HUMAN RESOURCE DEVELOPMENT CENTRE

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ANIMAL CELL CULTURE

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1. Principle of the method – Cell culture is a method allowing maintaining the life of cells, tissues or organs separated from multicellular organism, in conditions in vitro (Latin for "glass" in a broader sense means in a cell-culture dish).

2. Historical development of the method.

In early 1865 Claude Bernard /Bernhard, "The stability of the internal environment [the milieu intérieur] is the condition for the free and independent life"/ formulates the hypothesis that living organisms are characterized by the ability to maintain specific conditions within their bodies that are not affected by changes in the environment. This concept led to the assumption that cells, removed from the body, are likely to continue their growth and proliferation if placed in conditions, similar to those under which they exist in the multicellular organism. This assumption is the basis for the first experiments in growing cells outside organism carried out by Wilhelm Roux back in 1885. He was the first to show that cells, taken from chicken embryo, may retain their vitality for a few days, if stored in warm saline solution.

Claude Bernard, (1813-1878)

Wilhelm Roux, (1850-1924)

Ross Harrison (1870-1959)

More than 20 years later – in 1907, Harrison succeeds in culturing part of a frog embryo's nerve tube in frog lymph, achieving this way not only preservation of the vitality of neurons, but also the formation of axons thereof. For these experiments Harrison has put pieces of tissue in frog serum and after clotting, mounted them in a "hanging drop" glass slide /Fig. 1/. Thus he created the opportunity for direct observation of the nerve-cell outgrowth, settling a controversy concerning the formation and nature of nerve fibres.

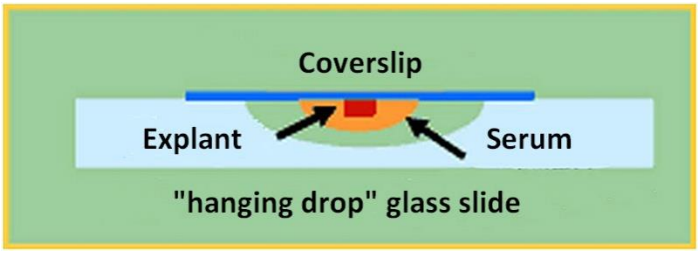

Figure 1. Schematic representation of the primary cell culture, developed by Harrison

Besides the exceptional importance of these experiments for neuroscience, tissue culture derived by Harrison meets the modern requirements for primary cell culture, so he is considered as the creator of the method of cell culture.

Despite the intriguing results of Alexis Carrel (Carrel, 1913, "On the Permanent Life of Tissues Outside of the Organism"), which show that cells can retain their vitality for a long period of time if maintained in aseptic conditions and in the presence of sufficient nutrients, the method of cell culture did not find wide application due to frequent bacterial contamination. Only after the Second World War, when antibiotics become widely applied and various companies start the commercial production of sterile culture media, sera and culture vessels, the method experience rapid development:

- In 1943 Wilton R. Earle creates the first established (permanent stable) cell line from mouse fibroblasts (L cells).

- In 1951 George Gey and coleagues create HeLa cell line - the first type of human cancer cell to be cultured continuously for experiments. The line is derived from cervical cancer cells and now is the oldest and most commonly used human cell line;

- Harry Eagle (1955), explores systematically nutritional needs of cells grown in in vitro conditions and develops the first formulation for cell culture media, known today as Eagle's minimal essential medium (EMEM);

- Hayflick and Moorhend, (Hayflick L., Moorhead P.S. 1961, “The Serial Cultivation of Human Diploid Cell Strains”) characterize cellular senescence in primary human cells and show that normal human fibroblasts die after a certain number of divisions;

- In 1965 Harris and Watkins (Harris H. and Watkins J.F. 1965, “Hybrid cells derived from mouse and man: Artificial heterokaryons of mammalian cells from different species”) cause viral fusion of cells from mouse and man and obtain the first heterokaryons of mammals.

These experiments create the base for development of hybridoma technology - a method for producing large numbers of monoclonal antibodies;

- In 1975 and Köhler and Milstein (Köhler G. and Milstein C. 1975, "Continuous cultures of fused cells secreting antibody of predefined specificity") receive first hybridomas secreting monoclonal antibodies;

- James Thomson (Thomson J.A. et al., 1998, “Embryonic stem cell lines derived from human blastocysts”) isolates the first five lines of human embryonic stem cells – H1, H7, H9, H13 and H14;

- In 2006 Shinya Yamanaka (Takahashi, K. and Yamanaka, S. (2006). "Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors") shows that differentiated cells can be “returned back” to pluripotent state after transfection with the genes for four transcription factors (Oct4, Sox2, cMyc, and Klf4). These cells are now known as induced pluripotent stem cells or iPS cells.

3. Requirements of the cells to the in vitro environment

Different types of cultured cells require some specific conditions, but generally, the artificial environment in which the cells are cultured in vitro always consists of a specific physicochemical environment (gases, pH, osmotic pressure and temperature) and physiological environment - medium that supplies the essential nutrients (carbohydrates, amino acids, vitamins, minerals), growth factors and hormones.

Physicochemical environment

Temperature

The optimal temperature for cell culture usually follows the body temperature of the donor organism from which the cells were isolated. Some variations are also accepted, depending on the anatomical specificity of the donor tissue (e.g., temperature of the skin is 35^{o}C and is usually lower than the temperature of skeletal muscle). To ensure optimal growth, most mammalian cell lines are maintained at 36°C to 37°C. For insect cells, 27°C is the most favorable temperature. Overheating above 30°C lowers the viability of insect cells and they do not recover even after they are returned to 27°C. Avian cell lines need higher temperatures for optimal growth - 38.5°C while cell lines derived from cold-blooded animals stand a wide temperature range between 15°C and 26°C.

Osmotic pressure

Since biological membranes are semi permeable the correct osmotic pressure of culture media is essential for cell survival. Osmotic pressure of 290 mOsm/kg, similar to the osmotic pressure of human plasma is considered best for human cell cultures. For most mammalian cells osmotic pressure of 260-320 mOsm/kg is suitable to ensure isotonic environment. Isotonicity of the medium is maintained by the dissolved salts. The main ions which must be present in the medium are: Na^+, Ca^{2+}, K^+, Mg^{2+}, Cl^-, phosphate and bicarbonate. Sulphates are often, added to the medium in concentrations up to 0,9 mM, although the supply of sulfur can be done at the expense of the amino acids methionine and cysteine. The yield of cells is often dependent upon the ratio of various ions, especially Na^+ and K^+ ions.

pH

For optimal growth different types of cell lines require a pH in the range 6.9-7.4. Using optimal pH can save usage of more expensive sera and glucose. While most normal mammalian cell lines grow well at pH 7.4, insect cell lines such as Sf9 and Sf21 grow optimally at pH 6.2. Usually the medium is buffered by the CO_2/HCO_3 system:

$$CO_2 + H_2O \rightleftarrows H_2CO_3 \rightleftarrows HCO_3^- + H^+$$

To maintain the appropriate pH the bicarbonate concentration in the medium should be in equilibrium with the CO_2 in the atmosphere. Most cultures are grown in media containing 23 mM bicarbonate and 5% CO_2 in the atmosphere. The disadvantage is the necessity for continuous supply of CO_2 in order to prevent quick alkalization of the medium. When CO_2 is not available the optimal pH can be sustained by growing cells in tightly closed vials in media containing organic buffer, usually HEPES /10-25 mM/ in tightly closed vessels.

Practically the evaluation of pH is made by the color of the dye phenol red added to the medium (Fig. 2).

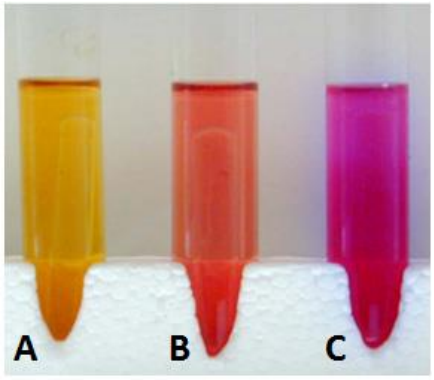

Figure 2. Change in color of the culture medium depending on the pH. A) yellow-orange color corresponding to acidic pH; B) tomato red color – pH 6.8 to 7.4; C) violet shades – alkaline pH

Gas balance

The main gases which the cells need are CO_2 and O_2. O_2 is an indispensable element for the growth of mammalian cells. In small-scale cultures the demand for O_2 is provided by the air. The usual gas mixture is 95% air and 5% CO_2. In large-scale cultures aeration must be provided, because the solubility of oxygen in the culture medium is very low (7 parts of O_2 per 1 million parts medium). The need for CO_2 was reviewed above.

Substrate for anchorage dependent cultures

To ensure cell adhesion to the solid support most often negatively charged polystyrene is used as a substrate. In addition, the plastic can be coated with various polymers for improving growth characteristics of the culture. Commonly different components of the extracellular matrix like fibronectin, laminin or gelatin are coated on the surface of the laboratory vessels. In table 1 various substrates for anchorage dependant cells are discussed.

Table 1

SUBSTRATE	COMMENTARY
Glass	Cheap, reusable, good optical properties (allows observations)
Polystyrene	Relatively inexpensive, disposable, charged surface, which ensures a good grip of the cells. There are differences in uptake between the products of different companies
Polytetrafluoroethylene	Hydrophilic (charged) for general use, hydrophobic (no charged) for growing macrophages and some tumors

Other substrates in use - PVC, polycarbonate, stainless steel, titanium etc.

Microcarriers

Microcarriers are used to create a large area in a small volume. In microcarrier culture, cells grow as monolayers on the surface of small spheres (Fig. 3) that are usually suspended in culture medium by gentle stirring. By using microcarriers in simple suspension culture, yields of up to 200 million adherent cells per milliliter are possible. Monitoring and sampling

microcarrier cultures is simpler than with any other technique for producing large numbers of anchorage-dependent cells.

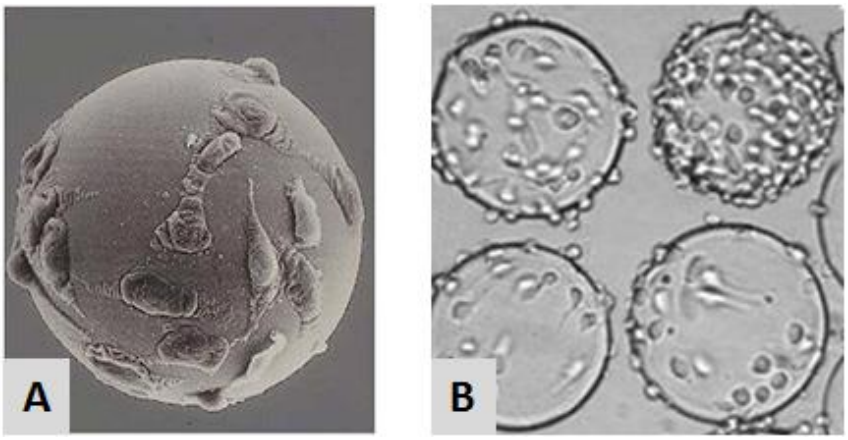

Figure 3. Cells growing on the surface of micro carriers - A) scanning and B) phase contrast microscopy

Microcarriers can be prepared by a wide variety of materials: Plastics, (polystyrene, polyethylene, polyester, polypropylene), glass, acrylamide, silica, silicone rubber, cellulose, dextran, collagen (gelatin), and glycosaminoglycans.

The most important parameters of microcarriers are:

1. Size. The diameter of the different carriers varies from 10 μm up to 5 mm. The smaller are best suited for stirred cultures, whereas the higher sedimentation rates of the larger make them suitable packed beds. The smaller the carriers, the larger the surface that can be used by cells to attach. The size of 100–300 μm and a very narrow size distribution for good mixing and equal sedimentation are ideal for microcarriers.

2. Density. Microcarrier must have a density just above the medium density that will enable them to remain in suspended state under weak stirring - about 1,02g - 1,04g /cm^3

3. Charge. Carriers are usually positively or negatively charged. However, non-charged carriers are also available. These are normally coated with proteins from extracellular matrix or peptides derived from them. Protein-coated microcarriers cannot be autoclaved as the protein coat is destroyed.

4. Transparency. Microcarrier transparency is important for simple cell observation in a light microscope. For example for vaccine production, it is important to see the morphology of cells directly on the carrier to find the right moment to infect the cells or harvest the virus.

5. Toxicity. The material used for microcarrier production should be non-toxic for the cells.

Currently, several types of commercially available microcarriers are produced including alginate-based (GEM, Global Cell Solutions), dextran-based (Cytodex, GE Healthcare), collagen-based (Cultispher, Percell), and polystyrene-based (SoloHill Engineering) microcarriers. They differ in their optical properties, porosity, specific gravity and surface charge.

Three-dimensional (3D) substrate

In the living organism cells are usually embedded in a complex three-dimensional extracellular matrix that and rarely do have the opportunity to attach to planar, rigid substrates used in conventional cell culture. Design of artificial 3D environments for cells aims at creating an environment that would mimic the physical and chemical properties of the natural extracellular matrix found in living tissues. Such scaffolds should be non-toxic, preferably biodegradable in time and must allow cell attachment and migration as well as diffusion of vital nutrients. The presently used 3D substrates for cell culturing could be divided into two groups – three-dimensional systems made of artificial polymers, and three-dimensional culturing systems based on natural extracellular matrix polymers.

Physiological environment

Nutrients

Culture conditions vary widely for each cell type, but the artificial environment in which the cells are cultured invariably contains liquid medium that supplies the essential nutrients: source of energy, amino acids, vitamins, minerals, lipids, and precursors of nucleic acids.

1. Source of Energy. A major source of energy in the culture medium is glucose. It is used in concentrations between 5-20 mM. However, glucose has the disadvantage that it's conversion into lactate can greatly reduce the pH of the medium, which has an inhibitory effect on the cells. Another major source of energy is glutamine - e.g. 30% of the energy needs of human diploid fibroblasts can be satisfied by glutamine. It is added in the medium at a concentration of 0.7 to 5 mM, which slows down the conversion of glucose to lactate. In some cases, the glucose may be substituted with galactose or fructose, which also reduces the amount of lactate.

2. Amino acids. Amino acids are the building blocks of proteins, and thus are obligatory ingredients of all known cell culture media. Essential amino acids must be included in the culture media as cells cannot synthesize these by themselves. In cell culture, 13 amino acids

are considered essential: arginine, cysteine, glutamine, histidine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, valine and leucine. L-glutamine is particularly important. L-glutamine provides nitrogen for NAD, NADPH and nucleotides and serves as a secondary energy source for metabolism. L-glutamine is an unstable amino acid that, with time, converts to a form that cannot be used by cells, and should thus be added to media just before use. Caution should be used when adding more L-glutamine than is called for in the original medium formulation since its degradation results in the build-up of ammonia, and ammonia can have deleterious effect on some cell lines. Supplements like glutamax are more stable and can replace glutamine for long term culturing of slow cells. For some cell lines, specific amino acids must be added in larger amounts, since they have a favorable effect on their growth - eg. serine for lymphoblastoid cells.

Nonessential amino acids may also be added to the medium to replace those that have been depleted during growth. Supplementation of media with non-essential amino acids stimulates growth and prolongs the viability of the cells.

3. Vitamins. Although the concentrations of these components are very low, the availability of vitamins is extremely important, since the cells usually cannot synthesise the them. Vitamins act as co-factors for many enzymes and are essential for their function. The absence of vitamins in culture may lead to decrease in cell growth, cell death or loss of productivity. Serum is the major source of vitamins in cell culture, however, media are also enriched with different vitamins making them suitable for a particular cell line. The B group vitamins are most commonly added for growth stimulation.

4. Trace elements. These micronutrients are essential for many biological processes, e.g. the maintenance of the functionality of enzymes. For animal cells Ca, Cu, I, Fe, Mn, Mo, Zn, Se, Cr, Ni, V, As, Si, Sn are considered indispensable. When media is supplemented with serum, these elements are delivered from the serum. When working with serum-free medium, they should be added in the basic formulation of the medium.

5. Lipids. In medium supplemented with serum lipids are supplied by the serum, as it contains a sufficient quantity of two classes of proteins transporting lipids - albumin (carries the free fatty acids) and lipoproteins (transport phospholipids, cholesterol and triglycerides). In serum free medium lipids like cholesterol and linoleic acid should be added.

6. Precursors of nucleic acids. Usually cells can synthesize purines and pyrimidines. In many cases, however, the addition of such precursors increases the yield of cells. Typically, the

medium is supplemented with adenine, hypoxanthine, cytidine, uridine and thymidine at concentrations around 10^{-5} - 10^{-7}M.

Feeder layer

Some cells (stem cells, hematopoietic cells, primary cells freshly taken from original tissue, etc.) need feeder (nourishing) layer composed of other cells which provide specific factors stimulating survival and growth of cultured cells. Cells of the feeder layer are treated so that their DNA is damaged (UV irradiation, mitomycin C, etc.). This treatment ensures that although alive they cannot divide.

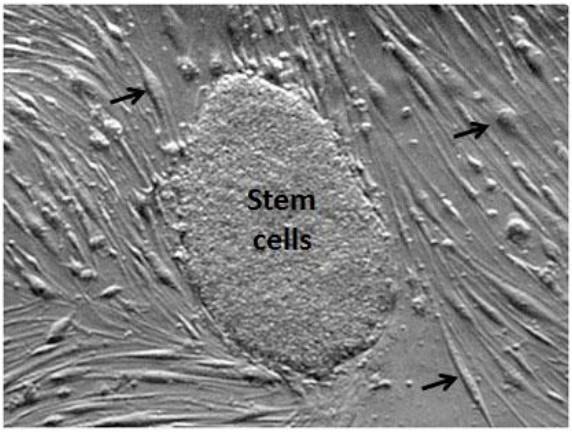

Figure 4. Phase contrast microscopy of human embryonic stem cells (stem cells) cultured on feeder layer of fibroblasts (arrows).

4. Types of cell cultures

Nowadays tissue culture has gained popularity as the general term describing the removal of cells, tissues, or organs from an animal and their subsequent survival for more than 24 hours into an artificial environment conducive to growth. This environment usually consists of a suitable glass or plastic culture vessel containing a liquid that supplies the nutrients essential for survival and growth. This general term can be refined by outlining three types of cultures taking into account the type and their mutual organization:

1. Cell culture - Culture of single, growing cells, which are not organized in the form of tissue.
2. Tissue culture - Culture of pieces of tissue, maintained in conditions in vitro, that allow differentiation and preservation of the tissue architecture and/or some of the tissue functions. Often this culture is referred also as explant culture.
3. Organ culture - the culture of organ rudiments, parts of an organ, or the whole organ, maintained in conditions in vitro, that allow differentiation and preservation of architecture and/or some organ functions.

When choosing the type of animal culture for scientific experiments, it should be taken into account that each type has its advantages and limitations. The main disadvantage of tissue and organ cultures is the limited lifetime in in vitro conditions. After the initial trauma caused by explantation and series of following necroses, a population of viable and proliferating cells remain in the organ and tissue culture. These cells retain cell to cell contacts and largely maintaine the histological and biochemical differentiation characteristic of the source organ or tissue. For example, in isolated glands a new glandular structures may be formed. Similarly, the periphery of the lung tissue explants is able to form new small bronchi. When culturing pieces of skin, or esophagus, a multilayer epithelial differentiation arises, similar to that in a multicellular organism. Hormone dependent tissues retain their usual sensitivity, and endocrine organs continue to secrete specific hormones. In embryonic tissue morphogenesis processes in vitro are very similar to those observed in vivo. These similarities to the situation in vivo demonstrate the advantages of tissue and organ cultures when used in experiments clarifying the processes at tissue or organ level. As noted, the main disadvantage of these cultures is that in most cases the cells show limited growth or remain in steady state, which defines the limited survival time of the culture in vitro.

Cell cultures, which consist of separate cells, lose their characteristic histological architecture and biochemical specificities of the tissue. Therefore, they are not suitable to study processes specific to the tissues or organs. These cultures, however, are appropriate for studies of virtually all processes that take place at the cellular level. They retain the ability to proliferate, which provides for the preparation of large quantities of cells required for most biochemical studies. Populations of already characterized cells can be stored indefinitely by freezing. This allows for a variety of tests on the same model system, without a time limit for implementation of experiments. These advantages make cell cultures much preferred and more widely used for research than tissue and organ cultures. Therefore the focus in the following paragraphs will be on cell cultures.

Cell cultures

To date, numerous cell cultures with great variety in their specific characteristics have been created. This diversity is due to differences in type of the parental cells, the type of multicellular organism from which they are isolated, the developmental stage of the donor organism (embryonic or mature) and the disease status (normal or malignant) of the tissue. However, despite these differences, cell lines show some similar features, when grown in in

vitro conditions. These similarities are used for combining them in specific types of cell cultures. Accepted approach for classification is based on two main parameters - the mode of growth and the duration of cultivation under in vitro conditions.

According to the way in which the isolated cells grown in the laboratory vessel two types of cell cultures can be defined:

- Adherent (monolayer) cell cultures;
- Suspension cell cultures.

Depending on the duration for which a cell line can be grown in a laboratory vessel the cell cultures are subdivided to:

- Primary cell culture;
- Cell line (secondary cell culture);
- Permanent (continuous) cell line.

Adherent (monolayer) cell cultures

Cultures which grow adherent to the bottom of the laboratory vessel in the form of a single layer of cells are defined as adherent or anchorage-dependent cultures. In addition to nutrients, these cells need surface for which the newly formed cells to adhere. If the culture completely fills the bottom of the container, the cell growth ceases despite the presence of sufficient nutrients (Fig. 5). This phenomenon is known as ”contact inhibition of growth”. It is characteristic of normal cells in a multicellular organism, primary cell cultures and certain permanent cell lines. For example, the murine fibroblasts from the 3T3 cell line grow rapidly, but after forming dense monolayer (100% confluence - a concept reflecting the ratio between the surface occupied by the cells and the whole surfaces of the laboratory vessel) division stops. It is estimated that the dense monolayers of 3T3 cells formed in a Petri dish with diameter of 6 cm contains about 10^6 cells. The number of cells involved in the formation of dense monolayer can vary extensively and depends on the properties of the cell line. To maintain growth in adherent cell cultures continuous subculturing (passaging) is necessary in order to provide new free surface in addition to nutrients.

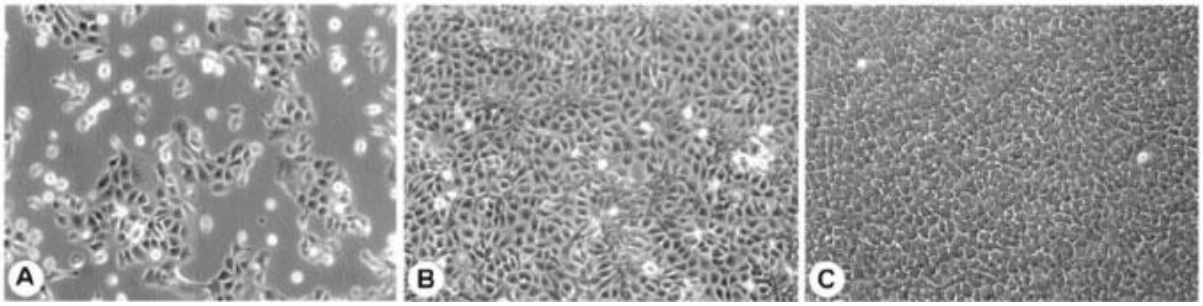

Figure 5. Phase contrast images of growing adherent culture. A – 45% confluent culture; B – 95% confluent culture; C - 100% confluent culture

Caner cells do not possess contact inhibition of growth. In many cases, they also grow as adherent cultures, but reaching confluence does not prevent their proliferation and they continue multiplying by climbing over one another to form multiple layers (Fig. 6). These cells as well as the cells from monolayer cultures are designated as anchorage-dependant cells or cell lines. As noted above, this term is not relevant to the characteristics normal or cancerous cells.

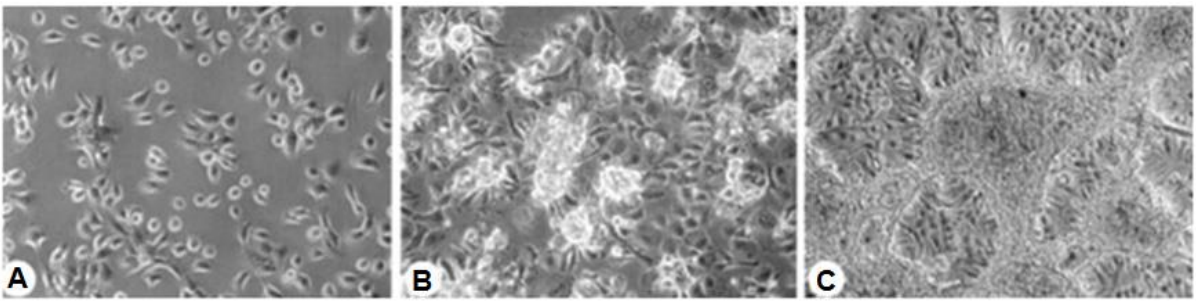

Figure 6. Phase contrast images of growing cancerous anchorage-dependant culture. A – 40% confluent culture; B – 95% confluent culture; C - 100% confluent culture

The ability of the anchorage-dependent cells to attach to the bottom of the laboratory vessel depends on the nature of the material from which the vessel is made, the presence of specific factors in the culture medium and the properties of the cells. To assess this ability the parameter "attachment efficiency" is used. It is calculated as the ratio between cells captured to the substrate and the total planted cells, expressed as a percentage and measured after a specified period of time. For example, if $6x10^4$ cells are planted, and after an hour $5x10^4$ cells have attached, the attachment efficiency for this culture is $5x10^4/6x10^4 \times 100 = 83.3\%$. Measurement of this parameter allows assessing the qualities of the various materials used to manufacture labware for cell culture, the role of individual adhesion molecules (fibronectin, collagen, vitronectin etc.) and changes in cell surface after various treatments of the cells.

The cells of the anchorage-dependent permanent cell lines usually lose the typical morphology of cells from which they originate. This change is due to the in vitro transformation (see permanent cell lines) and adaptation to the new conditions. These cells acquire the morphological characteristics of one of the two major cell types – *epithelial-like* and *fibroblast-like* cells (Fig. 7).

Epithelial-like cells resemble the morphology of epithelial cells in multicellular organisms. They are polygonal in shape and grow attached to a substrate in discrete patches in

which the neighboring cells are in close contact. In most cases, these cells have large nuclei and the nuclear-cytoplasmic ratio is high. If the origin of the cell culture is known to be from epithelial cells, or the line has defined functions, specific to this histological type, then the cell line can be referred as epithelial instead of epithelial-like.

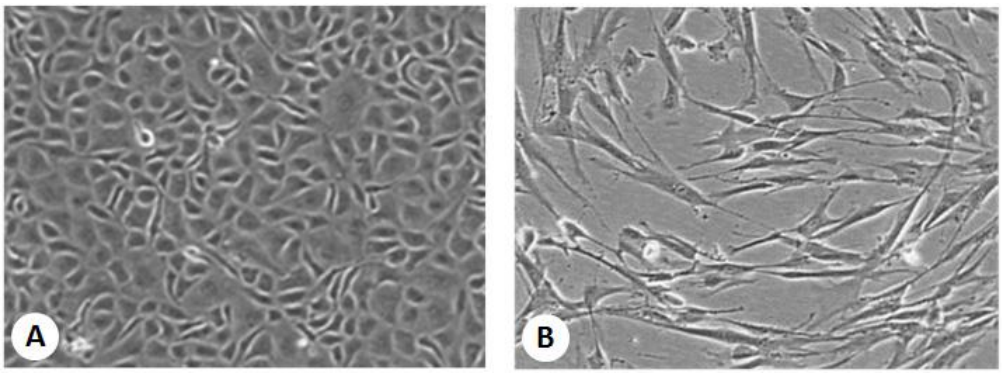

Figure 7. Representative images from epithelial-like (a) and fibroblast-like (B) cell lines

The *fibroblast-like cells* have morphology similar to the morphology of the genuine fibroblasts. Usually they have an elongated body shape with pointed ends and grow in a layer in which individual cells have only a small number of contacts. Nuclear cytoplasmic ratio is smaller compared with that of epithelial-like cells.

Suspension cell cultures

Cultures in which cells or small aggregates of cells grow freely floating in media are called *suspension* or *lymphoblast-like cultures* (Fig. 8). Typical for these cells is that their growth does not depend on the formation of contact with the substrate and do not have the property of contact inhibition. For their growth they only need enough nutrients and do not depend on free surface for attachment as monolayer cell cultures. In suspension growing most common Usually in suspension grow cell lines derived from hematopoietic cells (e.g. lymphoblast cell lines MOLT, RAJI, etc.).

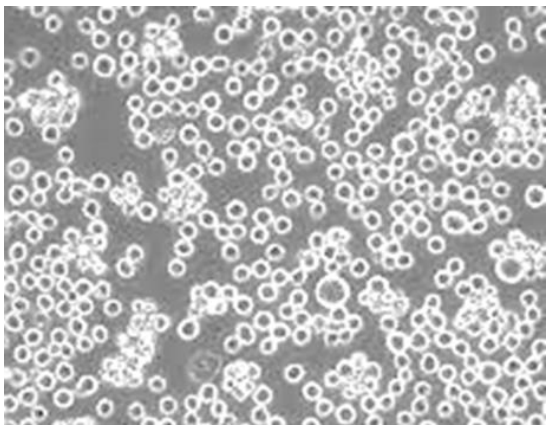

Figure 8. Phase contrast image of suspension (lymphoblast-like) culture

Some monolayer cultures can be converted into a suspension by two approaches - selection or adaptation. The method of selection is based on the fact that in every monolayer culture a population of loosely attached cells exists. Upon reaching a dense monolayer, these cells can be separated from the rest by gentle shaking, collecting the culture medium and subsequent separation of the cells by centrifugation. The procedure must be repeated several times and over a long period of time, since a large amount of collected cells are not able to grow in suspension but are removed from the monolayer because at the time of collection have been in mitosis. It is known that during mitosis, the cells are rounding and are weakly attached to the substrate. After sufficient cycles of selection of such weakly adherent cells it is possible to obtain a population of viable cells, which grow and divide in the form of a suspension. The selected cells can settle at the bottom of the beaker, but does not attach and spread on it.

The method of adaptation is based on the same principle as the preparation of suspension culture by selection, but in this case, the selection is directed to cells forcibly maintained in suspension by mechanical stirring. Briefly, the method consists of disaggregation of the cell monolayer by treatment with trypsin and the chelating agent EDTA that binds divalent (e.g. calcium and magnesium) and trivalent cations. The suspended cells are cultivated in medium without Ca^{2+} and Mg^{2+} with continuous mechanical stirring. Adaptation is considered successful when cell suspension shows a steady increase in the amount of cultured cells. The obtained suspension cultures are usually kept under constant agitation of the culture medium because of the possibility of reversion to a anchorage-dependent growth.

The use of monolayer or adherent cell culture depends on the specific goals of the experiment. For morphological studies, the most suitable are the monolayer cell cultures which permit quick and easy preparation of samples form cultured cells directly on the slides or even

on coverslips. These samples are very economical and are suitable for observing live cells, or fluorescence studies, autoradiography and histochemical examinations which require preservation of the cell structures. However, in cases where large amount of starting cell material is necessary (e.g. purification of specific cellular protein), suspension cell cultures are more convenient because of the possibility to obtain large amount of cells in a small volume of culture medium.

Primary Cell Culture

Primary cell culture is the initial culture of cells derived directly from fresh tissue that has survived in conditions in vitro for more than 24 hours. The tissue is taken by dissection, observing aseptic conditions. When working with small animals, they can be washed with 70% ethanol or other antiseptic solution. In the case of a human biopsy, the samples are collected in medium containing twice the concentration of antibiotics used in normal culture media. This is particularly important when making preparations of tissues having contact with the environment - respiratory, digestive and genitourinary tract. The tissue to be grown aseptically is cut into pieces with a volume of about $1mm^3$. They can be cultured directly (Fig. 9, A) or be subjected to further treatments to obtain single cell suspension (Fig. 9, B).

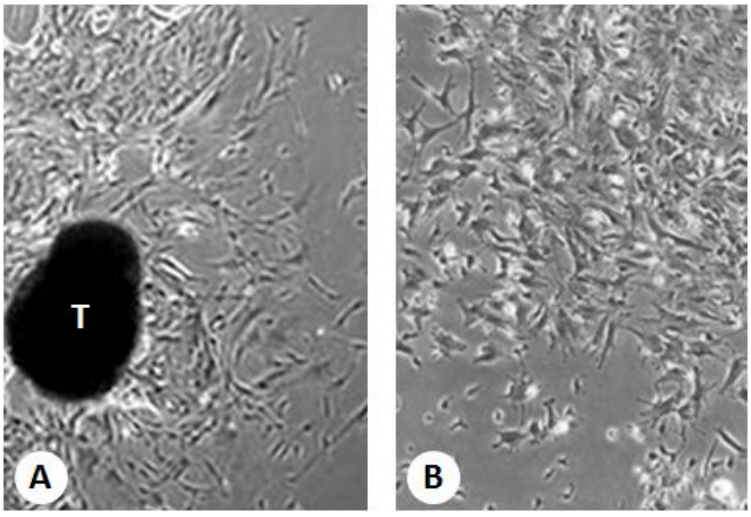

Figure 9. Primary cell culture growing from (A) piece of human tumor (T) and (B) single cell suspension, obtained after disaggregation of solid tissue

The most commonly used method for preparation of cell cultures from compact tissue is destruction of the tissue to individual cells prior to culturing. This can be achieved by mechanical disruption, treatment with enzymes that degrade the extracellular matrix or by

using chelating agents. Mechanical disruption of the tissue is carried out by passing through sieves with a certain diameter or homogenization but this method has a limited use. It is usually applied for the preparation of cultures of tissues in which intercellular contacts are weaker - lymphoid, neuronal or embryonic tissues.

More often, a combination of enzyme treatment (see Table 2), and chelating agents like EDTA (binds Ca^{2+} and Mg^{2+}) or EGTA (bind Ca^{2+}) is used.

Table 2. The most frequently used enzymes for tissue dissociation

Enzyme	Concentration	Comment
Trypsin	0.1 – 0.25%	Pancreatic enzyme, hydrolyses peptide bonds between basic amino acids, often used in combination with EDTA and collagenase, quickly inactivates by serum
Pronase	0.005%	Group of enzymes from Streptomyces griseus, which act faster and give better disaggregation of the tissue to single cells than trypsin, cannot be inactivated, therefore the termination of digestion is achieved by dilution or washing of the cells
Collagenase	0.15%	Hydrolyses more effectively the extracellular matrix proteins than proteins from plasma membrane, needs Ca^{2+} for its action
Elastase	0.05%	The only pancreatic enzyme that digests elastin – the integral part of the connective tissue, quickly inactivates by serum
Hyaluronidase	0.1%	Degrades the polysaccharide components of the extracellular matrix

Some primary cells, like spleen cells, hematopoietic cells, breast cancer cells and embryonic stem cells require certain nutrients and growth factors that support the adaptation to in vitro conditions and/or preservation of the undifferentiated state. In this case, the use of a feeder (nourishing) cell layer previously plated on the bottom of the vessel and irradiated with gamma irradiation is often used. These cells cannot divide, but continue their development and secrete growth factors necessary for the survival of cells from the primary culture**.** Nowadays,

when many of the growth factors can be purchased, nourishing layer can be replaced with the addition of appropriate growth factor to the culture medium.

Cell line (secondary cell culture)

Upon reaching a dense monolayer, anchorage-dependent primary cell cultures must be sub-cultured (passaged) in several new laboratory vessels, in order to provide fresh nutrients and growing space. Suspension cultures should be passaged when acidification of culture medium occurs shortly after addition of fresh medium – an indication for high cell density and quick exhaustion of the nutrients.

A culture of cells obtained from a primary culture after the first passage is known as cell line or secondary cell culture. The cells of the cell line as well as the cells of the primary cell culture remain diploid and retain many specific properties of the respective cells in the multicellular organism. Often, the secondary culture is a mixed population of several types of cells which participated in the formation of the original tissue. Furthermore, as a result of the new conditions of growth, some of their characteristics may be unstable. To clear the variations in these cells a selection or cloning is usually performed. The most frequently used methods for selection are as follows:

- Selective treatment with enzymes - if a mixed culture of fibroblasts and epithelial cells is incubated briefly with trypsin, the fibroblasts are the first to detach from the substrate, while the epithelial cells remain adherent, because of the stronger cell contacts. By repeating this this treatment pure epithelial cells line can be obtained.

- Use of specific inhibitors - certain substances are toxic to certain cell types - e.g., iodoacetic acid is more toxic to fibroblasts than epithelial cells.

- Transplantation – cancer cells can be selected and purified by alternating passaging in vivo (injecting into a laboratory animal) and in vitro.

- Centrifugation - cells can be separated according to their size by density gradient centrifugation. Thus, it is possible to separate not only different cell types, but also subpopulations of the same cells which are in different stages of the cell cycle.

- Fluorescence-activated cell sorting (FACS) - this is a specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. It is a useful scientific instrument as it provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest.

- Cloning - in cell culture, the term cloning means creating a cell culture in which all cells are derived from a single cell. This means that all the cells in the cell clone are genetically identical. This method is ensures elimination of variations in the cell lines. Practically cloning can be accomplished in several ways. The most commonly used method is cloning by limiting dilution (dilution cloning). In this case, the cell suspension is diluted so that, one volume unit statistically contains one cell. The corresponding volumes are plated in 96 well plates. Given enough time surviving cells proliferate and give rise to single colonies and later - cell clones. Cloning can be performed by manual separation of single cells using micromanipulator, but this method is too laborious and only limited amount of colonies can be obtained.

Initially, researchers thought that once a cell line has been established, it can grow and multiply indefinitely, preserving their specific characteristics. This concept has been revised when in 1961 Heyflik and Moorhead isolate the diploid line WI38 of human embryonic lung cells. The existence of these cells in vitro is limited to about 50 population doublings. Then, although the cells remain diploid and do not show malignant changes, the proliferation declines and the cell line fades away. Further studies on other cell lines showed that the observed phenomenon called “Hayflick limit” is a result of increased cellular senescence and programmed cell death. This limitation is related to the length of the chromosome telomeres and applies for all the cell lines isolated from normal tissues of vertebrates. The period during which the cell line shows reduced proliferation rate is referred to as “crisis”. It is characterized by abnormal mitotic figures, detachment of the cells from the substrate and the formation of multinucleated or giant cells. During this massive degeneration of the cell culture it is possible a small number of cells to survive as a result of in vitro transformation. Such cells can become a source of a new cell line, which is able to survive in vitro indefinitely. It is referred to as a permanent (continuous, immortal) cell line.

Permanent (continuous) cell line

Cell line formed by the transformed cells that have the ability to survive in conditions in vitro indefinitely, is referred to as permanent or continuous cell line. As mentioned above, the transformation can take place spontaneously, but it can also be induced by treatment of the cell lines with chemical carcinogens, radiation, transfection with oncogenes or oncogenic viruses. These treatments are often applied for immortalization of various cell lines. For example, the permanent murine cell line L is obtained after treatment with the mutagen methylholantren. The inheritable change that occurs in cells of the permanent cell line spontaneously or intentionally, is referred to as “in vitro transformation” in order to be

distinguished from malignant transformations that may occur in a multicellular organism. The concept of in vitro transformation does not correspond to the concept of cancerous transformation, because immortalized (in vitro transformed) cells are not always capable of forming tumors after reinjection in laboratory animals. This is the safest and most commonly used test for malignant transformation. For example, permanent fibroblast cell line BHK21, which can proliferate in vitro indefinitely, retains even a certain degree of contact inhibition. BHK21 cells can be transformed into cancerous after treatment with polyoma virus or SV40. Cells from permanent cell lines undergo substantial changes and typically lose the characteristics of the cells from which they originate. These changes affect their karyotype, which most often become aneuploid. For example, chromosome number in L929 cells varies from 56 to 241, the largest percentage cells having 66 chromosomes.

Some of the most widely used cell lines are presented in Table 3.

Table 3. Examples of some of the most widely used permanent cell lines

Cell line	Origin	Comment
Epithelial and epithelial-like cell lines		
MDCK	dog, kidney	Initially the line has been fibroblast-like, but in the process of sub-culturing has become epithelial-like. Used for production of veterinary vaccines.
CHO-K1	hamster, ovary	Widely used for expression of recombinant proteins.
BeWo	human, choriocarcinoma	The first human cell line from endocrine cells. Secretes a number of placental hormones.
HeLa	human, cervical cancer	The most widely used human cell line. Aneuploid. Can grow in suspension.
WISH	human, amnion	Used for preparation of polio virus and adenovirus. Used to distinguish virulent from non-virulent strains of pox virus.
Fibroblast and	**fibroblast-like**	**cell lines**

BHK21	hamster, kidney	Can be induced to suspension growth. Clone 13 is widely used for production of veterinary vaccines.
WI38	human, embryonic lung	Diploid cells. Limited growth in vitro.
COS1, COS3, COS7	monkey, kidney	Widely used for expression of recombinant proteins.
BALB/3T3	mouse, embryo	Used to study of tumorigenicity and viral transformation.
F9	mouse, embryonal carcinoma	Can be differentiated after treatment with specific agents
Limphoblasts and limphoblastoid cell lines		
Daudi	human, Burkitt's lymphoma	Used for studies of leukemia
HL60	human, promyelocytic leukemia	Express surface receptors for FC and complement. Differentiate into monocytes. secrete immunoglobulins
Namalva	human, Burkitt's lymphoma	Used for production of α interferon
P3/NS1/1-Ag4-1	mouse, spleen	Used for hybridomas

5. Applications of cell culture technique

Today cell culture is the major tool used in modern biology, providing outstanding model systems for studying the physiology and biochemistry of cells, the effects of drugs and toxic compounds on the cells and mutagenesis and carcinogenesis. It is also used in drug screening and development and large scale manufacturing of biological compounds like vaccines, therapeutic proteins and other bioactive products. Some of the major advantages of using cell culture in research are outlined below:

The ability to grow cells in a petri dish allows cell biologists to design experiments employing live cells. With the help of various microscopic techniques, it became possible to

observe and study live cells. Furthermore, new methods for delivery into living cells (microinjection, electroporation, transfection, etc.) of labeled macromolecules and tracking their intracellular localization and metabolism were developed (Fig. 10). This has opened new opportunities for close correlation between morphological studies and cell biochemistry, physiology and genetics.

Cell culture allows conducting of better defined experiments when assessing the impact of different physical and chemical factors on the fundamental life processes occurring in the cell. Of significance in these experiments is that it eliminates the mediating effect of the various systems in multicellular organism and homogeneous population of genetically

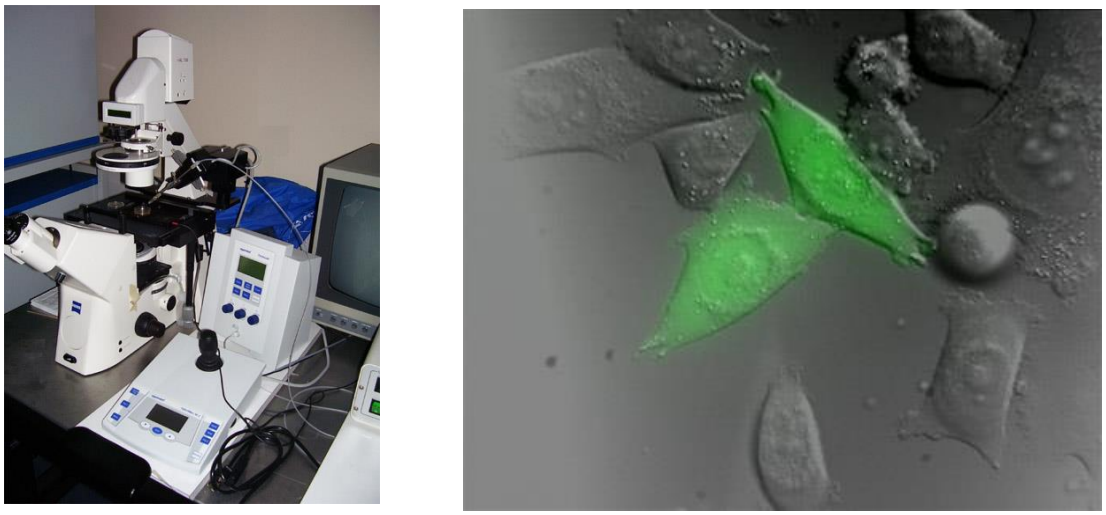

Figure 10. Example of microscope equipped with a system for microinjection and image of two cells, micro-injected with green dye.

identical cells can be used. In addition, the assessment of the cells viability during the entire experiment is possible and last but not least, the method is very economical. When working with cell cultures, statistically significant results can be obtained using very small amounts of cells. For example, if an experiment requires the use of 100 mice to obtain statistically valid results, the same statistical reliability can be achieved with the use of the 100 cell cultures grown on glass slides or even coverslips. This is an important advantage, eliminating many ethical issues arising from the need to use large groups of animals. These advantages over studies using whole animals, define cell culture as a experimental system similar to cultures of microorganisms.

It is particularly important that after development of this method experiments with human cells, that are otherwise ineligible, became possible.

On the basis of cell culture, a number of methods with important applications in research and industry were developed. Such method is **somatic hybridization**, which allows fusion of somatic cells derived from the same or even different animal species to yield respectively intraspecific (e.g. mouse fibroblasts + mouse lymphoblasts) or cross-species (mouse + man; chicken + rat, mosquito + man etc.) cell hybrids. For the preparation of hybrid cells it is necessary to mix the two starting cell cultures and treat them with agents inducing membrane fusion of adjacent cells (Fig. 11). Most commonly used fusogenic agents are inactivated Sendai virus, lysophosphatidylcholine (lysolecithin) or polyethylene glycol. Immediately after fusion, the resulting cells have mixed plasma membrane and cytoplasm but still preserve separate nuclei of the parental cells. Since the genetic material is still separated, these cells are not true hybrids, and are defined as heterokarions. Formed heterokarions can die or proceed to mitotic division which leads to formation of the true hybrid cells - synkarions in which the chromosomes of the source cells reside in a common nucleus. Hybridization of somatic cells is widely used in the field of cell biology, genetics, cancer biology and virology. For example, using this method, the fluid nature of plasma membrane was confirmed unambiguously. Important data for regulatory processes controlling

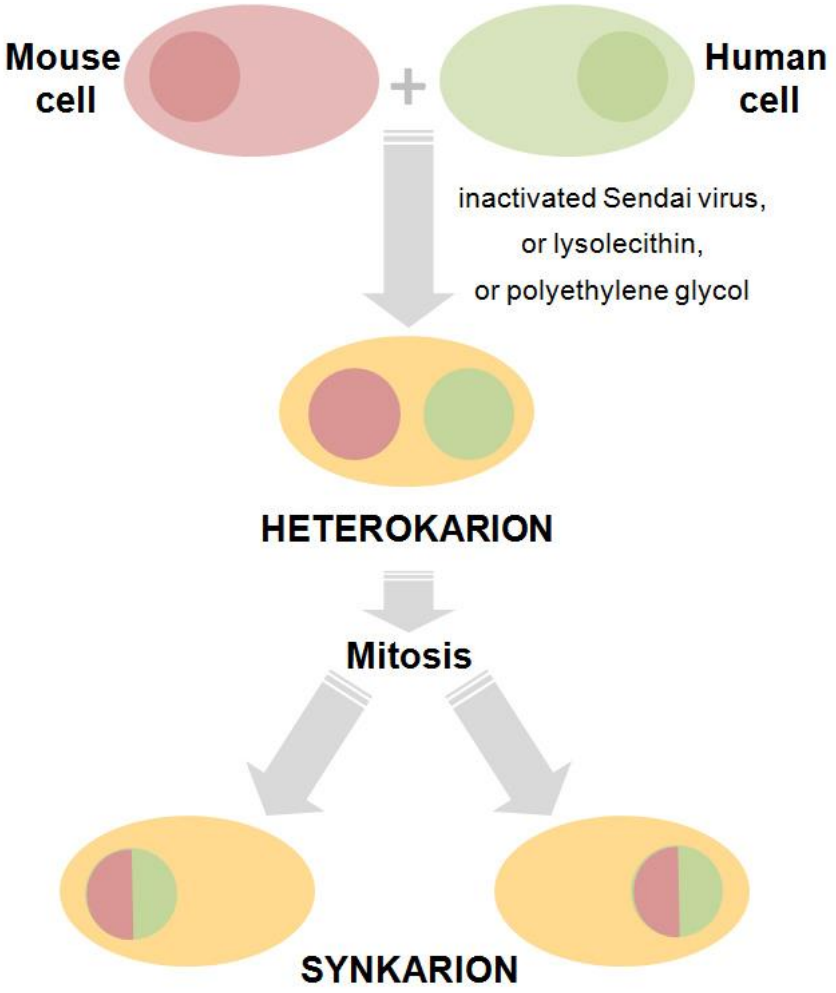

Figure 11. Schematic representation of the process of cell fusion

condensation of chromosomes and factors that control transition through interphase and mitosis is obtained by fusion of cells residing in different phases of the cell cycle. Hybridization of dividing cells and cells in interphase (G1 or Go) leads to premature chromosome condensation (PCC) in interphase nucleus. This phenomenon is widely used in the study of chromosomes in non-dividing cells; in the study of the structural units of DNA replication (replicons); and the identification of chromosomal disruptions caused by radiation or different chemical agents.

Analysis of the human genome is also possible with the aid of cell hybrids. The analysis is based on the selective loss of chromosomes occurring during cell division of synkarions, obtained after fusion of human and mouse cells. In the resulting clones of hybrid cells various groups of human chromosomes are retained. Ideal for determining the localization of genes in specific chromosomes would be hybrids retaining a single human chromosome, but unfortunately obtaining of such clones is very time consuming and largely unpredictable. Therefore, more commonly a sets of hybrid clones containing various combinations of human

chromosomes are used. In each hybrid clone, human chromosomes, identified by standard cytogenetic techniques, are matched to the set of human proteins expressed from each of the clones. Comparing different clones it is possible to establish a correlation between the loss of particular chromosome and specific human proteins. Thus, the chromosomal localization of numerous human genes has been determined before their accurate nowadays analysis, performed by sequencing and in situ hybridization.

In 1976, based on cell culture and somatic hybridization, a new method for production of antibodies derived from one clone B lymphocytes (**monoclonal antibodies**) has been developed (Fig. 12). B lymphocytes or B cells are a type of lymphocyte with the ability to secrete antibodies. Such cells can be isolated from the spleen of the immunized laboratory animals. Their lifetime in vitro is very limited and soon after isolation from the body they die. This disadvantage can be overcome if B lymphocytes are fused with cancer cells having the ability to survive in cell culture indefinitely. From the resulting fused cells only those with preserved useful qualities - to secrete antibodies (derived from B cells) and to grow in cell culture indefinitely (derived from myeloma cells) were selected. These are the true hybridomas, which give rise to clones, each of which is a stable source of a specific type of monoclonal antibodies. This method eliminates the intrinsic limitations in the quantity and variability of the obtained up till now polyclonal sera from immunizate animals and proves to be very suitable for industrial purposes.

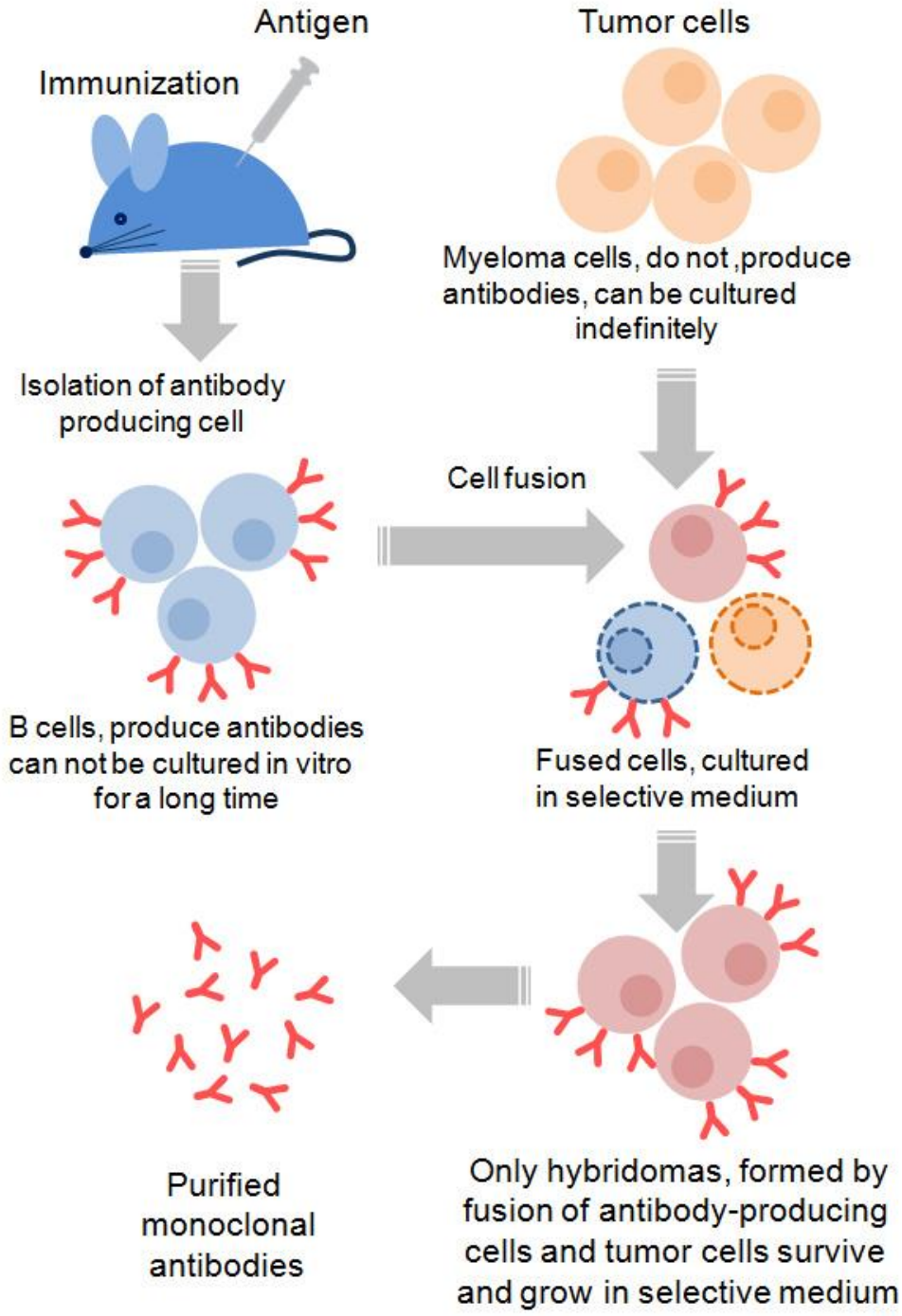

Figure 12. Schematic representation of the basic steps of the hybridoma technology for obtaining monoclonal antibodies

An interesting and very promising experimental direction based on the methods of cell culture and somatic hybridization is cellular fragmentation and cellular reconstruction (Fig. 13). Fragments suitable for these experiments are prepared by treating cells with actin disrupting agents (e.g. cytochalasin B) and centrifugation. As a result, the cell separates into two fragments - nucleus with a very small part of the cytoplasm – a structure called karyoplast

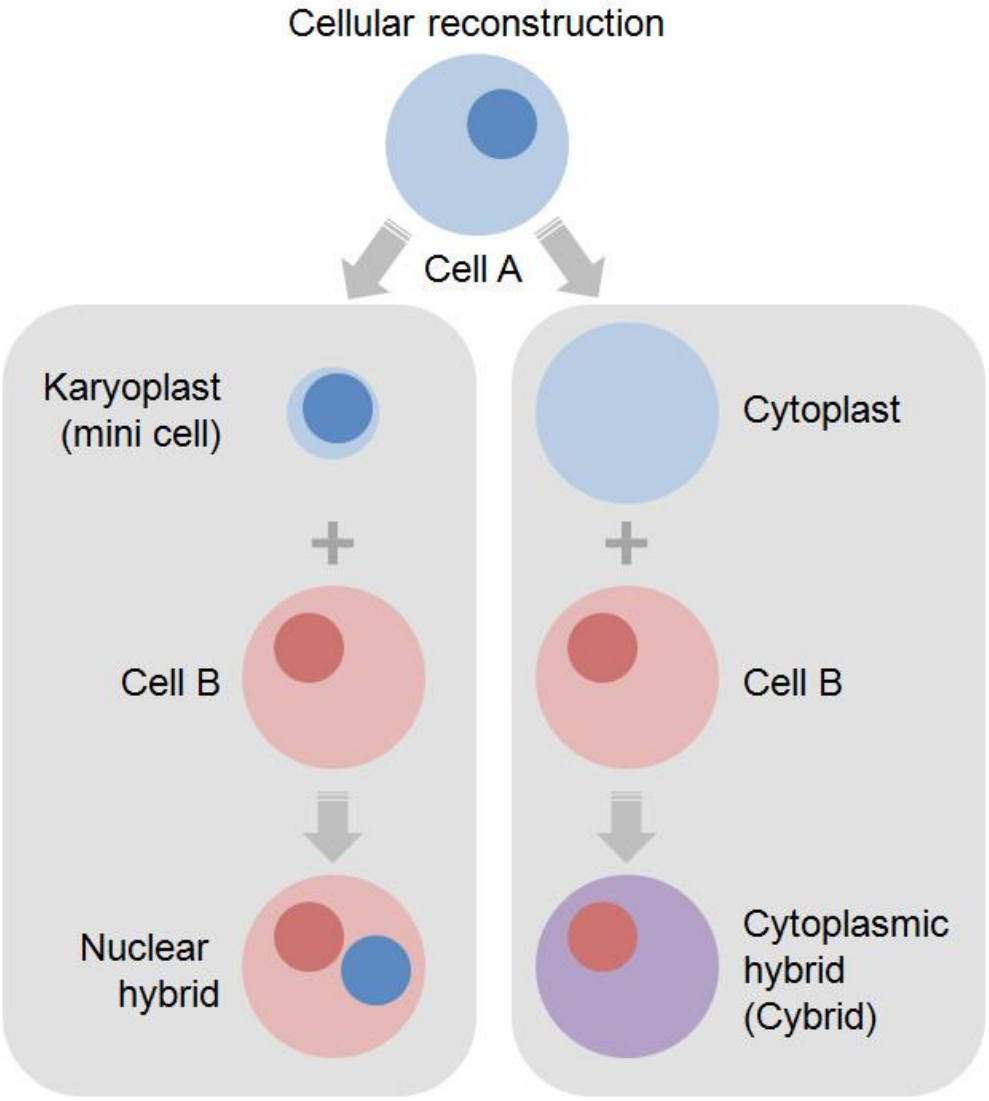

Figure 13. Diagram of cellular fragmentation and possible ways of cellular reconstruction.

or mini cell, and the rest of the cytoplasm – a nuclear-free cell named cytoplast. The time for which both fragments remain viable does not exceed two days, but they may be subjected to fusion with other normal cells, resulting in reconstructed cells (nuclear hybrids and cybrids) that are viable and can divide. The study of the cybrid cells and their progeny can be useful in the studies of various important questions of cell biology such as the communications between the nucleus and cytoplasm, and especially the role of the cytoplasm in the regulation of nuclear activity; regulation of gene expression and differentiation; influence of nuclear genes on mitochondrial activity; dependency of viral replication of nuclear and cytoplasmic factors and others.

Of particular interest is a specific type of cell reconstruction known as **somatic cell nuclear transfer (SCNT)**. This is a laboratory strategy for creating a viable embryo carrying

the genome of existing organism and is often called cloning. The process involves transferring the nucleus of a somatic cell (e.g. fibroblast) in the cytoplasm of an enucleated egg (an egg that has had its own nucleus removed; analogous to the cytoplast). Once inside the egg, the somatic nucleus is reprogrammed by egg cytoplasmic factors to become a zygote (fertilized egg) nucleus. The zygote is stimulated with a shock and begins to divide. Development proceeds normally and after many mitotic divisions, this single cell forms a blastocyst (an early stage embryo with about 100 cells) with an identical genome to the original organism (i.e. a clone). The resulting blastocyst can be used to generate embryonic stem cells with a genetic match to the nucleus donor (therapeutic cloning), or can be implanted into a surrogate mother to create a cloned individual, such as Dolly the sheep (reproductive cloning).

Based on the method of cell culture and the discovery of stem cells a method allowing assaying the role of individual genes at the level of the whole multicellular organism has been developed. The so-called **"transgenic" experiments** permit a segment of DNA containing a gene sequence that has been isolated from one organism to be introduced into a different organism (Fig. 14). This non-native segment of DNA may either retain the ability to produce RNA or protein in the transgenic organism or alter the normal function of the transgenic organism's genome. In the classical transgenic models, which are based on random DNA integration, the transgene could end up anywhere in the host genome. In more accurate techniques like “knock-in” (substitution of endogenous gene with an altered variant) and “knock-out” (inactivation of endogenous gene by replacement or disruption) the transgene is targeted, meaning it is inserted into a specific locus in the target genome via homologous recombination and behaves as its endogenous analog. Briefly, the method consists of genetic manipulation, wherein the transgene is selectively introduced into mouse embryonic stem cells by transfection (carried out using calcium phosphate, electroporation or lipophilic agents). Next, the transfected cells are selected and implanted into normal host embryo (blastocyst stage) by microinjection. The chimeric blastocyst is than transfered to a surrogate mother where transfected cells proliferate together with the normal cells of the embryo and participate in the formation of different tissues and organs of the developing mouse. The resulting organism is formed by both normal and manipulated cells and thus is referred to as chimeric. Usually, the manipulated cells participate in the formation of the chimeric’s mice reproductive system and enable the transmission of the transgene in germ cells. By breeding chimeric mice it is possible to obtain homozygous generation with respect to the transgene. These are the true

transgenic mice where all of their cells contain the transgene. Genetically modified mice are commonly used for research or as animal models of human diseases.

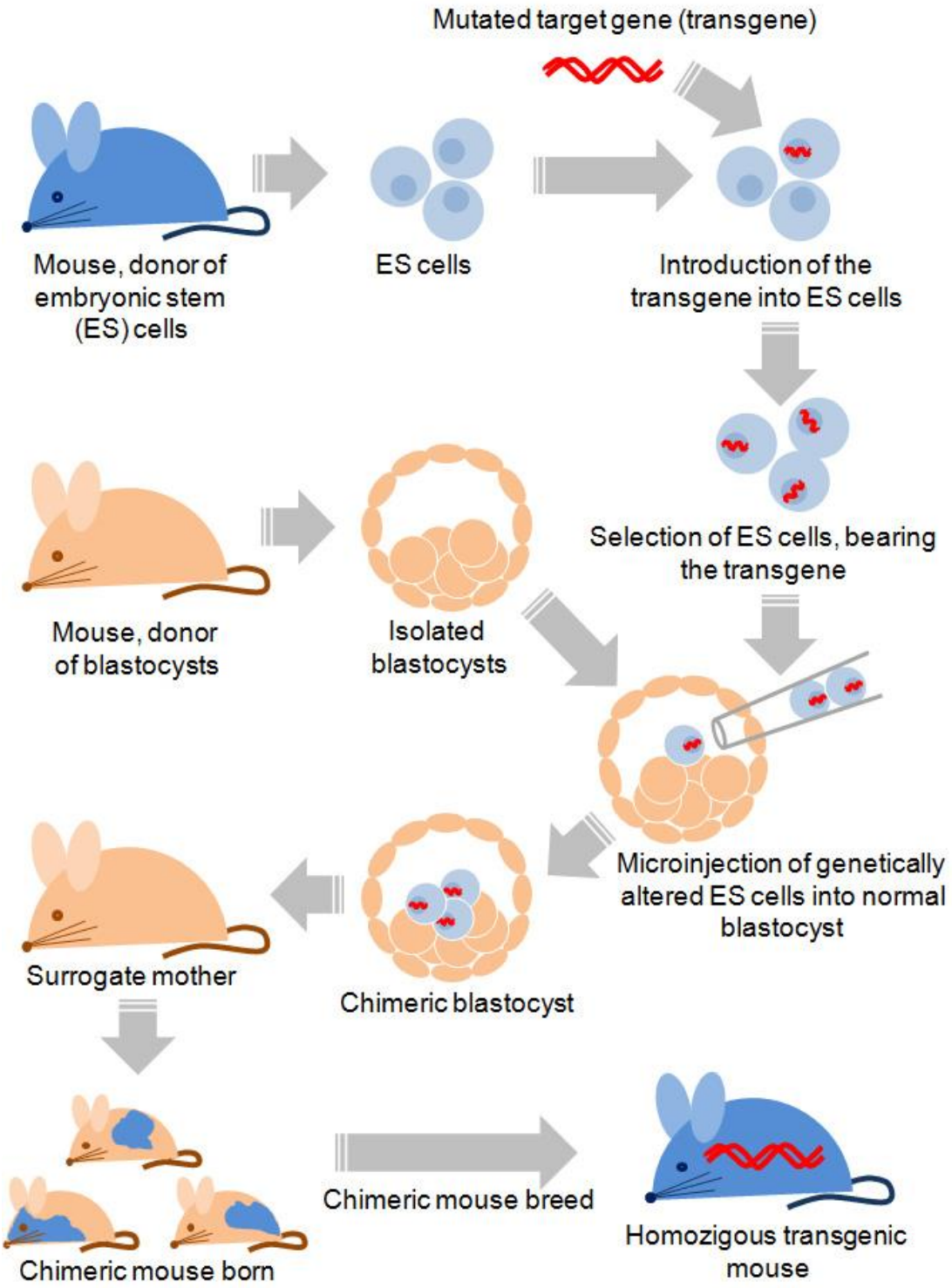

Figure. 14. Schematic depiction of the main steps in production of transgenic mouse

In addition to the basic research, the method of cell culture is widely used in human practice. The progress in virology in the last three decades is largely based on the possibility of growing viruses in cultured cells. This has important practical significance not only for the identification and study of viruses, but also for their use in the preparation of vaccines. **Cell-based vaccine** is a type of vaccine developed from mammalian cell lines rather than embryonic chicken eggs. The main benefit is the ability to rapidly produce vaccine supplies during an impending pandemic. Other benefits are the avoidance of egg-based allergy reactions, elimination the need for embryonated chicken eggs from managed, biosecure flocks and the reduction of the potential for contamination by viable and nonviable particulates.

Cell culture is one of the major tools used in determining the **cytotoxicity** - testing and studying the mechanism of action of different substances which can be used as medications, detergents, cosmetics, insecticides, preservatives and others. Although the results obtained from studies on cell cultures should not be transferred directly to draw conclusions for the entire multi-cellular organisms, the use of cells deliver from suffering a large amount of laboratory animals and allows experimentation on human material.

Cell cultures are an ivaluable **source of biologically active substances**. Mammalian cells synthesize a vast range of biopharmaceuticals that cannot be expressed by recombinant microbial techniques. Some examples of such animal cell culture products of medical/pharmaceutical importance are presented in Table 4

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Table 4

Cell culture product	Applications
Plasminogen activators	
Tissue type plasminogen activator Urokinase plasminogen activator Recombinant plasminogen activator	Acute myocardial infarction, pulmonary embolism, vein thrombosis, acute stroke
Interferons	
Interferon α	Anticancer, immunomodulator
Interferon β	Anticancer, antiviral
Interferon γ	Anticancer, immunomodulator

Blood clotting factors	
Factors VII, VIII, IX, X	Hemophilia, blood clotting agents
Hormones	
Human growth hormone	Growth retardation in children
Somatotropin	Chronic renal insufficiency
Follicle stimulating factor	Treatment of infertility
Human chorionic gonadotropin	Treatment of infertility
Others	
Erythropoetin	Antianaemic agents
Interleukin 2	Anticancer, HIV treatment
Tumor necrosis factor	Anticancer
Granulocyte stimulating factor	Anticancer
Carcinoembryonic antigen	Diagnosis and monitoring cancer patients