Animal Cells in Culture Protocols

Created: Saturday, 29 September 2007

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Information

Animal cells were first grown in vitro early in the twentieth century. In 1912, Alexis Carrel began growing bits of chick heart in drops of horse plasma. The cells at the edge of the explant divided and grew out of the plasma clot. The explants died within a few days, and Carrel reasoned that their death was due to exhaustion of nutrients. He found that cells from a given explant could be maintained indefinitely if they were periodically subdivided and fed with a sterile aqueous extract of whole chick embryos.

In the early 1950s, Earle (2) developed a technique for dissociating cells of a whole chick embryo from each other with trypsin. When this suspension of single cells was mixed with plasma and embryo extract and placed in a sterile glass container, the cells adhered to the glass and divided to form a primary culture. The primary culture contained a variety of cell types including macrophages, muscle fibers, etc. Some types of cells grew to a monolayer, a thin sheet of cells one layer in thickness, that covered the entire bottom surface of their culture vessel and then stopped dividing. The cells could then be redispersed with trypsin and planted in new culture vessels containing fresh medium. These secondary cultures contained fewer cell types than did the primary cell cultures, as many of the differentiated primary cells had not divided and were diluted out upon transfer. Often, secondary cultures were composed entirely of spindle-shaped cells called fibroblasts because of their similarity to cultured connective tissue. Cells derived from kidneys and from certain carcinomas had a polygonal appearance in culture. Because of their tissue of origin, they and other cells with similar morphology are called epithelial.

Purpose

Cell culture is essential for growth of viruses because they are obligate intracellular parasites; they cannot replicate in any cell-free medium, and they require living cells from a suitable host within which to multiply. Animals such as mice and embryonated avian eggs may be used for the propagation of viruses, but for various reasons (time, cost, uniformity, ease of handling, animal welfare, etc.), the propagation of most viruses in cultured living cells is the method of choice today. Cell culture is also used in recombinant DNA technology and many in vitro assays. In fact most of what we know about the function and regulation of mammalian genes comes from studying genes transfected into cell culture (3).

Theory

Cells may be grown in vitro in several ways. Organ cultures, if carefully handled, maintain their original architecture and functions for several days or sometimes weeks. Slices of organs (which are actually tissue cultures) consisting of respiratory epithelium have been used to study the histopathogenesis of infection by certain respiratory viruses that can only be grown outside their natural host in complex organized tissue. The term tissue culture was originally applied to explants of tissue embedded in plasma. The term subsequently became associated with the culture of cells in general and is now obsolete in its original sense. Cell culture is the term most correctly used today. It refers to tissue dissociated into a suspension of single cells by both mechanical and enzymatic means. After being washed and counted, the cells are diluted in growth medium and allowed to settle onto the flat bottom surface of a glass or specially treated plastic container. Most types of cells adhere quickly, and under optimum conditions they will undergo mitosis and cell division about once a day until the surface is covered with a confluent cell monolayer. Such cells constitute a primary culture. These cells are usually removed from the surface on which they are growing with a combination of trypsin and a chelating agent such as EDTA, counted, diluted, and replated in new containers with fresh medium. This is called a secondary culture.

There are three main types of cultured cells. The difference in these types lies in the number of times the cells can divide.

1. Primary cell cultures.

When cells are taken freshly from animal tissue and placed in culture, the cultures consist of a wide variety of cell types, most of which are capable of very limited growth in vitro, usually fewer than ten divisions. These cells retain their diploid karyotype, i.e., they have the chromosome number and morphology of their tissues of origin. They also retain some of the differentiated characteristics that they possessed in vivo. Because of this, these cells support the replication of a wide range of viruses. Primary cultures derived from monkey kidneys, mouse fetuses, and chick embryos are commonly used for diagnostic purposes and laboratory experiments.

2. Diploid cell strains.

Some primary cells can be passed through secondary and several subsequent subcultures while retaining their original morphological characteristics and karyotype. Subcultures will have fewer cell types than primary cultures. After 20 to 50 passages in vitro, these diploid cell strains usually undergo a crisis in which their growth rate slows and they eventually die out. Diploid strains of fibroblasts derived from human fetal tissue are widely used in diagnostic virology and vaccine production.

3. Continuous cell lines.

Certain cultured cells, notably mouse fetal fibroblasts, kidney cells from

various mammalian species, and human carcinoma cells, are able to survive the growth crisis and undergo indefinite propagation in vitro. After several passages, the growth rate of the culture slows down; then isolated colonies of cells begin to grow more rapidly than diploid cells, their karyotype becomes abnormal (aneuploid), their morphology changes, and other poorly understood changes take place that make the cells immortal. The cells are now "dedifferentiated," having lost the specialized morphology and biochemical abilities they possessed as differentiated cells in vivo. Continuous cell lines such as KB and HeLa, both derived from human carcinomas, support the growth of a number of viruses. These lines and others derived from monkey kidneys (e.g., Vero), mouse fetuses (L929), and hamster kidneys (BHK) are widely used in diagnostic and experimental virology. Continuous cell lines have been established from many types of vertebrate and invertebrate animal tissues and are available from the American Type Culture Collection.

The development of antibiotics during World War II simplified long-term animal cell culture by minimizing the problems of bacterial and fungal contamination. Eagle made another important discovery in the 1950s when he determined the minimal nutritional requirements of cultured cells (1). He began by showing that HeLa and mouse L cells would grow in a mixture of salts, amino acids, vitamins and cofactors, carbohydrates, and horse serum. By eliminating one component at a time, he then determined which nutrients were essential for cell growth. His minimum essential medium (MEM) contains 13 amino acids (human tissue in vivo requires only eight), 8 vitamins and cofactors, glucose as an energy source, and a physiological salt solution that is isotonic to the cell. The pH is maintained at 7.2 to 7.4 by $NaHCO_3$ in equilibrium with CO_2. The pH indicator phenol red is usually incorporated into the medium; it turns red-purple if the medium is basic, yellow if the medium is acidic, and remains red-orange if the pH is in the right range. Serum in concentrations of 1 to 10% must be added to the medium to provide the cells with additional poorly defined factors, without which most cells will not grow. Most mammalian cells are incubated at 37°C; avian, reptilian, and arthropod cells may grow best at higher or lower temperatures. If cells are grown in vessels open to the atmosphere, their incubator must be humidified and contain an increased CO_2 concentration. Some nonvolatile phosphate or substituted sulfonic acid buffers (HEPES, TES) eliminate the requirement for incubators to be gassed with CO_2. Tightly sealed containers also eliminate the need for CO_2. Other conditions required for growth of cells in culture are a negatively-charged solid surface (some tumor cells can be adapted to grow in suspension) and a minimum concentration of 2×10^5 to 5×10^5 cells per ml of medium or cm^2 of growth area.

With the advent of cell culture, many animal viruses have been propagated in vitro, and hundreds of previously unknown viruses have been isolated and identified. The discovery of the adenoviruses, echoviruses, and rhinoviruses, for example, is directly attributable to the use of cultured cells, as is the revolution in the diagnosis of viral diseases and the development of poliomyelitis, measles, rubella, and other vaccines.

PROTOCOL

A. Culture of Primary Chick Embryo Fibroblasts (CEF)

Materials

10- to 12-day-old embryonated eggs

Forceps and scissors

Sterile petri dishes

Sterile 125-ml Erlenmeyer flask with magnetic stir bar

Sterile 25-cm^2 flasks containing MEM plus 10% fetal calf serum

Sterile 0.5% trypsin in Saline A

Sterile 15-ml centrifuge tubes containing 0.5 ml of serum

Hemacytometers

1-ml and 10-ml pipettes

Sterile Saline A

Procedure

1. Disinfect the surface of the egg over the air sac. With scissors or the blunt-end of a forceps, break the shell over the air sac. Sterilize forceps by dipping in alcohol and flaming. Cool forceps, then peel away the shell over the air sac, sterilize forceps again, and pull back the shell membrane and chorioallantoic membrane to expose the embryo.

2. **Resterilize** the forceps, grasp the embryo **loosely** around the neck, and remove the entire embryo from the egg to a sterile petri dish.

3. Using two forceps or a scissors plus a forceps, decapitate and eviscerate the embryo. Mince the embryo carcass into very small fragments with a scissors.

4. Add about 10 ml of sterile Saline A to tissue fragments in the petri dish, swirl **gently** for **1 to 2 minutes** to resuspend and wash fragments, and carefully pour entire contents into a 125-ml Erlenmeyer flask. Tilt flask, allow fragments to settle, and gently decant saline. Discard saline.

5. Add **10 ml of sterile warm trypsin** solution to fragments in flask, cover, and stir **slowly** with magnetic bar for **5 to 10** minutes. Tilt flask, allow fragments to settle, and pour the trypsin-cell suspension into a 15-ml centrifuge tube containing 0.5 ml of serum. The serum contains a trypsin inhibitor that will prevent further damage to cell membranes by the enzyme.

6. Add 10 ml of sterile warm trypsin to fragments and repeat step 5. At

the end of this second treatment, the size of tissue fragments will be greatly reduced and a large number of single cells should be suspended in trypsin. (Note: it is preferable to treat the tissue with multiple short applications of trypsin; however, if time is a limitation, for example in a lab class, this method will work).

7. Visually balance volume in centrifuge tubes (transfer liquid if necessary) and centrifuge at 1,500 rpm for 10 minutes. Carefully decant and discard supernatant, resuspend **pooled** cell pellets in a total of 5 ml of MEM. (Resuspend the pellet in one tube, then transfer the suspension to the second tube and resuspend that pellet.) Mix well for counting in a hemacytometer. Be sure to keep your cell suspension sterile.

8. In most hemacytometers, each heavily etched square (surrounded by double or triple lines and containing either 16 or 25 smaller squares) is 1 mm on each side and 0.1 mm deep. Therefore, the area is 1 mm^2 and the volume is 0.1 mm^3 (Fig. 1). After centrifugation, the cells should be packed in a tight pellet at the bottom of each tube.

9. Place the cover slip on top of the hemacytometer, bridged on the two glass arms beside the etched pattern. Add one drop of evenly-suspended cell suspension to the groove in the hemacytometer stage and allow it to fill the chamber under the cover slip. Examine with the 10X objective. If there are too many cells to count (>200), make a 1:10 dilution of the cell suspension in MEM (0.1 ml of cell suspension plus 0.9 ml of MEM) for counting.

The following example shows how to convert your cell count to the concentration of cells in your original suspension. Assume you made a 1:10 dilution, then counted a total of 168 cells in one 16-square grid:

$$168 \times 10^4 \times 10 = 1.68 \times 10^7 \text{ cells/cm}^3$$

What does each term mean?
168 is the number of cells in the grid
x 10^4 converts to cells per ml (10^4 is the number of 0.1 mm^3 in 1 cm^3 (1 ml))
x 10 accounts for the dilution
1.68 x 10^7 cells/ml(cm^3) is the number of cells in your original suspension

Note: if a really accurate count is needed it is customary to count more than one 1-mm^2 grid, then take the average of the number of grids counted.

10. Calculate what volume of the original cell suspension you will need to add to the growth medium in the flask to give between 2 x 10^5 and 8 x 10^5 cells/ml (~5 x 10^5 cells/ml). (Hint: if you have 5 ml of growth medium in the flask, you need a total of 5 ml x 5 x 10^5 cells/ml = 2.5 x 10^6 cells.) Add the appropriate volume of **original cell suspension** (not the 1:10 dilution, if you made one) to the medium in your flask.

11. Be sure to examine cell cultures both macroscopically and microscopically each day. Actively growing cells produce acidic metabolic

by-products and their medium becomes yellow, and thus the pH of the medium may need to be adjusted by the addition of a drop or two of 7.5% $NaHCO_3$. If floating (dead) cells and debris are present or the color of the medium indicates a basic pH, the medium should be changed.

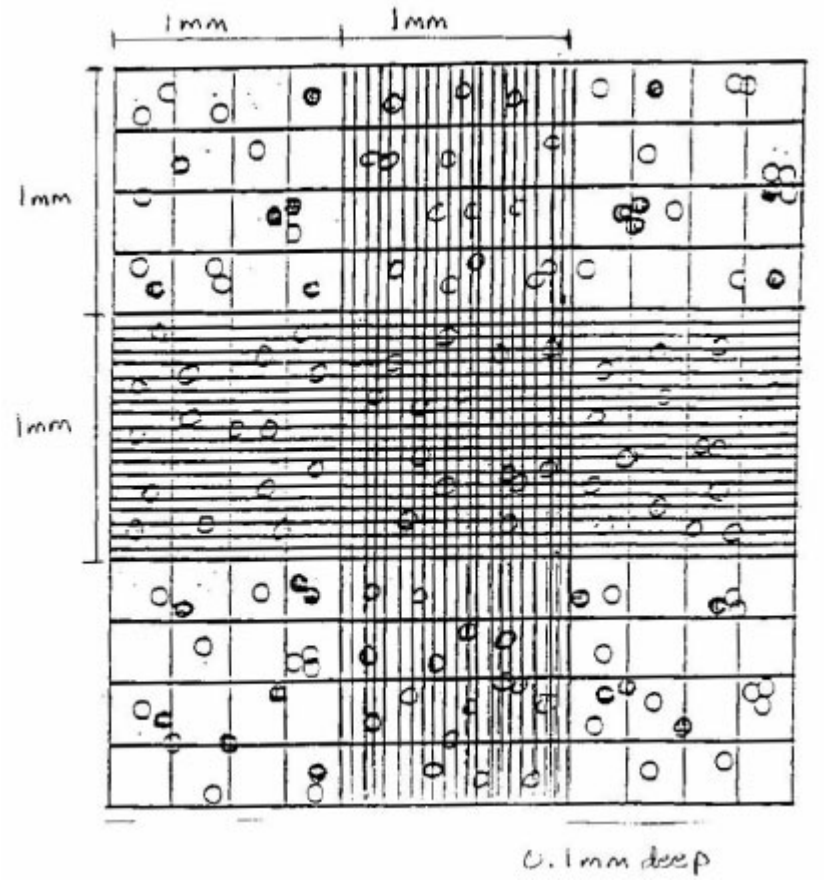

FIG. 1. Hemacytometer (improved Neubauer counting chamber).

B. Transfer of Cell Cultures

After cultured cells have formed a confluent monolayer on the surface of their culture vessel, they may be removed from the surface, diluted, and seeded into new vessels. If the initial culture was primary, the new cultures are called secondary and are likely to consist of fewer cell types. Removal of cells from glass or plastic surfaces may be by either physical methods—scraping with a sterile rubber policeman—or chemical methods—proteolytic enzymes or chelating agents—or a combination of the two. After removal, cells are pipetted up and down against the bottom of the flask to break up clumps, diluted, and counted. Primary cultures can usually be diluted 1:2 or 1:3 for secondary culturing, and after one becomes familiar with the growth characteristics of a certain cell type, counting can usually be dispensed with.

The same procedure can be used to transfer both primary cells and a continuous cell line, removing the cells from flasks with a mixture of trypsin and EDTA in physiological saline (STE stands for saline, trypsin, EDTA).

Procedure

1. Examine the cells growing in a 25-cm^2 flask with an inverted microscope to see if they have formed a confluent cell monolayer. If there are sufficient cells, pour off the medium.

2. Wash the monolayer with 2 ml of Saline A. Rinse well without shaking (shaking produces bubbles) and pour off. Repeat.

3. Add 1.0 ml of STE to the flask and incubate at 37°C for 2 to 10 minutes with STE covering the cells. Observe periodically to determine when cells are loosened from plastic. (Note: STE will contain a pH indicator and should have a pH of 7.0 to 8.0. Below pH 7.0 trypsin is inactive. A pH above 8.0 is damaging to cells.)

4. When cells are seen to detach from the surface upon shaking or jarring against the heel of your hand (you can check in the microscope), add 4 ml of fresh growth medium with 10% serum and suspend cells by pipetting up and down a few times. Count cells in a hemacytometer, calculate the volume of additional medium needed to bring the cell concentration to ~$5x10^5$ cells/ml, and add this volume to the cell suspension.

5. Seed an appropriate volume for the size of flask, or 1 ml of cell suspension into each well of a 24-well cluster dish, or 5 ml of cell suspension into a new 25-cm^2 plastic flask.

C. Preservation of Cultured Cells by Freezing

Viability of viruses and bacteria is preserved during freezing, but original attempts to preserve animal cells by freezing resulted in cell death. This was first thought to be due to laceration of cell plasma membranes by ice crystals, but more recent evidence suggests the cause may be osmotic changes during freezing which give rise to irreversible changes in lipoprotein complexes in intracellular membranes. In any event, the answer to animal cell preservation has proved to be the addition of glycerol, ethylene glycol, or dimethyl sulfoxide (DMSO) to the medium and slow freezing, ideally at a cooling rate of one Celsius degree per minute. Cells must be stored at -70°C or lower (ideally in liquid nitrogen at -196°C), and when they are recovered, thawing must be rapid. With careful technique, 50 to 80% of the cells of a healthy culture will survive freezing.

Procedure

1. Remove the confluent cell monolayer from the culture flask by the method described in the cell transfer procedure. Suspend cells in added MEM, transfer to tubes containing fetal bovine serum, and centrifuge at 1,500 rpm for 5 to 10 minutes. After centrifugation, resuspend cells in 0.5 ml of cold medium containing 10% serum and 10% DMSO and place in a small cryotube.

2. Immediately place tubes in an ice bath. They will then be transferred to an insulated container and cooled to –20°C at a rate of -1°C per minute (if you do not own such a container, cells can be stored on ice for 1/2 hour then moved to a -70°C freezer in a box with lots of Kimwipes around the tubes), after a few days these cells can be moved from -70°C to liquid nitrogen for permanent storage. Cells stored at -70°C will not remain viable as long as cells stored in liquid nitrogen. If cells are to be stored in liquid nitrogen, they must be placed in sealed ampules or cryotubes.

3. To recover cells, remove tubes from -70°C or liquid nitrogen and place directly in 37°C water bath. Note: liquid nitrogen can cause burns, therefore, care should be taken when handling liquid nitrogen and tubes should be held in your fingers for as brief a period of time as is possible. When thawing is barely complete, add contents of the tube to a 25-cm^2 flask containing 5 ml of medium with 10% serum. The culture medium should be changed approximately 4 hours later (after cells have attached) to minimize the time of exposure to DMSO at 37°C.

Formulations of Media and Solutions

Saline A

Ingredient	g/l
NaCl	8.0
KCl	0.4
$NaHCO_3$	0.35
Glucose	1.0
Phenol red	0.05

Add distilled H_2O to 1 liter. Filter sterilize. Saline A is usually prepared as a 10X solution and stored at -20°C.

Saline-Trypsin-EDTA (STE)

Ingredient	g/l
Trypsin (1:250)	0.5
EDTA (disodium salt)	0.2
NaCl	8.0
KCl	0.4
$NaHCO_3$	0.35
Glucose	1.0

Phenol red	0.05

Add distilled H_2O to 1 liter. Dissolve trypsin first by slowly stirring in ~200 ml H_2O (avoid denaturation). Add remaining ingredients and filter sterilize. STE is usually prepared as a 10X solution and stored at -20°C.

Dulbecco's Phosphate Buffered Saline (PBS)

Ingredient	g/l
$CaCl_2.2H_2O$	0.133
$MgCl_2.6H_2O$	0.1
KCl	0.2
KH_2PO_4	0.2
NaCl	8.0
Na_2HPO_4	1.15

Add distilled H_2O to 1 liter. Osmolality (mOsm/kg H_2O) = 289 $\pm$ 5%. pH = 7.3 $\pm$ 0.3.
This saline may be made without Ca^{+2} and Mg^{+2} salts for washing cells.

Minimum Essential Medium Eagle (MEM)

Ingredient	g/l
$CaCl_2.H_2O$	0.265
$MgSO_4$	0.09767
KCl	0.4
NaCl	6.8
NaH_2PO_4	0.122
L-arginine.HCl	0.126
L-cystine.HCl	0.0313
L-glutamine	0.292
L-histidine.HCl.H_2O	0.042
L-isoleucine	0.052
L-leucine	0.052

L-lysine.HCl	0.0725
L-methionine	0.015
L-phenylalanine	0.032
L-threonine	0.048
L-tryptophan	0.010
L-tyrosine 2Na.2H_2O	0.0519
L-valine	0.001
Folic acid	0.001
myo-Inositol	0.002
Niacinamide	0.001
D-Pantothenic acid (calcium)	0.001
Pyridoxal.HCl	0.001
Riboflavin	0.0001
Thiamine.HCl	0.001
Glucose	1.0
Phenol red	0.011
$NaHCO_3$	2.2

Add distilled H_2O to 1 liter. Filter sterilize. Minimum essential medium Eagle is usually purchased as a preweighed mixture or as a sterile solution. The above formulation has Earle's salts.

SAFETY

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the ASM Curriculum Recommendations: Introductory Course in Microbiology and the Guidelines for Biosafety in Teaching Laboratories.

COMMENTS AND TIPS

This section is to evolve as feedback on the protocol is discussed at ASMCUE. Please contact the project manager for further information.

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