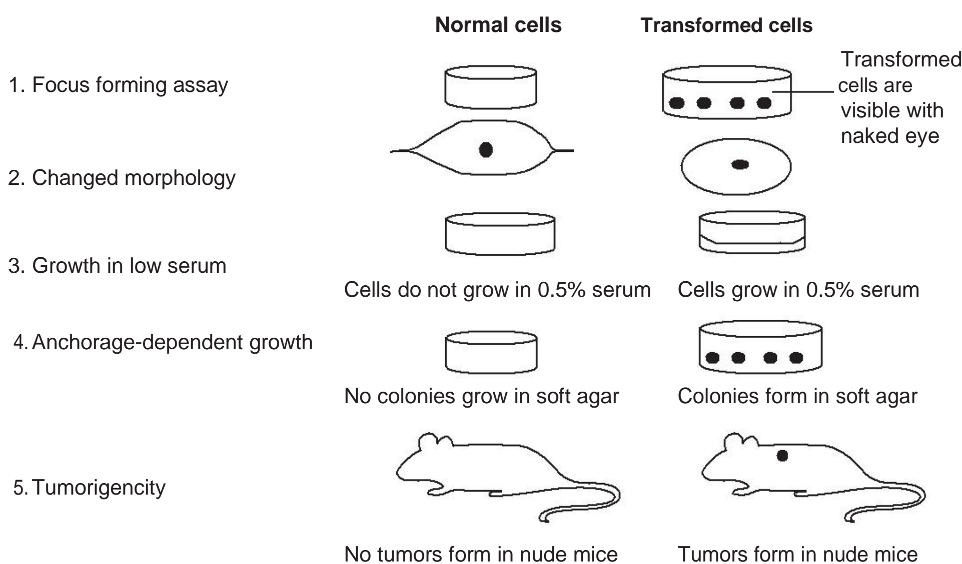

Figure 8-15 Techniques used to demonstrate transformed cells in culture.

in soft agar, growth of colonies from single cells in such a medium indicates that the cells have become transformed.

Tumorigenicity in 'nude' mice: The ultimate criterion for cell transformation is tumorigenicity – the ability to generate tumors. This can only be demonstrated in vivo and is measured experimentally using a special kind of mouse called the 'nude' mouse (also called the athymic mouse). The nude mouse is a development mutant which has lost its ability to produce a thymus gland and therefore has a defective immune defence system. It can therefore serve as a host for the growth of tumorigenic cells originating from any species, because these cells will not be recognized as foreign by the animal's immune response. The rate of tumor formation and the number of tumors formed when cells are injected under the skin of immune deficient mouse gives an indication of tumorigenicity.

8.8.4 Organ and Histotypic Culture

Organs cannot be propagated, but pieces of organs can be cultured on artificial medium. Care should be taken to handle organ culture in such a way that tissue dose not get damaged. Therefore, organ culture technique demands more tactful manipulation than tissue culture. The culture media on which organ is cultured are the same as described for cell and tissue culture. However, it is easier to culture embryonic organs than the adult animals. Methods of culturing embryonic organ and adult organs differ. A major deficiency in tissue architecture in organ culture is the absence of a vascular system, limiting the size (by diffusion) and potentially also the polarity of the cells within the organ culture. When cells are cultured as a solid mass of tissue, gaseous diffusion and the exchange of nutrients and metabolites become limiting. The dimensions of individual cells cultured in suspension or as a monolayer are such that diffusion is rapid but aggregates of cells beyond 250 m diameter (5000 cells) start to become limited by diffusion and at or above 1.0 mm (~ 2.5×10^5cells) central necrosis is often apparent. To alleviate this problem, organ cultures are usually placed at the interface between the liquid and gaseous phases to facilitate gas exchange while retaining access to nutrients.

8.8.4.1 Historical Development

Tissue culture experiments were reported as early as 1885 by Roux, when he maintained the medullary plate of chick embryo in warm saline for a few days. Later, Loeb (1887) cultured fragments of adult rabbit liver, kidney, thyroid and ovary on plasma clots inside a test tube. The histopathological structure of the tissue remains unaltered for 72 h. For successful organ culture, it is imperative to keep the organ at the interface between liquid and gaseous phases so as to maintain gas exchange while retaining access to nutrients. In 1929, Fell and Robinson introduced the 'watchglass technique', when organ rudiments or organs were grown on the surface of a clot consisting of chick plasma and chick embryo extract contained in a watchglass.

This became a standard technique for morphogenetic studies of embryonic organ rudiments and the method was later modified to investigate the action of hormones, vitamins and carcinogens in adult mammalian tissues. The main disadvantage of this technique is that explants often liquefy the clot and sink into a pool of liquefied medium. For this reason the technique was superseded in the 1940s by the agar clot technique, which uses agar instead of clotted plasma.

8.8.4.2 Organ Culture on Plasma Clots

A plasma clot is prepared by mixing five drops of embryo extract with 15 drops of plasma in a watchglass placed on a cotton wool pad. The cotton wool pad is put in a petridish. Time

to time cotton is moistened so that excessive evaporation should not occur. Thereafter, a small piece of organ tissue is placed on the top of plasma clot present in the watchglass. In the modified technique the organ tissue is placed into raft of lens paper or ryon. The raft makes it easy to transfer the tissue; excess fluid can also be removed.

8.8.4.3 Organ Culture on Agar

Solidified culture medium with agar is also used for organ culture. The nutrient agar media may or may not contain serum. When agar is used in medium, no extra mechanical support is required. Agar does not allow the support to liquefy. The tumors obtained from adults fail to survive on agar media, whereas embryonic organs grow well. The ingredients of the media are: agar (1% in basal salt solution), chick embryo extracts and horse serum in the ratio of 7:3:3.

8.8.4.4 Organ Culture in Liquid Media

The liquid media consist of all the ingredients except agar. When liquid media are used for organ culture, generally perforated metal gauze or cellulose acetate or a raft of lens paper is used.

8.8.4.5 Whole Embryo Culture

During 1950s, Spratt studied how metabolic inhibitors affect the development of embryo in vitro. Old embryo (40 h) was studied upto another 24–48 h in vitro until it died. For embryo culture a suitable medium is prepared and poured into a watchglass, which is then placed on moist absorbent cotton wool pad in petridishes. For the culture of chick embryo, eggs are incubated at 38 °C for 40–42 h so that a dozen of embryos could be produced. The egg shell sterilized with 70% ethanol is broken into pieces and transferred into 50 ml of balanced salt solution (BSS). The vitelline membrane covering the blastoderm is removed and kept in petridish containing BSS. With the help of a forcep the adherent vitelline membrane is removed. The embryo is observed by using a microscope so that the developmental stage of blastoderm could be found out. The blastoderm is placed on the medium in the watchglass placed on sterile absorbent cotton wool pad in petridishes. Excess BSS is removed from medium and embryo culture of chick is incubated at 37.5 °C for further development. We can subdivide organ culture in two basic types:

1. histotypic culture;
2. organotypic culture.

8.8.4.6 Histotypic Culture

When one characterized cell line is propagated at high density in the presence of appropriate extracellular matrix and soluble factors then such a culture is called *histotypic*. For example vascular endothelial cells will form capillary tubules in the presence of appropriate soluble factors when grown in a collagen matrix. Homologous cell interactions occur because the cells are cultured at high density. An alternative approach is the use of cellulose sponges coated with extracellular matrix components such as collagen. Cells may penetrate the sponge and form glandular structures.

8.8.4.7 Organotypic Culture

It is not possible to study heterologous cell interactions in a histotypic culture. When cells of different lineages are recombined to create a tissue-like structure then we call it *organotypic culture*.

To begin with, the simplest is to maintain a co-culture of two cell types. For example co-culture of epithelial cells and fibroblast clones derived from mammary gland allow the

epithelial cells to differentiate functionally. This has been demonstrated by their ability to produce milk proteins in optimal hormonal environment. This functional differentiation is preceded by the formation of characteristic structures, such as three-dimensional cords in which fibroblast cells organize themselves into bundles that are then enveloped by epithelial cells.

The first working '*organoid*' reported, was an artificial liver surgically implanted into the peritoneal cavity of rat. The organoid was made from a combination of cells, growth factors, collagen and Gore-tex fibers. These organoids could potentially be used to replace the diseased organ or deliver genetically altered cells into a patient's body.

8.8.5 Three-dimensional Culture and Tissue Engineering

Cells in culture need a three-dimensional architecture for maintaining their differentiated phenotype in vitro. Maintaining the correct architecture means reproducing the molec-ular and supramolecular complexity of the in vitro extracellular environment, which has led to the development of a new branch of science – ***in vitro tissue engineering***. Tissue engineering is an emerging field that allows us to look into the future of medicine, one in which doctors may be able to routinely repair or replace failing or aging body parts. Such engineering is made possible by years of research into processes by which cells grow and on the possibility of the restoration of differentiation function of cells in vitro. Tissue engineering refers to the manipulation, manufacture or alteration of cells, tissues or organic substances for human implantation. This technology is totally dependent on the ability to manipulate cell behavior in such a way as to produce ***three-dimensional tissues*** that can be used to repair damage in tissues and organs. Using this technology, it will one day be possible to regenerate or replace damaged tissues with laboratory-grown parts such as bone, cartilage, blood vessels and skin.

8.8.5.1 Concept of Tissue Engineering

Artificial skin: Skin was the first organ to be artificially grown. In early 1970s, irradiated 3T3 fibroblast cell line, was grown with keratinocytes which constitute 90% skin cells. 3T3 was found to stimulate the growth of skin cells and helped them to differentiate into epidermis. Three biotechnology companies are developing artificial skin, mainly for treating burn patients. The supporting materials for these skin equivalents are natural or synthetic. Bovine collagen type I (acid extracted) as well as polygalactic acid (PGA) have been used for growing skin from neonatal foreskin. Synthetic polymers allow cells to grow into skin without scars; hence are a potential organ developer. Many industries are involved in creating allogenic grafts. Another company 'Organogenesis' seems to have created a ***'living skin equivalent (LSE)'*** comprising of dermis and epithelial layer. The

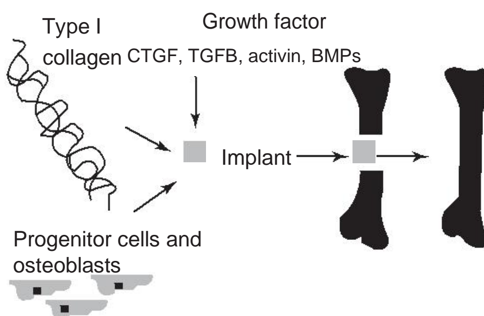

Figure 8-16 Bone growth and repair by tissue engineering.

Table 8-8 | Vaccines expressed in mammalian culture systems

Gene expressed	*Cell culture system*	*Expression vector*	*Comment*
HBsAg C-127 cell line	Mouse transformed vector	Bovine Papilloma virus	Phase I/II clinical trails
EBV gp40	C-127 cell line	Bovine Papilloma virus vector	Preproduction development stage
HSVgD and gB	CHO cell line	DHFR amplified vector	Phase II clinical trials
HBsAg-PreS1	CHO cell line	DHFR amplified vector	Fully licensed vaccine
HIVgp160	CHO cell line	DHFR amplified vector	Phase I/II clinical trials

support for growth is provided by collagen matrix. These LSE grafts elicit no immunogenic response in host and are eventually replaced by host cells.

Artificial cartilage: Cartilage tissue replacement holds promise in tissue engineering owing to lack of vascularization. The chondrocyte cells which are the precursors of cartilage are combined with PGA or collagen and are grown in culture. Given a suitable microenvironment, artificial cartilage can grow. Tissue engineering is a new field based upon a relatively simple concept: Start with some building material (e.g. extracellular matrix or biodegradable polymer), shape it as needed, seed it with living cells and bathe it with growth factors. When the cells multiply, they fill up the scaffold and grow into three-dimensional tissue, and once implanted in the body, the cells recreate their intended tissue functions. Blood vessels attach themselves to the new tissue, the scaffold dissolves and the newly grown tissue eventually blends in with its surroundings.

Tissue engineering frequently involves stem cells, a kind of premature cell first isolated from the body in 1992. Implanting stem cells in the appropriate location can generate everything from bone to tendon to cartilage. Although there is growing excitement in the field of tissue engineering, it is still in its infancy. Success will largely depend on the ability of scientists to figure out complex cellular interactions, then intervening with the right scaffold material and exact growth factors and cells.

8.8.5.2 | Future Importance

What would be important in future:

1. delivery of cells or delivery of regenerative growth factors;
2. delivery of autologous cells (including stem cells);
3. delivery of allogenic cells (including stem cells);
4. delivery of regenerative growth factors.

8.9 | Application of Animal Cell Culture

8.9.1 | Cell Culture Based Vaccines

A vaccine is an important immunizing agent. Vaccines are prepared either by dead organisms or attenuating live microorganisms. These are chemical substances prepared from the proteins (antigen) of other animals, which confer immunity to a particular virus. Cell culture based vaccines are purer and safer than existing vaccines. The existing vaccines contain antigens obtained after treatment of animals with particular bacteria or virus causing the disease, are used. This causes ‘overloading’. In cell culture based vaccines, cells are cultured with the specific bacteria or virus and only those antigens or

antibodies that are specifically required against that disease are injected into the human; hence they are considerably purer. Cell culture gained eminence after the large-scale production of vaccine. By using animal cell technology we are able to produce very potent antigens. The antigen vaccine derived from cell culture system stimulates both humoral and cell-mediated immunity.

8.9.1.1 Steps of Vaccine Production

Cell cultures principally used for virus vaccine production are prepared from monkey kidney or chick embryos. Human diploid cells are extensively used. They are grown as monolayers, which are infected with the appropriate virus. After incubation with the virus, the culture fluid is harvested and the cells are removed by filtration.

Production of vaccine is a complex process, involving rigorous quality control and safety procedures. Tissue culture substrates are carefully examined to exclude contamination with infectious agents from the source animal or in case of diploid cells, to exclude cells with abnormal characteristics. Monkey tissues are tested exhaustively for agents like ***Herpes simplex virus*** or ***Simian virus***, ***Tubercle bacilli*** and ***Mycoplasma.*** Human cells are subjected to karyological examination to exclude cultures with resembling transformed cells or malignant cells.

Industrial uses of animal cells are mainly to produce vaccines. A brief description of the number of cells required to be cultivated to produce a single dose of vaccine would be helpful. For example, for Polio vaccine, 2×10^4 host cells are sufficient to produce a single dose of vaccine. This can be supplied by a cell culture volume of 0.0001 l. Similarly for Rabies, 4×10^6 host cells would require 0.005 l of animal cell culture; for HSV, 2×10^7 host cells would require 0.03 l of animal cell culture; and for FMDV, 2×10^7 host cells would require 0.01 l of animal cell culture.

8.9.2 Somatic Cell Genetics

Somatic cell genetic studies have following three aims:

1. Study the behavior of heterokaryons following fusion of parent cells with different properties.

2. Study the behavior of hybrid cells derived from heterokaryons with the view to eliciting information about interaction between different genes.

3. Studies with a view to mapping loci on different chromosomes have been done using tissue culture.

Cultured human cells have been studied for more than 25 years by the methods of somatic cell genetics, an invaluable approach to gene mapping, and other studies of human chromosomes. To study gene mapping, investigators have fused mammalian cells with rodent cells in the presence of chemical substances, for example ***polyethylene glycol,*** which enhance the frequency of fusion of the cells in the incubation mixture.

8.9.2.1 Somatic Cell Fusion

Fusion of different types of cells has been successfully achieved in animals to produce hybrid cells. This technique is useful because it helps to study the control of gene expression, differentiation, to know the problem of malignancy, viral replication, gene mapping and production of hybridomas for antibody production.

The cell fusion in animals has been studied within the body, for example myoblasts fusing to form multinucleate muscle fibers. They can also be fused in vitro to produce binucleate heterokaryons (cells fuse, but two types of nuclei do not fuse) or uninucleate

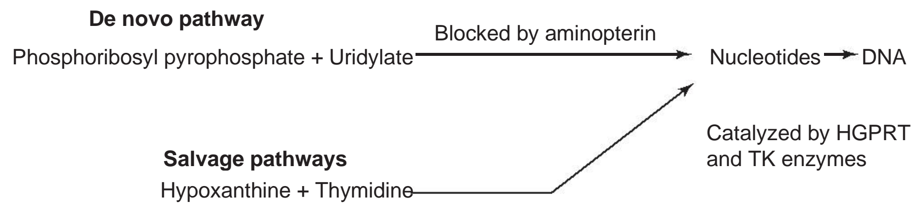

Figure 8-17 De novo and Salvage pathways used by mammalian cells.

hybrid cells. Fusion can be achieved by incubating a suspension of two cell types with an inactivated enveloped virus called Sendai virus or with polyethylene glycol, both of which promote the fusion of plasma membranes. In this way the plasma membranes, cytoplasm and nuclei of two separate cells are brought together in a single hybrid cell.

In the early1970s, L.D. Frye and M. Edidin used heterokaryons to study the fluidity of membrane proteins. In a classic experiment they fused a mouse fibroblast with a human fibroblast, generating a mouse–human heterokaryon, which was then exposed to fluorescein- and rhodamine-tagged antibodies specific for the MHC molecules that are confined to separate halves of the heterokaryon's plasma membrane.

A heterokaryon initially is multinucleated, having two to five separate nuclei. In the course of cell division, the nuclear membranes disintegrate and a single large nucleus is formed containing the chromosomes of both parent cells. At this stage the hybrid cell is unstable. After fusion the hybrid cells must be separated from unfused parent cells (e.g. A and B cells). When Sendai virus or polyethylene glycol is the fusing agent, only a small percentage of the cells actually fuse, and some of the fused cells are homogeneous A–A or B–B cells rather than the desired A–B hybrid. In order to select for the hybrid cells, a selective medium called HAT is employed. HAT selection depends on the fact that mammalian cells can synthesize nucleotides by two different pathways: the *de novo* and the *salvage* pathways. The de novo pathway, in which a methyl or formyl group is transferred from an activated form of tetrahydrofolate, is blocked by ***aminopterin***, a folic acid analog. When the *de novo* pathway is blocked, cells utilize the salvage pathway, which bypasses

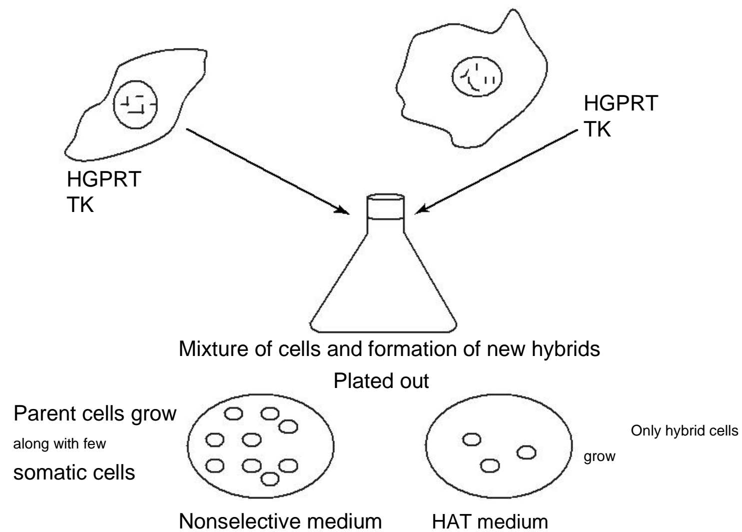

Figure 8-18 Cellular events in somatic cells and hybrid cells in HAT medium.

the aminopterin block by converting purines and pyrimidines directly into DNA. The enzymes catalyzing the salvage pathway are ***hypoxanthine-guanine phosphoribosyl transferase*** (HGPRT) and thymidine kinase (TK).

The problem is solved by plating the mixture on ***selective*** growth medium, which allows the hybrid cells to grow but simultaneously discourages or inhibits growth of the parental human and mouse cells. One of the most useful selective medium is HAT medium, which includes the drug ***aminopterin*** to block the major pathway for synthesis of deoxyribonucleotides; ***hypoxanthine***, a purine precursor; and ***thymidine***. Cells can grow in the presence of aminopterin only if they have the enzymes HGPRT and TK to catalyze utilization of the two precursors in a 'salvage' pathway of nucleotide synthesis.

When TK-deficient mouse (or human) cells are mixed with HGPRT-deficient human (or mouse) cells, neither parent can grow when plated out on HAT medium. The only cells that grow to produce colonies on HAT medium are $TK^{+}/HGPRT^{+}$ somatic cell hybrids, which received a functional TK gene from one parent and a functional HGPRT gene from the other parent. Colonies or cells can be isolated from HAT medium and these can be established separately in culture. The development of tumors or of malignant cancerous growths in the organism can also be made via studies of cellular transformation in cultured cells. Tumors grow without restraint and invade tissues near their site of origin: If the tumor is malignant, the cancer spreads to distant sites away from the original focus of growth or undergoes metastasis. Transformed cells in culture display modified properties that distinguish them from normal cells, and they can be considered analogs of tumorous growths in the organism. Normal cells in culture stop dividing upon making physical contact with each other, so that a confluent single layer of cells is produced in the culture dish. Transformed cells may continue to migrate and to multiply indefinitely, so that mounds of cells in disordered heaps are produced on solid substratum. Normal cells do not multiply in media made semisolid by the addition of agar or methylcellulose, whereas transformed cells can proliferate in semisolid media. The transformed phenotype is heritable, as they produce descendant cells like themselves.

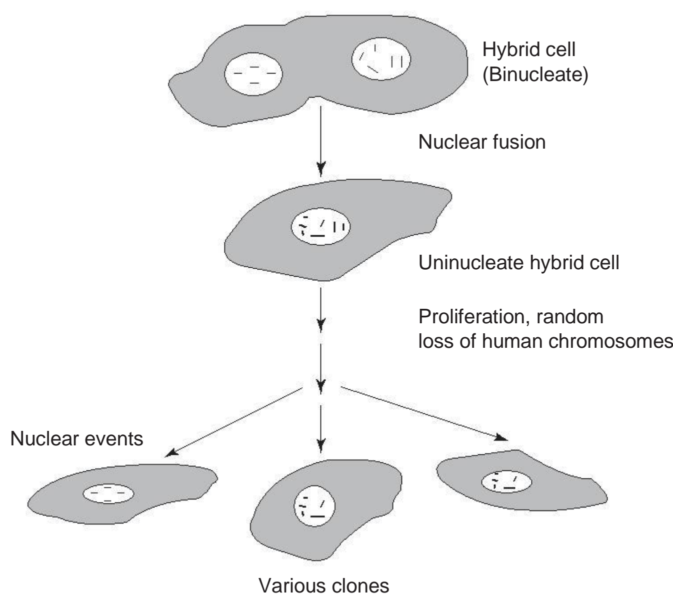

Figure 8-19 | Nuclear events occurring in cells and hybrid cells in HAT medium.

8.9.3 | Valuable Products from Cell Culture

From cultured animal cells several valuable products such as human monoclonal antibodies and biochemicals can be produced on a large scale. More interestingly, the genetically engineered baculovirus-infected animal cells are also used in a bioreactor. To fulfil the process several 'perfusion systems' have been developed that retain the cells in the bioreactor at the time of replacement of conditioned medium with fresh medium. As a result, there is increase in cell density and in turn cell productivity. For commercial production on a large scale, cell culture system and scaling up of process are required. Therefore, 'master cell banks' (MCBs) are established to meet out the demand. The MCBs are used to develop master working cell bank (MWCB) which meets the demand of production system. After several subculturings, the MWCB is regularly checked for any kind of change occurring in cells. Thus, the large-scale cultures are the source of all valuable products which are produced in a bioreactor. Some of the important products that are produced from animal cell cultures are: (i) enzymes (asparaginase, collagenase, urokinase, pepsin, hyalurodinase, rennin, trypsinand tyrosine hydroxylase); (ii) hormones (luteinizing hormone, follicle-stimulating hormone, chorionic hormone and erythropoietin); (iii) vaccines (foot and mouth disease vaccine, vaccines for influenza, measles and mumps, rubella and rabies); (iv) monoclonal antibodies; (v) interferons; (vi) tissue plasminogen activator (t-PA).

8.10 | Stem Cell Culture

Stem cells are defined by virtue of their functional attributes. Stem cells of a particular tissue are defined as undifferentiated cells (i.e. lacking certain tissue-specific differentia-tion markers), are capable of proliferation and are able to self-maintain the population. On being induced they can produce a large number of differentiated functional progeny. They are also able to regenerate the tissue after injury. Cells or population of cells actually fulfiling all these criteria at a given instance are called ***actual stem cells***, while those not

Table 8-9 | A list of applications of cell culture

Cell type	***Process investigated/product made***
Monocytes and macrophages	Pinocytosis and phagocytosis
Blood lymphocytes	Karyotype analysis for detection of genetic defects in humans
Normal and transformed fibroblasts	Surface adherence properties of normal and malignant cell membranes.
Kidney tubule epithelial cells	Differentiation of monolayers; electrical and vectorial transport of solutes; monoclonal antibody production
Myeloma cells and ˇ-lymphocytes dopamine	Purification and characterization of specific membrane proteins, e.g. ¿- and ˇ-adrenergic receptors
Kidney epithelial cells	To investigate relationship between membrane polarity and budding
Transformed leucocytes, fibroblasts and other lymphoblastoid cells	To study properties of envelope RNA-viuses
Transformed Hela cells, mammalian cells	Cells are infected with Sendai virus to produce ¸ˇ and interferon, respectively
Mouse fibroblasts	Radiation therapy and the design of radiosensitizers and radioprotectors
Primary monkey kidney cells	Acute and chronic toxicity testing and metabolism of xenobiotics; vaccine production
Fibroblasts, mammalian brain cells	Production of polio vaccines; hormone secretion; to identify chemicals capable of including chromosome aneuploidy

Table 8-10 | List of cell culture products

Products	***Application***
Erythropoietins	
Erythropoietin-¿	Anaemia resulting from cancer and chemotherapy
Erythropoietin-ˇ	Anaemia secondary to kidney disease
Human growth hormones	
hGH	Human growth deficiency in children, renal cell carcinoma;
Somatotropin	chronic renal insufficiency, Turner’s syndrome
Monoclonal antibodies (therapeutic)	
Anti-lipo-polysaccharide	Treatment of sepsis
Murine anti-idiotype/human	
B-cell lymphoma	B-cell lymphoma
Monoclonal antibodies (diagnostics)	
Anti-fibrin 99	Blood clot
Tem-FAb (breast)	Blood cancer
PR-356 CYT-356-in-111	Prostate adenocarcinoma
Plasminogen activator	
Urokinase type plasminogen activator	Acute myocardial infarction, acute stroke, pulmonary embolism, deep vein thrombosis
Tissue type plasminogen activator	Recombinant plasminogen activator
Vaccines	
HIV vaccines (gp120)	AIDS prophylaxis and treatment
Malaria vaccines	Malaria prophylaxis
Polio vaccines	Poliomyelitis prophylaxis

actually expressing these capabilities at a particular moment in time, though they possess these capabilities, are termed *potential stem cells*. It may be possible for a stem cell to cease proliferation, that is become quiescent, in which case it is not an actual stem cell. During embryonic development, the *primordial stem cells* proliferate and migrate from the base of the allantois. Primordial germ cells are potentially immortal in that they are self-renewing population of cells. Initial tests of cultured primordial germ cells have revealed that they are pluripotent, that is they can form embryoid bodies that undergo further differentiation in vitro (Figure 8-16).

Differentiation can be defined as a qualitative change in the cellular phenotype. It may be recognized by change in morphology of the cell or by changes in enzyme activity. Being a qualitative change, the cell can be differentiated only relative to another cell, and during its lifetime it is capable of undergoing several differentiation events. Differentiation event is indicated by the presence of a novel protein, but it also involves changes in the repression/activation of the genome. Therefore, cells developing from a primitive stage to functional competence may undergo many differentiation events, each linked to a novel change in the gene-activation pattern. Even before a cell actually differentiates, its destiny is fixed in a process called *commitment*. All the cells are not committed to a single development fate but are able to give rise to only a limited number of different cell types.

Maturation is a quantitative change in the cellular phenotype. Hence the degree of maturation could be measured on a quantitative scale, for example amount of a specific protein per cell. A differentiated cell matures as a function of time to form a competent cell for that particular tissue.

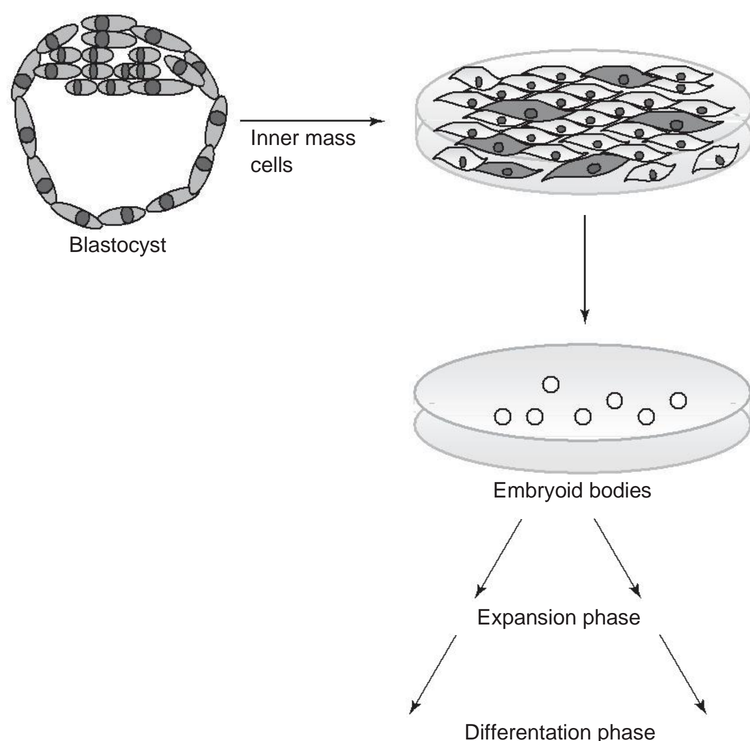

Figure 8-20 Schematic representation of the stages in the differentiation of cells.

Proliferation is a process which involves sequential pattern of cyclic changes in gene expression leading to cell division.

Stem cells have two major characteristics:

1. They are capable of dividing and renewing themselves for long periods.
2. They are unspecialized and can give rise to specialized cell types under controlled micro-environments.

Stem cells lack any tissue-specific structures that allow them to perform specialized functions. A stem cell has no cross-talk, hence it cannot work in conjunction with surrounding cells to pump blood through the body (like a heart muscle cell) nor can it carry molecules of oxygen through the bloodstream (like a red blood cell) or fire electrochemical signals to other cells that allow the body to move or speak (like a nerve cell). However, unspecialized stem cells can give rise to specialized cells, including heart, muscle cells, blood cells, or nerve cells. Stem cells may replicate themselves many times, this is called *proliferation*. The cells are said to be capable of *long-term self-renewal*, that is the population of stem cells can proliferate for months in the laboratory, but this results in a population of cells which continue to be unspecialized, like the parent stem cells.

Two types of stem cells can be identified based on its origin: (i) embryonic stem cell and (ii) adult stem cell.

Embryonic stem cells are derived from embryos. These cells were first isolated from mice in 1981, and until recently, scientists have used only animal embryonic stem cells in research. In November 1998, two groups published the results of their work on human stem cells from embryos or fetuses. In both cases, the embryos and fetuses were donated

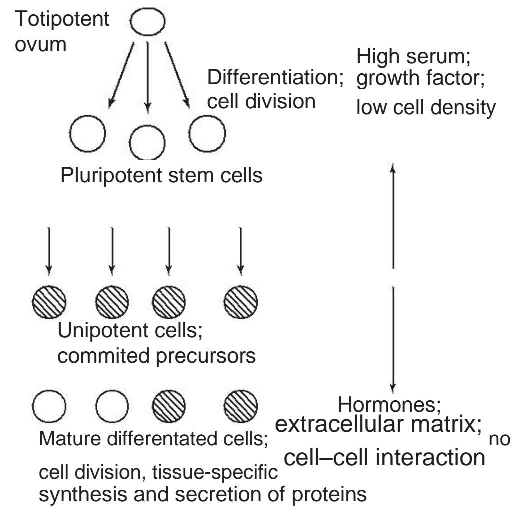

Figure 8-21 Embryoid bodies that undergo further differentiation.

for research purposes following a process of informed consent. University of Wisconsin researchers derived stem cells from 1-week-old embryos, also called blastocysts, produced via in vitro fertilization (IVF) for the treatment of infertility.

Embryonic stem cells develop from eggs that have been fertilized in vitro or via IVF and are not derived from eggs fertilized in a woman's body. The embryos fr5om which human embryonic stem cells are derived are typically four or five days old and are a hollow microscopic ball of cells called the *blastocyst*. The blastocyst includes three structures: the ***trophoballast,*** which is the layer of cells that surrounds the blastocyst; the ***blastocoel***, which is the hollow cavity inside the blastocyst; and ***the inner cell mass***, which is a group of approximately 30 cells at one end of the blastocoel. Because the stem cells are located within the embryo, the process of removing the cells destroys the embryo. Another potential source of embryonic stem cells is ***somatic cell nuclear transfer*** (SCNT). In SCNT, the nucleus of an egg is removed and replaced by the nucleus from a mature body cell, such as a skin cell. The cell created via SCNT would be allowed to reach the 1-week (blastocyst) stage and the stem cells would then be removed, an example of this is Dolly, the sheep that was first produced using this method in 1996.

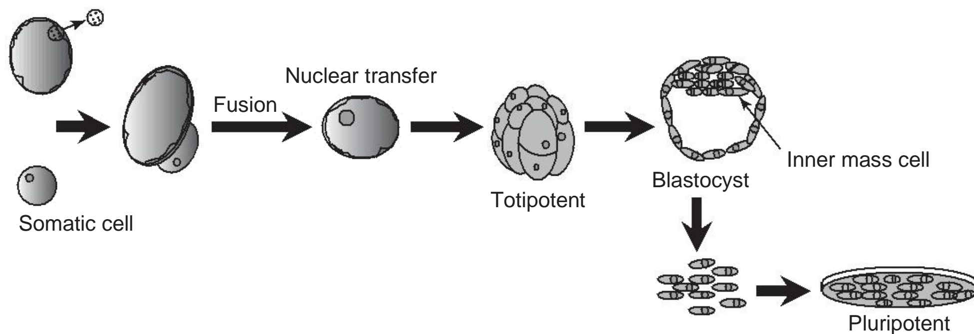

Figure 8-22 Stem cell versus somatic cell nuclear transfer.

Regular laboratory tests are recommended to check whether:

1. The cells grown in the laboratory are healthy and still in an undifferentiated state.
2. Surface-markers, specific for undifferentiated cells, are present on the cells.
3. A protein Oct-4 (Octamer-4) that is a stem cell marker is being expressed. Oct-4, a transcription factor belonging to POU family, is known to be active in the early embryonic stage of an organism. Oct-4's primary function is to keep an embryo in an immature state. It prevents the cells in the embryo from differentiating into tissue-specific cells. While Oct-4 is operating, all the cells in the embryo remain identical, but when Oct-4 shuts off, the cells begin growing into differentiated cells, for example heart or liver tissue.
4. Karyotyping is done to ensure whether the chromosomes are damaged to detect genetic mutations in the cells.
5. To retain the pluripotency of the cells by
   cross-checking whether they can replicate after freezing and thawing;
   allowing the cells to differentiate into specific cells in cell culture;
   checking their ability to form *teratoma* in nude mice (T-cell compromised mice). Teratomas contain both differentiated or partly differentiated cell types indicating that the embryonic stem cells are capable of differentiating into multiple cell types.

Adult/somatic stem culture: An adult stem cell is an undifferentiated cell found among differentiated cells in a tissue or organ, can renew itself, and can differentiate to yield the major specialized cell types of the tissue or organ. Their primary role in a living organism is to maintain and repair the tissue in which they are found.

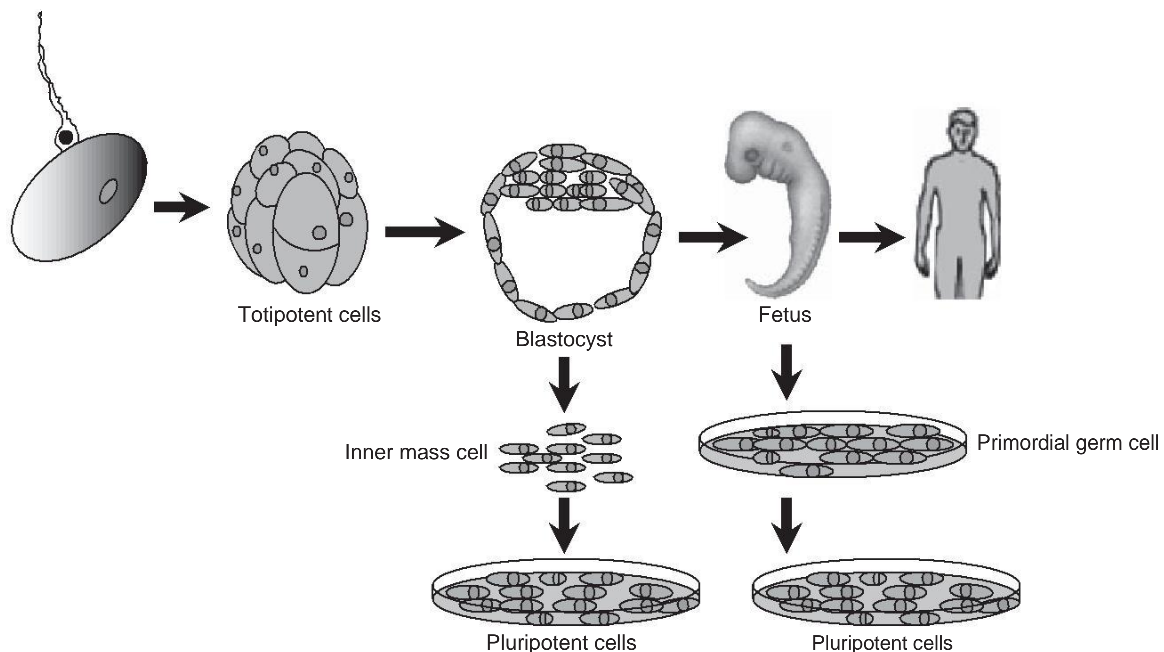

Figure 8-23 | Schematic representation of isolation of pluripotent cells from blastocyst and cord blood.

In the 1960s, researchers discovered that the bone marrow contains at least two kinds of stem cells. One population, called *haematopoietic stem cells*, forms all the types of blood cells in the body. A second population, called the *bone marrow stromal cells*, is a mixed cell population that generates bone, cartilage, fat and fibrous connective tissue. In 1990s, the scientists discovered that the adult brain does contain stem cells that are able to generate the three major cell types of the brain – *astrocytes* and *oligodendrocytes*, which are non-neuronal cells, and *neurons,* or nerve cells, unlike the earlier belief that new nerve cells could not be generated in the adult brain.

Stem cells from one tissue may be able to give rise to cell types of a completely different tissue, a phenomenon known as plasticity. Examples of such plasticity include: blood cells becoming neurons, cells that can be made to produce insulin, and hematopoietic stem cells that can develop into heart muscle. Therefore, exploring the possibility of using adult stem cells for cell-based therapies has become a very active area of investigation by researchers.

Research on adult stem cells has recently generated a great deal of excitement. Scientists have found adult stem cells in many more tissues than they once thought possible. This finding has led scientists to ask whether adult stem cells could be used for transplants. In fact, adult blood forming stem cells from bone marrow have been used in transplants for 30 years. Certain kinds of adult stem cells seem to have the ability to differentiate into a number of different cell types, given the right conditions. If this differentiation of adult stem cells can be controlled in the laboratory, these cells may become the basis of therapies for many serious common diseases.

In the hematopoietic system, a small population of pluripotent, undifferentiated stem cells typically give rise to committed precursor cells which progress toward terminally differentiated cells, loosing the capacity to divide as they reach the terminal stages. The fully mature, terminally differentiated cell will not normally divide. The use of human hematopoietic stem cells (HSC) isolated from umbilical cord blood as a source of cellular reconstitution following high-dose chemotherapy is now a common therapeutic modality for the treatment of malignancy. HSC transfusion is most effective in children and has limited application in adults, due to the low yield of HSC from cord blood. To obtain sufficient numbers of cells for applications of this therapeutic approach in adults, ex vivo expansion has been utilized to ensure successful engraftment and minimize the short-term effects of neutropenia and thrombocytopenia. Some companies have developed a serum-free, animal-protein-free medium, for example Stemline^{TM} Hematopoietic Stem Cell Expansion Medium, for the optimal expansion of cord blood HSC. It also promotes growth of $CD34^{+}$ hematopoietic stem cells derived from bone marrow, cord blood or mobilized peripheral blood. This medium supports high viable cell densities. The elimination of serum reduces performance variability in the medium and eliminates safety risks associated with possible adventitious agents in serum.

The various points below show the importance of stem cells:

1. Stem cells provide the opportunity to study the growth and differentiation of individual cells into tissues.
2. Stem cells could be used to produce large amounts of one cell type to test new drugs for effectiveness and chemicals for toxicity.
3. Stem cells might be transplanted into the body to treat disease (diabetes, Parkinson's disease) or injury (e.g. spinal cord). The damaging side effects of medical treatments might be repaired with stem cell treatment. For example, cancer chemotherapy

destroys immune cells in patients, thereby making it difficult to fight off a broad range of diseases; correcting this adverse effect would be a major advance. Before stem cells can be applied to human medical problems, substantial advances in basic cell biology and clinical technique are required.

Conclusion: There is significant interest in studying stem cells, both to elucidate their basic biological functions during development and adulthood as well as to learn how to utilize them as new sources of specialized cells for tissue repair. Whether the motivation is basic biology or biomedical application, the progress will hinge upon learning how to better control stem-cell function at a quantitative and molecular level. There are several major challenges within the field, including the identification of new signals and conditions that regulate and influence cell function, and the application of this information toward the design of stem-cell bioprocesses and therapies. Both of these efforts can significantly benefit from the synthesis of biological data into quantitative and increasingly mechanistic models that not only describe, but also predict, how a stem cell's environment can control its fate. Early models formulated on the assumption that cell fate was decided by stochastic, cell-intrinsic processes have gradually evolved into hybrid deterministic–stochastic models with increasingly finer molecular resolution that accounts for environmental regulation. As our understanding of cellular control mechanisms expands from the cell surface and toward the nucleus, these efforts may culminate in the development of a stem-cell culture program, or a series of signals to provide to the cells as a function of time to guide them along a desired developmental trajectory. Cell-based therapies, which are used to treat disease, are often referred to as ***regenerative or reparative medicine***.

8.11 | Apoptosis

Apoptosis means 'programmed cell death'. Its role in cell population control during growth and development suggests that there are inherent cellular mechanisms that lead the cell to destruction. It is a form of cell death which is different from any other form of cell death – necrosis. It can be distinguished by a characteristic pattern of morphological and molecular changes. Owing to rapid dehydration, cells that are round in shape become convoluted or elongated and diminished in size. Chromatin condensation and the loss of chromatin structure, which occurs simultaneous to cell shrinkage, starts at the nuclear periphery and is followed by nuclear fragmentation. Nuclear fragments along with constituents of cytoplasm (including intact organelles) enveloped in plasma membrane detach from the dying cell as blebs and are eventually thrown out as ***apoptotic bodies***. In vivo the apoptotic bodies are phagocytosed by macrophages, hence evoking no inflammatory response.

Increase in cytoplasmic Ca^{2+}concentration and activation of proteases known as caspases as well as one, or several, endonuclease(s) that degrades DNA at the inter-nucleosomal (linker) sections are also very characteristic events of apoptosis. Thus, the products of DNA degradation are discontinuous and of the size of nucleosomal and oligonucleosomal DNA sections that generate a very characteristic 'ladder' pattern during gel electrophoresis. Also typical is activation of some genes; the latter suggests that products of such genes are required for apoptosis to occur. However, exceptions are noted, and several cell types (so-called apoptosis primed cells) may undergo apoptosis when protein synthesis is inhibited, for example in the presence of cycloheximide. Even

during advanced stages of apoptosis, the structural and functional integrities of the plasma membrane, mitochondria and lysosomes are generally preserved. Thus, considering all the changes that occur in a cell undergoing apoptosis, the most characteristic feature of this process is active participation of the cell in its demise. The cell activates a cascade of molecular events which result in orderly degradation of the cell constituents with minimal impact on the neighboring tissue.

The study of apoptosis is important in many disciplines such as embryology, immunol-ogy, endocrinology and oncology, as death plays an important role in tissue development. The pattern of events in death by suicide is so orderly that the process is often called programmed cell death (PCD). The cellular machinery of programmed cell death turns out to be as intrinsic to the cell as, say, mitosis. PCD is also called apoptosis.

8.11.1 Reasons for Cell Suicide

8.11.1.1 PCD is Needed for Proper Development

Examples are commonly seen in the resorption of the tadpole tail at the time of its metamorphosis into a frog; this occurs by apoptosis. The formation of the fingers and toes of the fetus requires the removal of the tissue between them, usually by apoptosis. The sloughing off of the inner lining of the uterus (the endometrium) at the start of menstruation occurs by apoptosis. The formation of the proper connections (synapses) between neurons in the brain requires that surplus cells be eliminated by apoptosis.

8.11.1.2 PCD is Needed to Destroy Cells that Represent a Threat to the Integrity of the Organism

Cells infected with viruses: One of the methods by which cytotoxic T lymphocytes (CTLs) kill virus-infected cells is by inducing apoptosis (Figure 8-20B).

Cells of the immune system: As cell-mediated immune responses wane, the effector cells must be removed to prevent them from attacking body constituents. CTLs induce apoptosis in each other and even in themselves. Defects in the apoptotic machinery is associated with autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis.

Cells with DNA damage: Damage to its genome can cause a cell to disrupt proper embryonic development leading to birth defects. Also the cell can become cancerous. Cells respond to DNA damage by increasing their production of p53. p53 is a potent inducer of apoptosis but how it participates in apoptosis is under scrutiny. It hastens the death of potentially dangerous cells. Mutations in the *p53* gene, producing a defective protein, are commonly found in cancer cells and are frequent in breast, lung and colon cancers. Radiation and chemicals used in cancer therapy induce apoptosis in some types of cancer cells. *p53* and other tumor-suppressor proteins have been dealt with in Chapter 13, Section 13.8.4.

The vital decision of the cell to commit suicide is often because of the altering of the balance between the withdrawal of positive signals that is signals needed for continued survival, and the receipt of negative signals.

8.11.1.2 *Withdrawal of Positive Signals*

The continued survival of most cells requires that they receive continuous stimulation from other cells. Some cells require continued adhesion to the surface on which they are

growing. Some examples of positive signals are growth factors for neurons. Interleukin-2 (IL-2) is an essential factor for the mitosis of lymphocytes.

8.11.1.4 | *Receipt of Negative Signals*

The increased levels of oxidants within the cell and the damage to DNA by these oxidants or other agents like UV light, X-rays as well as chemotherapeutic drugs are some of the negative signals. Molecules that bind to specific receptors on the cell surface and signal the cell to begin the apoptosis program are called death activators. These death activators include tumor necrosis factor-alpha (TNF-α) that binds to the TNF receptor, lymphotoxin (also known as TNF-β) that also binds to the TNF receptor, Fas ligand (FasL) – a molecule that binds to a cell-surface receptor named Fas (also called CD95).

8.11.2 | *The Mechanisms of Apoptosis*

Cells are known to commit suicide by three different mechanisms by apoptosis. First, there are signals arising within the cells. Second, cells are triggered by death activators, binding to the receptors, for example TNF-α lymphotoxin, Fas ligand (FasL). Lastly, it may be triggered by dangerous reactive oxygen species.

8.11.2.1 | *Apoptosis Triggered by Internal Signals*

The intrinsic or mitochondrial pathway: In a healthy cell, the outer membranes of its mitochondria express the protein Bcl-2 on their surface. Bcl-2 is bound to a molecule of the protein Apaf-1. Internal damage to the cell (e.g. from ROS – reactive oxygen species) causes Bcl-2 to release Apaf-1, which keeps cytochrome-c from leaking out of the mitochondria. The released cytochrome-c and Apaf-1 bind to molecules of caspase 9. The resulting complex of cytochrome-c, Apaf-1, caspase 9 and ATP is called the apoptosome (Figure 8-20A). These aggregate in the cytosol. All caspases are proteases, and their function is to cleave proteins – mostly each other – at aspartic acid (Asp) residues. Caspase 9 is one of a family of over a dozen caspases. Caspase 9 cleaves and, in so doing, activates other caspases. The sequential activation of one caspase by another creates an expanding cascade of proteolytic activity (rather like that in blood clotting and complement

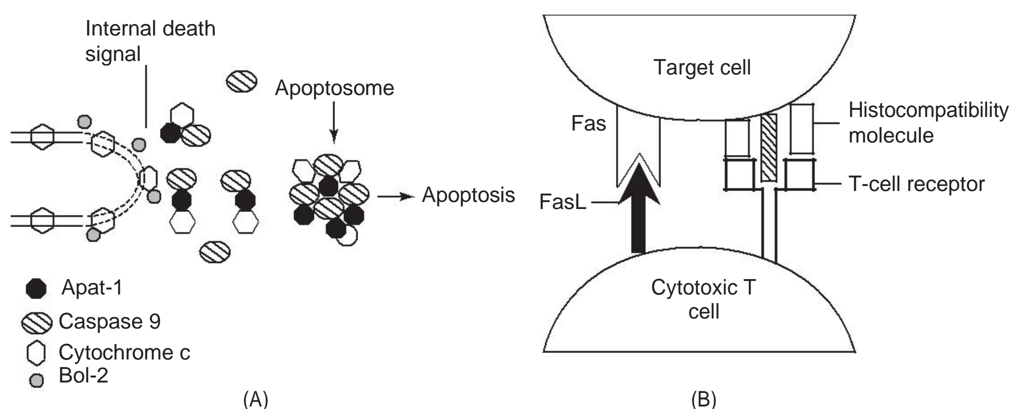

Figure 8-24 | (A) Mechanism of cell death. (B) Cytotoxic cells inducing their target (virus-infected cells) to commit suicide.

Table 8-11 | Morphological differences and similarities between apoptosis and necrosis

	Differences		
	Apoptosis	***Necrosis***	***Similarities***
1. Nuclei	Dense condensation of chromatin	Preceeded by irregular cell clumping	Damage occurs in both
2. Cytoplasmic organelles	Intact	Disrupted	Secondary damage in apoptosis
3. Cell membrane	Blebbing, apoptotic bodies formed	Blebbing and loss of integrity	Changes seen in both
4. Cell volume	Cells shrink	Cells swell	Changes not detectable
5. In tissues	Single cell affected	Groups of cells affected	In epithelia, superficial cells are apoptotic and in groups
6. Tissue response	None	Inflammation	–

activation) which leads to digestion of structural proteins in the cytoplasm with subsequent degradation of chromosomal DNA and phagocytosis of the cell.

8.11.2.2 | Apoptosis Triggered by External Signals

The extrinsic or death receptor pathway: Fas and the TNF receptor are integral membrane proteins with their receptor domains exposed at the surface of the cell. Binding of the complementary death activator (FasL and TNF, respectively) transmits a signal to the cytoplasm that leads to activation of caspase 8. Caspase 8 (like caspase 9) initiates a cascade of caspase activation leading to phagocytosis of the cell. As seen in Figure 8-20B, when the cytotoxic T cells recognize (bind to) their target, they produce more FasL at their surface. This binds with the Fas on the surface of the target cell leading to its death by apoptosis. The early steps in apoptosis are reversible – at least in *C. elegans*. In some cases, final destruction of the cell is guaranteed only with its engulfment by a phagocyte.

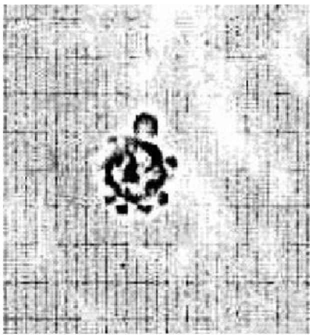

Figure 8-25 | Cell blebbing in an apoptotic cell.

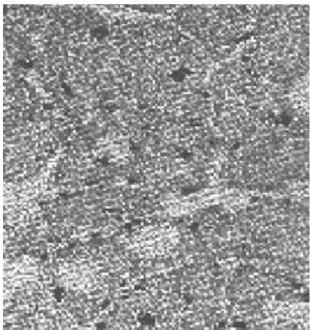

Figure 8-26 | TUNEL-labeling tissue section. Black spots are the TUNEL positive cells.

8.11.2.3 | Apoptosis-inducing Factor (AIF)

Neurons, and perhaps other cells, have another way to self-destruct that unlike the two paths described above does not use caspases. AIF is a protein that is normally located in the intermembrane space of mitochondria. When the cell receives a signal indicating that it is time to die, AIF is released from the mitochondria (like the release of cytochrome-c in the first pathway), migrates into the nucleus and binds to DNA, which triggers the destruction of the DNA and cell death.

There are two ways in which cells die. Either they are killed by injurious agents or they are induced to commit suicide.

8.11.2.4 | Death by Injury

Cells that are damaged by injury, such as by mechanical dam-age or by exposure to toxic chemicals, undergo a characteristic series of changes. The cells and their organelles, like mito-chondria, swell because the ability of the plasma membrane to control the passage of ions and water is disrupted. Therefore, the cell contents leak out, leading to inflammation of surrounding tissues.

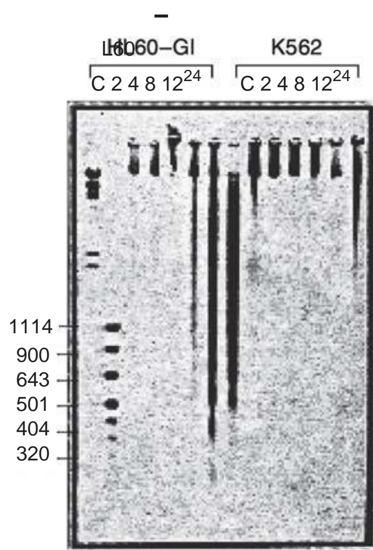

Figure 8-27 DNA ladder as seen in HL60 and absent in K562.

8.11.2.5 *Death by Suicide*

Cells that are induced to commit suicide shrink, have their mitochondria break down with the release of cytochrome-c, develop bubble-like blebs on their surface, have the chromatin (DNA and protein) in their nucleus as degraded, small, membrane-wrapped fragments. The phospholipid phosphatidylserine, which is normally hidden within the plasma membrane, is exposed on the surface. This is bound by receptors on phagocytic cells like macrophages and dendritic cells which then engulf the cell fragments. The phagocytic cells secrete cytokines that inhibit inflammation.

8.11.3 *Histochemical Detection of Apoptosis*

Demonstration of the products of chemical reactions at tissue level for detection of cell damage has an advantage in that the investigator simultaneously obtains morphological data and information on the localization of the reaction products. The fragmentation of nuclear DNA that occurs in apoptosis can be detected by labeling the newly formed free ends of DNA, followed by the examination of the label under a light microscope. A technique has been described for the histochemical detection of chromosomal breaks based on a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP biotin nick end-labeling (TUNEL) of fragmented nuclear DNA in situ. A variant of this method, in situ end-labeling (ISEL) of the fragmented DNA differs from TUNEL in its use of DNA

Table 8-12 Biochemical detection of specific DNA damage for demonstration of apoptosis

	Differences		
	Apoptosis	***Necrosis***	***Similarities***
1. Nuclear DNA damage ladders on gels	Nucleosomal and/or 50–300 kb fragments	Random smears on gels	Takes place in both, but more in apoptosis
2. Nuclear gene expression	Usually needed	Not needed	Not needed in cells 'primed' for apoptosis
3. Mitochondrial DNA damage	Spared	Occurs early	–
4. Enzyme activation	Necessary	Not necessary	Lysosomal DNase and proteases are activated in necrosis
(a) DNases	Necessary		
(b) Proteases	Frequent		
(c) Transglutaminase			
5. Membrane function	Intact	Loss of function	–
6. Cell internal milieu	Slightly acidic (pH 6.4)	Acidic	Both acidic
(a) pH	Often increases	Always increases	Seen in both
(b) Ca^{2+}	May be intact	Defective	
(c) Na^+/K^+ pump			

polymerase in place of the TdT. Because of this similarity, only the TUNEL method is described here. It can be used on tissue sections or on cell suspensions. In addition, a commercial kit has been developed by Oncor, called Apoptag, which utilizes this technique.

The oligonucleosome-sized DNA fragmentation associated with apoptosis consists of small protein-free DNA double-strand breaks. It is clear that an endonucleolytic pathway is activated in apoptosis and results in the cleavage of the genome, first into large DNA fragments that vary from 300 to 50 kb in size, then into 180 bp multiples. Together with morphological changes characteristic of the process of apoptosis, the 180 bp periodicity of the DNA fragments visible on agarose gels stained for DNA is a tell-tale sign of this mode of cell death. It is believed that the DNA ladder formation by low-molecular-weight DNA fragments results from the nucleolytic cuts in linker DNA between nucleosomes within chromatin; the DNA derived from one nucleosome and the linker region is about 180 bp long. Cuts which lead to oligosome-sized fragments result in multiples of 180 bp. The long-term stability of the extracted DNA also adds favor to these procedures because the DNA samples can be analyzed weeks after extraction. However, nucleosomal ladders are not essential for demonstration of apoptosis, since the process can occur without this type of DNA fragmentation. The 300 kb fragments are considered to be generated by activation of an endogenous endonuclease or topoisomerase II molecule located at specific sites in chromatin, perhaps the nuclear matrix, whereas the 50 kb fragments and the accompanying DNA ladders are generated by endonucleolytic activity which appears to increase during apoptosis. The various sizes of the very large fragments have been suggested to be related to the higher order of folding of chromatin into chromosomes.

8.12 Containment Levels

"Containment" can be defined as the action of managing hazardous and lethalagents like bacteria, viruses, mycoplasmas and recombinant DNA in a controlled manner, well within the limits of the laboratory. This clearly indicates that infections must be curtailed either by providing physical barriers or by developing procedures that help restrict the exposure of the worker to the microorganisms.Designer laboratories are necessary to work with micro-organisms and are called "Containment laboratories". Four different levels of containment are recognized by statutory bodies based on the degree of risk from the hazardous agents used. They may also be referred to as Containment Level [CL] or Biological Safety Levels [BSL] 1-4.

8.12.1 *Containment level 1 [CL-1]*

Here, the harmful agents are either toxigenic or allergenic but are not capable of causing infection. Nonetheless, the laboratory personnel and students must be given adequate training to use the laboratory. Also, the design of the laboratory should be such that the doors are always closed, no eatables should be allowed inside the laboratories. Mouth pipetting by students must never be allowed. The surfaces, hands and the areas should be disinfected with 70% alcohol prior to any work. Lab coats and other protective garments should always be worn while working with viable materials.

8.12.2 ***Containment level 2*** [CL2]

Biosafety Level 2 [BSL-2] is appropriate for work relating to agents that pose reasonable threat to the environment and the students. The persons using the laboratory must have specialized training for handling virulent agents, and should be supervised by scientists experienced in handling infectious agents and procedures; Limited access to the laboratory is a requisite to avoid contamination. The airflow should be maintained as an inward flow of air is necessary only when work is progress. All procedures pertaining to good lab practices should be defined and distinct. Any procedure dealing with infectious aerosols must be conducted in safety cabinets or isolators. Separate hand washbasins should be made in the immediate vicinity, preferably at the exit. The tap must be operated without being touched with hand. Laboratory coats or gowns with buttons or fasteners must be worn before entering and removed while leaving the laboratory. Remember, the coats should not be left in the same storage area that is meant for personal clothing to avoid contamination. The working bench areas should be waterproof, water resistant, rust proof, resistant to acids, disinfectants and easy to clean. A reasonably high standard of supervision of the work should be maintained. As a precaution, use sterilized gloves, but in case there are financial constraints it is preferable to spray 70% alcohol before touching any items like paper, pen, phone, computer, note books, equipment should also be wiped with 70% alcohol swab. The biological waste generated after working also needs sterilization on a regular basis, preferably by autoclaving. Biological agents should be stored safely. Overcrowding in the laboratories must be avoided to avoid accidental spillage.

8.12.3 *Containment level /Biosafety Level 3*

Negative air pressure to external air and/or other parts of the laboratory surrounding or building is mandatory for a CL 3 laboratory. Therefore, a continuous inward airflow into the laboratory has to be maintained by proper engineering of the design of the laboratory. This is essential when working with the biological agents. It is extremely important to ensure the continuous supply of fresh air and temperature controls to maintain the comfort factors of the workers. This category is appropriate to teaching, research, clinical, diagnostic, or production facilities where work is executed with agents that may cause severe or potentially fatal disease even through inhalation. Procedures pertaining to manipulation of hazardous materials must be confined to the Biological Safety Cabinets or other physical containment devices. Students or other personnel desiring to use the facility must be specifically trained to handle potentially pathogenic and lethal agents, where they must be under the strict vigil of scientists who are themselves competent in handling infectious agents and associated procedures. A CL-3 laboratory design has to be an improvement on CL2 with specially engineered features like ensuring airflow of outside air through a HEPA filter *via* an independent ducting system. Extracting the inside air to the outside air with a fan and HEPA filter fixed in a wall or window of the laboratory may maintain the negative pressure. The exhaust air from the biological safety cabinet to the outside air through a HEPA filter can also be ducted. Proper maintenance of HEPA filters, ducts, seals and the cabinets should be thorough and tested at six monthly intervals depending on the frequency of use. Some laboratories have mechanical air supply system, in that case the supply and extract airflows must be interlocked to avert positive pressurization of the laboratory in the event of a failure of the extract fan. Reverse airflows should be prevented at all costs by integrating a good

ventilation system. Engineers' priority should be to make simple, uncomplicated systems to ensure all safety features of the laboratory when arranging heating and ventilation, keeping in mind the heat generated by equipment. Particularly, the influx of cold air coupled with the

placement of the ventilation outlets and extracts can have a significant effect on the performance of safety cabinets

Needles or similar sharp objects used in the laboratory should not be broken, bent or recapped into disposable syringes', rather they should be carefully disposed off in puncture-resistant containers meant for disposal. Needles may also be autoclaved before finally being transported to decontamination area. The hazardous non-disposable material must be retained in a double walled container for transference to an area for decontamination or for autoclaving. Judicious use of glassware is a must and if broken by accident it should strictly not be handled directly. Rather, it must be removed using tongs, forceps or a brush and dustpan. Plasticware should be used where ever possible.

8.12.4 *Containment Level 4/ Biosafety Level 4*

This is restricted to hazardous agents that area threat to the individual as aerosol-transmitted laboratory infections or any life-threatening disease for which there are no vaccines or treatments, or an agent with unidentified risk of transmission. Any source that has a close or identical antigenic relationship to the agents necessitating BSL-4 containment must be handled at this level until adequate data is acquired either to approve continued work at this level, or re-designate the level. Laboratory personnel must have explicit and exhaustive training in handling extremely perilous infectious agents. Students are best not allowed in the CL 4 laboratories and the lab staff must appreciate the primary and secondary containment requirements of standard and exceptional practices, specialized maintenance of equipment, and specially designed characteristics of laboratories. The scientist/supervisor should be aware of the policies and the controls to access to the laboratory must be in accordance with the institutional guidelines.

There are two approved models for CL-4 laboratories:

1. **A Cabinet Laboratory** - All procedures with the agents must be performed in a Class III BSC; and
2. **A Suit Laboratory** - Personnel must wear a positive pressure supplied air shield suit.

The cabinets and the suit laboratories have distinctly engineered designs that confine the lethal agents within the cabinet simultaneously preventing the microorganisms from being disseminated into the environment.

Care must be taken to maintain the standard and special safety practices, equipment, and facilities. At the onset, at the entrance to the laboratory where infectious agents are to be manipulated, the universal biohazard symbol should be pasted. Inside the laboratory information about the laboratory's biosafety level must be glued that includes: the supervisor's name (or other responsible personnel), telephone number, and required protocol number justifying the entry, and time of entering and exiting the laboratory. The information regarding the lethal agent to be used must be posted in accordance with the institutional policy.

The experimental procedures should be approved by the Institutional Ethical committee and the protocols reviewed intermittently. The work surfaces should be decontaminated after even a splash of potentially infectious material and necessarily after completion of work with an appropriate disinfectant. The cultures, stocks, and other infectious agents should also be autoclaved before disposal. The facility to disinfect and autoclave has to be within the confines of the laboratory i.e. autoclave, chemical disinfection, incineration, or a -20°C to

store before incineration. Before the biological waste is exited from the laboratory rigorous monitoring of the materials to be disposed must done. All agents should be placed in a durable, leak proof container and secured for transport.

A Suit Laboratory must either be a separate building or a distinctly demarcated portion within the building. All doors must have locks in accordance with the institutional policies. The entry into the rooms of the CL 4 facility must be organized to safeguard of personnel by maintaining the one-way movement to exit through the chemical shower, inner change room (dirty), shower, and outer (clean) changing area. The entry must be through the airlock fitted with airtight doors. A positive pressure suit supplied with HEPA filtered breathing air has to be worn by the personnel who enter this area. Failure alarms and emergency backup must accompany the breathing air systems To decontaminate the surface of the positive pressure suit the worker must pass through a chemical shower before exiting the laboratory. Alternatively, a gravity fed supply of chemical disinfectant maybe used. An independent power supply to such a containment facility is desirable as the exhaust system, life support systems, alarms, lighting, entry and exit controls are detrimental to the life of the worker.

Keywords

Primary animal cell culture and cell line cultures
Growth media for animal cell culture
Preparation and maintenance of animal cell culture
Biology of cultured animal cells
Scaling-up of animal cell cultures
Synchronization of animal cells
Organ and histotypic culture
Animal cell culture based vaccines and other products
Stem cell culture
Apoptosis

Suggested Additional Readings

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Model Questions

For answers see the sections mentioned following the questions.

1. How would you proceed to isolate specific animal cells? (Section 8.4.1.1)
2. Enumerate some of the valuable products that can be obtained from animal cell culture. (Section 8.8.3)
3. What are the advantages and disadvantages of primary cell culture? (Section 8.2.2.1)
4. How can a cell line be established? (Sec-tion 8.2.3.2)
5. Describe some of the basic aseptic techniques. (Section 8.3.1)
6. Describe the physical methods of cell separation. (Section 8.4.5.1)
7. What are the basic constituents of animal cell culture media? Describe the importance of each constituent. (Section 8.5.2)
8. What is the importance of serum in cell culture? (Section 8.5.3)
9. How do you propose to characterize a cell line? (Section 8.6.3)
10. What are the advantages and disadvantages of serum-free media? (Section 8.5.5.2)
11. Explain the various phases of cell growth. (Section 8.6.6)
12. What are the characteristics of established cell lines? (Section 8.2.3.1)

13. Following are some of the bioreactors commonly used: Spinner culture reactors, air-lift reactors, membrane/hollow fiber reactors, continuous-flow stirred-tank reactors.

Which of these above-mentioned bioreactors match the following statements?

(a) Cell densities are usually low;
(b) may generate high shear forces which damage the cells;
(c) require large media reservoirs;
(d) present a high probability of microbial contamination;
(e) can be operated continually over a long period of time.

14. Discuss the importance of cell synchronization and the factors to be considered for it. (Section 8.7.2)

15. Why is cell cloning necessary? (Sec-tion 8.7.2.3)

16. What are the phenotypic properties of transformed cells? (Section 8.7.3.1)

17. Differentiate between:

(a) Vertical laminar flow hood and horizontal laminar flow hood. (Section 8.3.2.1)
(b) Pluripotent cells and totipotent cells. (Section 8.9)
(c) Organotypic and histotypic culture. (Section 8.7.4)

(d) Apoptosis and necrosis. (Tables 8-11 and 8-12)

18. Is tissue engineering helpful in replacing organs? If so, how? (Section 8.7.5)

19. Discuss the various applications of cell culture. (Section 8.8 and Table 8-9)

20. Cell suicide, a myth or reality? Explain reasons for cell suicide. (Section 8.10)

21. What is somatic cell fusion? Enumerate its importance. (Section 8.8.2.1)

22. What is meant by de novo and salvage pathways? Give one example of each. (Sec-tion 8.8.2.1)

23. Why is stem cell culture important? How do stem cells differentiate? (Section 8.9)

Answers

13. (a) Applies to spinner flasks, air-lift bioreactors, continuous-flow bioreactors.

(b) Spinner and stirred-tank cultures produce high shear forces.

(c) Membrane and hollow fiber reactors require large media reservoirs.

(d) All containers are susceptible to contamination.

(e) Hollow fiber bioreactors and continuous-flow stirred-tank reactors. These reac-tors, however, have to be periodically emptied, cleaned and recharged.