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# BASICS IN ANIMAL CELL CULTURE – PRINCIPLES AND APPLICATIONS

**T. Raja Swaminathan**
**Microbiology, Fermentation & Biotechnology Division,**
**E-mail: t.swaminathan@icar.gov.in**

Vertebrate cell cultures are *in vitro* models. The term in vitro refers to keeping entities of an organism outside the living body in an artificial environment, in contrast to in vivo, i.e. in the organisms. Primary cultures start from cells, tissues or organs taken directly from organisms. If a primary culture can be divided into new culture vessels and successfully propagated, it becomes a cell line. A cell line may be propagated a limited number of times, in which case it is finite, or indefinitely, in which case it becomes an immortal or continuous cell line.

Although animal cell culture was first successfully undertaken by Ross Harrison in 1907, it was not until the late 1940's to early 1950's that several developments occurred that made cell culture widely available as a tool for scientists. First, there was the development of antibiotics that made it easier to avoid many of the contamination problems that plagued earlier cell culture attempts. Second was the development of the techniques, such as the use of trypsin to remove cells from culture vessels, necessary to obtain continuously growing cell lines (such as HeLa cells). Third, scientists were able to develop standardized, chemically defined culture media that made it far easier to grow cells. These three areas combined to allow many more scientists to use cell, tissue and organ culture in their research. During the 1960's and 1970's, commercialization of this technology had further impact on cell culture that continues to this day. The overall result of these and other continuing technological developments has been a widespread increase in the number of laboratories and industries using cell culture today.

Any interaction of a toxic substance with an organism is initiated at the cellular level. From cells, alterations can translate to changes in tissue, organ function and finally impact on whole organism. Based on the central role of cells in the expression of toxicity, several cell lines or in vitro models have received regulatory acceptance by the organization as alternative to whole animal tests in health sciences. Besides their potential to replace or reduce animals in toxicity tests, cell lines have several advantages compared to whole animal tests. Large numbers of potentially toxic substances can be screened rather quickly in multiple-well plates, which can be analysed rapidly. As well, cells can help identify the mechanisms underlying a toxic response. If, for a particular purpose, a suitable continuous cell line can be used, a donor

animal is never again needed. Based on these reasons, the role of cell lines is expected to significantly increase.

**Fish cell line**

Fish cell lines have been useful in many areas of research. Originally developed to support the growth of fish viruses for studies in aquatic animal viral diseases, fish cell lines have grown tremendously in number covering a wide variety of species and tissues of origin and an array of applications. Fish immunology, physiology, genetics and development, toxicology, ecotoxicology, endocrinology, biomedical research, disease control, biotechnology and aquaculture are some of the areas in which fish cell lines have made significant contributions.

Fish cell lines being of poikilothermic origin, grow well at room temperatures without the need of thermo regulated incubators, furthermore, an amino acid-rich nutrient medium such as Leibovitz-15 that does not require $CO_2$ buffering has been successfully used with fish cell lines, thus $CO_2$ incubators are not necessary and cells can be grown conveniently in any undisturbed areas. Additionally, because of lower metabolic rates than eurythermic cells, fish cells can be maintained with little care for long periods of time.

In 1962, the first teleost cell line was reported in the literature. The first continuous fish cell culture (RTG-2) was originated over 20 year ago from rainbow trout gonad tissue. Subsequently, many other cell lines of poikilothermic origin have been developed. Most fish cell lines have been established and utilized for isolating, identifying, and studying viruses that cause economically important diseases. Consequently, the majority of these cell lines originate from species that are artificially propagated to some extent. Moreover, most such fish cell lines have been developed in North America or Europe. Nowadays, more fish cell lines are available from fishes indigenous to Asia, since aquaculture and fish farming are pursued on a large scale in this part of the world.

Wolf & Mann (1962) enumerated 61 cell lines originating from 36 fish species, representing 17 families. These cell lines were chiefly used for viral diagnostic purposes, and many had not been well characterized or previously reported. Fryer and Lannan (1993) have compiled a new listing of the fish cell lines reported in the literature that have been at least partially characterized. Recently Lakra et al (2010) made a comprehensive review on the characterized fish cell lines of both freshwater and salt water that have been developed after 1993.

Most fish cell lines originate from normal tissues, and embryos or normal fins are most frequently listed as the source of the tissue used in the primary culture. However, few cell lines were initiated from fish tumours, and in some cases, these cells remained tumorigenic *in vivo* following repeated in vitro passage. Traditionally, the chief uses of these cell lines were for detection and study of fish viruses and for diagnosis of the diseases caused by these agents. Today, fish cell cultures are increasingly utilized in research unrelated to disease, and with the recent identification of rickettsial fish pathogens, the diagnostic role of cultured fish cells has

also expanded. Along with the multiplying uses of fish cell culture is a concomitant increase in the need for guidelines for the health and maintenance of fish cell lines.

***Fish Cell Culture characteristics***

The physiology and the blood plasma constituents of teleost fishes are very much like those of terrestrial vertebrates; therefore the methodology for culture of cells and tissues is also similar. Most fish cell lines are readily propagated in vitro using unmodified media and techniques developed for mammalian cells, with appropriate adjustment of incubation temperatures to reflect the temperature range normal for the donor fish species. Also, osmolarity of the media must be adjusted upward for fishes of marine origin. Most important, fish tissue culture often requires less time for preparation and maintenance. Mammalian cell culture techniques need only be modified to reflect the lower incubation temperature requirements and the slower replication rates of the poikilothermic cells.

Appropriate incubation temperatures for cultured fish cells correspond to the normal temperature range of the fish species from which the cell line is derived. For lines from coldwater fishes, incubation temperatures range from 4-24°Cwith an optimal range of 15-21°C. For lines from warm water fishes, incubation temperatures range from 15-37°C, and the range of optimal incubation temperatures is 25-35°C.

**Cell Culture Systems**

Once in culture, cells exhibit a wide range of behaviors, characteristics and shapes. Some of the more common ones are described below. Two basic culture systems are used for growing cells. These are based primarily upon the ability of the cells to either grow attached to a glass or treated plastic substrate (Monolayer Culture Systems) or floating free in the culture medium (Suspension Culture Systems).

**Types of Cells**

Cultured cells are usually described based on their morphology (shape and appearance) or their functional characteristics.

There are three basic morphologies:

*Epithelial-like:* cells that are attached to a substrate and appear flattened and polygonal in shape.

*Lymphoblast-like:* cells that do not attach normally to a substrate but remain in suspension with a spherical shape.

*Fibroblast-like:* cells that are attached to a substrate and appear elongated and bipolar, frequently forming swirls in heavy cultures.

*Endothelial cells:* Endothelial cells are very flat, have a central nucleus, and are about 1-2 µm thick and some 10-20 µm in diameter.

*Other types:* Macrophages, neuronal cells, melanocytes, etc.

It is important to remember that the culture conditions play an important role in determining shape and that many cell cultures are capable of exhibiting multiple morphologies.

**Development of cell line**

***Primary Culture***

There are several ways with which monolayer cultures of fish cells may be initiated. This is a quick method that employs multiple explants of tissues of either fresh water or marine fish as the simplest way to produce monolayer cultures. When cells are surgically removed from an organism and placed into a suitable culture environment, they will attach, divide and grow. This is called a Primary culture. There are two basic methods for doing this. First, for Explants Cultures, small pieces of tissue are attached to a glass or treated plastic culture vessel and bathed in culture medium. After a few days, individual cells will move from the tissue explant out onto the culture vessel surface or substrate where they will begin to divide and grow.

The second, more widely used method speeds up this process by adding digesting (proteolytic) enzymes, such as trypsin or collagenase, to the tissue fragments to dissolve the cement holding the cells together. This creates a suspension of single cells that are then placed into culture vessels containing culture medium and allowed to grow and divide. This trypsinization method describes warm (1-2 hrs at $37^0$ C) and cold (4 ~ to 6~ overnight (16 hr) trypsinization of fish tissues which yields cultivable cells and small aggregates of cells for monolayer cultures. The disaggregated cells obtained by this procedure generally yield more uniform monolayer more quickly than do cultures initiated with minced tissues alone. This method is called enzymatic dissociation.

Before starting the preparation of primary culture, food should be withheld from donor fish for a day or more before use. Healthy specimens free of external lesions are preferred; otherwise there is risk of encountering systemically disseminated bacteria. Cells and tissues should be cultured at a temperature similar to the environmental temperature preferred by the donor species. Extended exposure of tissues from cold-water fishes such as salmon and trout to 30°C can be lethal. In contrast, many and perhaps most fish tissues remain viable even if held for a day or two on ice or at 4°C to 6°C. Internal tissues may be safely removed after thorough topical disinfection; this is conveniently done by total immersion of the fish for several minutes. A solution of liquid household bleach having 500 ppm available chlorine, or a 1:1000 dilution of a quaternary ammonium compound may be used. Excess disinfectant should be rinsed off with chlorinated tap water or sterile water and the surgical site sponged with 70% iso-propanol or ethanol. External tissues such as those of fins, gills, corneas and barbells are severely damaged by such disinfection. Consequently, such tissues should be excised first and decontaminated separately. Immersion for 1 hr in a solution containing 500 IU polymyxin B, 500 µg neomycin and 40 IU bacitracinis suggested, for these are bactericidal antibiotics.

***Subculturing***

When the cells in the primary culture vessel have grown and filled up all of the available culture substrate (called monolayer) they must be sub cultured to give them room for continued growth. This is usually done by removing them as gently as possible from the substrate with enzymes. These are similar to the enzymes used in obtaining the primary culture and are used to break the protein bonds attaching the cells to the substrate. Some cell lines can be harvested by gently scraping the cells off the bottom of the culture vessel. Once released, the cell suspension can then be subdivided and placed into new culture vessels. Once a surplus of cells is available, they can be treated with suitable cryoprotective agents, such as dimethylsulfoxide (DMSO) or glycerol, carefully frozen and then stored at cryogenic temperatures (below -130°C) until they are needed.

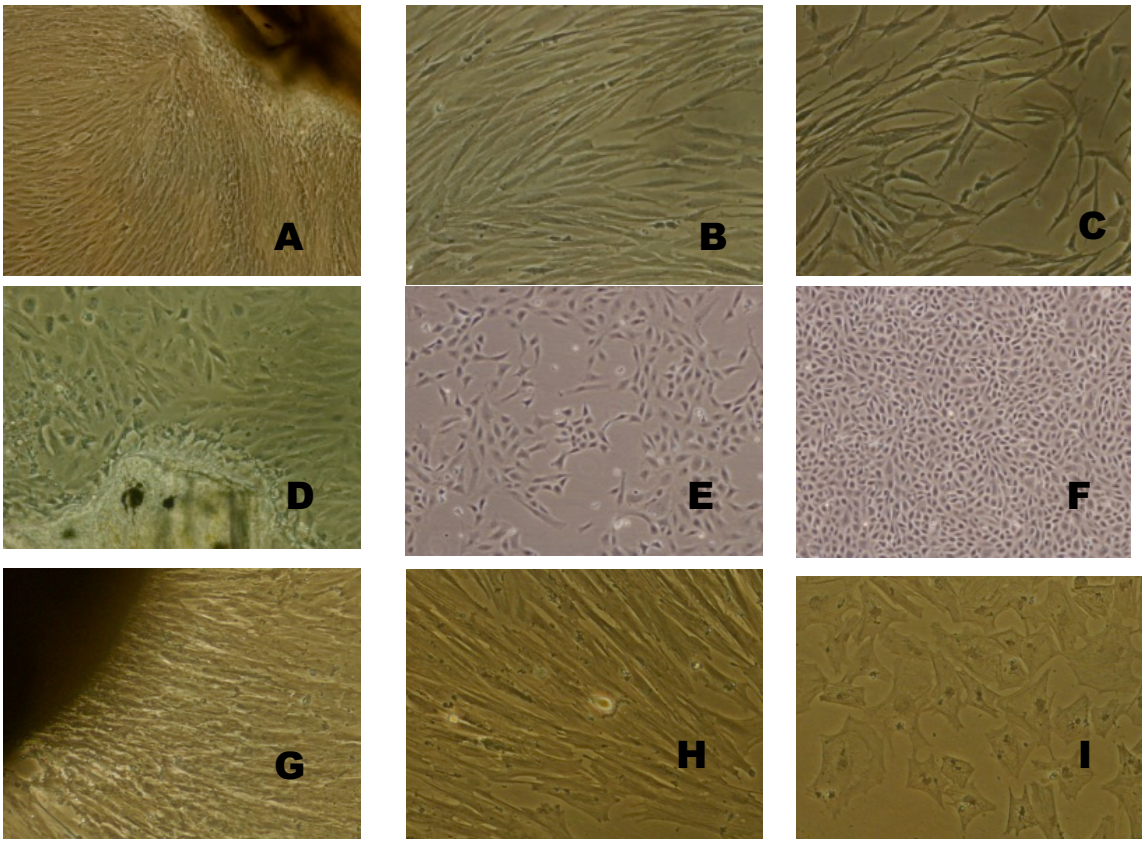


Fig.1. Phase contrast photomicrographs of rohu cells derived from Heart - (A, B, C), Fin - (D,E, F) and Swim bladder - (G, H, I). (A, D, G)-Primary culture on Day 5 following seeding tissue explant; (B, E, H) - subcultured at Passage 10; (C,F,I) - subcultured at Passage 30.

***Development of continuous cell lines***

Some cell lines may give rise to continuous cell lines. The ability of a cell line to grow continuously probably reflects its capacity for genetic variation, allowing subsequent selection. Genetic variation often involves the deletion or mutation of the p53 gene, which would normally arrest cell cycle progression, if DNA were to become mutated, and over expression of the telomerase gene. Possibly the condition that predisposes most to the development of a

continuous cell line is inherent genetic variation, so it is not surprising to find genetic instability perpetuated in continuous cell lines. The alteration in a culture that give rise to a continuous cell line is communally called *in vitro* transformation and may occur spontaneously or be chemically or virally induced. Immortalization means the acquisition of an infinite life span and transformation implies an alteration in growth characteristics (anchorage independence, loss of contact inhibition and density limitation of growth) that will often, but not necessarily correlate with tumorigenicity

Many (if not most) normal cells do not give rise to continuous cell lines. Normal human fibroblasts remain euploid throughout their life span and at crisis will stop dividing (around 50 generations), although may viable for 18 months. Human glia cells and chick fibroblasts behave similarly. Epidermal cells, on the other hand, have shown gradually increasing life span with improvements in culture techniques and may yet be shown capable of giving rise to continuous growth. Continuous cell line of lymphoblastoid cells is also possible by transformation with Epstein-Barr virus.

**Properties of finite and continuous cell line**

| **Properties** | **Finite cell line** | **Continuous cell line** |
|---|---|---|
| Ploidy | Euploid, eiploid | Aneuploid, heteroploidy |
| Transformation | Normal | Immortal, growth control altered and tumorigeneic |
| Anchorage dependence | Yes | No |
| Contact inhibition | Yes | No |
| Density limitation of cell proliferation | Yes | No |
| Mode 0of growth | Monolayer | Monolayer or suspension |
| Maintenance | Cyclic | Steady phase possible |
| Serum requirement | High | Low |
| Cloning efficiency | Low | High |
| Markers | Tissue specific | Chromosomal, enzymic, antigenic |
| Special function | May be retained (virus susceptibility, differentiation) | Often lost |
| Growth rate | Slow | Rapid |
| Yield | Low | High |
| Control parameters | Generation number, tissues specific markers | Stain characteristics |

**Characterization of Cell Lines**

In contrast to mammalian cells, are easier to maintain and manipulate, and unlike primary cultures, produce highly reproducible results. This ease of handling and simpler growth requirements makes cross-contamination of cell lines a more likely possibility, Proper characterization and identification of the cell lines are hence critical for scientific usage.

Authentication of a cell line is the sum of the process by which a line's identity is verified and shown to be free of contamination from other cell lines and microbes. Tests used to authenticate cell cultures include iso-enzyme analysis, antigenic markers, karyotyping/ cytogenetic analysis and more recently molecular techniques of DNA profiling. Whilst most of the techniques above are generalized tests and are applicable to all cell lines additional specific tests may also be required to confirm the presence of a product or antigen of interest.

**Cell line contamination**

Cell line contamination is a major drawback of main cell banks of the world and it has cost of losing important biological products or valuable research. The causative agents are different chemicals, invertebrates, bacteria, fungi, parasites, viral species and even other cell lines. Bacteria, fungi, parasite, viruses, invertebrates and mycoplasmas are main causative agents of cell line contamination.

The bacterial and fungal (including moulds and yeast) contamination of cell lines (except mycoplasmas) can be readily detected, as these organisms cause increased turbidity, shift in media pH (change in medium color) and cell destruction. Some reports have indicated that putative pathogens such as nanobacteria also will not be detected by this method.

In the case of mycoplasmas their cell line contamination is always undetected for many passages. They can proliferate within the cell, tolerate antibiotics and their growth always does not have any obvious microbial evidence like turbidity and pH changes or cytopathic effect. Their contamination also spreads quickly to the other cell lines

***Sources of contamination***

Another approach to cell culture contamination is sources of contamination. The sources of microbial culture contamination are different and may be grouped under four subjects.

- Contaminated cells, which are used as the primary starting material for cell culture.
- Glassware or apparatus, including storage bottles and pipettes
- Culture media (serum, basal cultural media containing heat-sensitive essential amino acids and vitamins, enzymes like trypsin, pronase and collagenase, and basic salt solutions).
- Airborne modes which can occur anytime the culture vessel is opened or contact is made with culture fluid through a defective culture vessel, stopper, or poor technique

***Cross-Contamination and Misidentification***

The problem of intra species and interspecies cross-contamination among cell lines has been recognized for half a century. For those scientists working on cell lines derived themselves or

received from a colleague, basic authentication tests such as STR profiling, iso-enzyme analysis, and contamination tests are readily available and should be routinely used. Transferring cell lines to colleagues should be avoided, or when it does occur, accompanied with comprehensive documentation verifying the integrity of the material or tests need to be repeated. Although cross-contamination of fish cells with other cell types has not been widely reported, conveyed the identification of a cell line dubbed Clone 1A believed to be derived from rainbow trout as being CHSE-214, a cell line derived from Chinook salmon embryos. Accordingly, awareness of good laboratory practices and careful vigilance with fish cell cultures as detailed by Lannan should be followed to avoid confusion of cell lines.

**Applications of cell culture**

Fish cell lines have been useful in many areas of research. Originally developed to support the growth of fish viruses for studies in aquatic animal viral diseases, fish cell lines have grown tremendously in number covering a wide variety of species and tissues of origin and an array of applications. Fish immunologies, physiology, genetics and development, toxicology, ecotoxicology, endocrinology, biomedical research, disease control, biotechnology and aquaculture are some of the areas in which fish cell lines have made significant contributions

***Toxicity Testing***

Cultured cells are widely used alone or in conjunction with animal tests to study the effects of new drugs, cosmetics and chemicals on survival and growth in a wide variety of cell types. Especially important are liver- and kidney-derived cell cultures.

***Cancer Research***

Since both normal cells and cancer cells can be grown in culture, the basic differences between them can be closely studied. Since, the normal cultured cells could be induced into cancer cells, the mechanisms that cause the change can be studied. Cultured cancer cells also serve as a test system to determine suitable drugs and methods for selectively destroying types of cancer.

***Virology***

One of the earliest and major uses of cell culture is the replication of viruses in cell cultures (in place of animals) for use in vaccine production. Cell cultures are also widely used in the clinical detection and isolation of viruses, as well as basic research into how they grow and infect organisms**.**

***Cell-Based Manufacturing***

Cultured cells can be used to produce many important products, like viral vaccines, genetically engineered protein of medicinal and commercial value and replacement of tissues and organs.

***Genetic Counseling***

Amniocentesis, a diagnostic technique that enables doctors to remove and culture fetal cells from pregnant women, has given doctors an important tool for the early diagnosis of fetal

disorders. These cells can then be examined for abnormalities in their chromosomes and genes using karyotyping, chromosome painting and other molecular techniques.

***Genetic Engineering***

The ability to transfect or reprogram cultured cells with new genetic material (DNA and genes) has provided a major tool to molecular biologists wishing to study the cellular effects of the expression of these genes (new proteins).

***Gene Therapy***

The ability to genetically engineer cells has also led to their use for gene therapy. Cells can be removed from a patient lacking a functional gene and the missing or damaged gene can then be replaced. The cells can be grown for a while in culture and then replaced into the patient. An alternative approach is to place the missing gene into a viral vector and then "infect" the patient with the virus in the hope that the missing gene will then be expressed in the patient's cells.

**Further reading**

Fryer J L, Lannan C N. 1994. Three decades of fish cell culture: a current listing of cell lines derived from fish. Journal of Tissue Culture Methods 16:87–94.

Freshney R I. 2005. Culture of animal cells: a manual of basic technique. Wiley, New Jersey.

Lakra W S, Swaminathan T R and Joy K P. 2010. Development, characterization, conservation and storage of fish cell lines: a review. Fish Physiology and Biochemistry. DOI 10.1007/s10695-010-9411-x.

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