Cell Culturing Techniques

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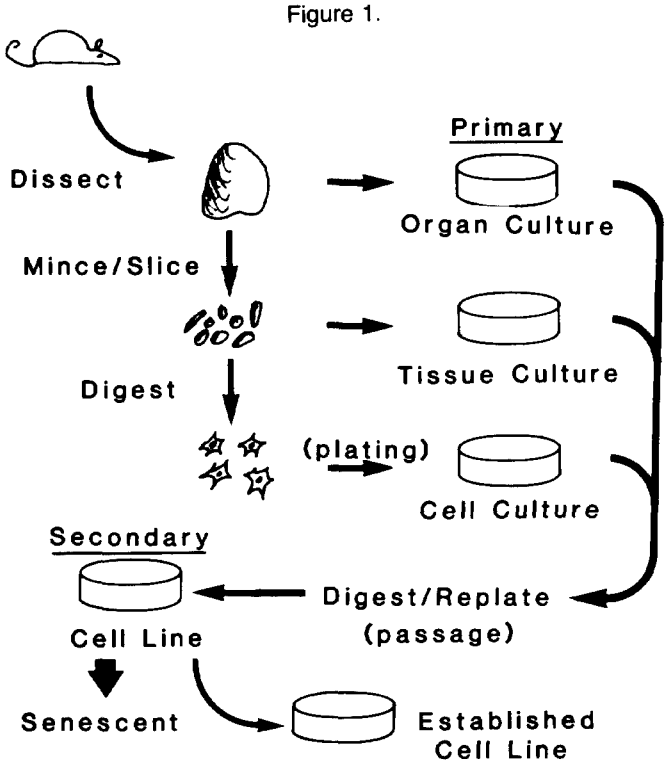
We plan to present a general overview of cell culturing. The goal is to discuss some of the definitions and terminology of cell culturing, fundamental equipment needs, as well as cell characteristics, and to compare the advantages and disadvantages of primary and cell line cultures. We will discuss some of the research questions that can be asked with cell culturing, as well as some of the problems incurred and the decisions that have to be made when one performs experiments with cell culturing. Hopefully, two things will be accomplished. For those of you who are not very familiar with cell culturing, we will give you an idea of the basics of cell culturing and stimulate interest, as well as questions. And for those of you who are experienced, maybe we will jog your memory and help you recall some details that can be shared with others. In addition, you may wish to identify points that need more detailed discussion or clarification.

Definitions and Terminology

First of all, some terminology must be established as there are a variety of in vitro approaches and corresponding terms. This will enable us to take a quick view of what cell culturing is about. Basically, no matter what type of cell or tissue culture you use, all cells come from an animal at some point. Invariably, some organ, such as muscle, heart, lung, or adipose tissue, is dissected out of the animal. This is illustrated in Figure 1. If you take the intact organ, place it in a culture dish or vessel, and provide for maintenance or preservation of the structural integrity and/or function, it is referred to as an *organ culture*. Organ cultures are not particularly new. People have been studying them since the 1870's. In this sense, tissue culture is not a modern science but has been around for a long time. When we take tissue, cells or organs out of the animal and put them directly in culture, it is referred to as a *primary culture*. These cultures are called primary until they are subcultured. Instead of culturing the whole organ, one could chop it up into pieces and culture those pieces. This has two advantages. The pieces are small enough (about 2 mm) to get diffusion of nutrients and gases from medium, and the architecture of the particular cell types and organization you might see in the original organ is still maintained. If one maintains the tissue structure, it is referred to as a *tissue culture*. These terms are loosely used. There are some suggested guidelines (Schaeffer, 1978) but many people refer to this as explant culture, not just tissue culture. They refer to tissue cultures as everything in general. Finally, one can digest the tissue chunks to isolate cells. One then places the cells in a culture dish, a process called *plating*, and the result is a cell culture. All three of these types of cultures (organ, tissue and cell) are called primaries. This is basically what most of us do with rats, chicks and pigs. If one does not want to go through the process of using a live animal every time, and prefers to work only with cells, one can take any of these primary organs, tissues or cell cultures and digest cells out of that culture. One uses the same enzymes (i.e., trypsin) that are used to digest tissue and to isolate cells. When these newly-digested primary cells are plated in another culture dish, it is referred to as a *passage*.

Figure 1.



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Once this passage has been performed, the result is called a *secondary culture*. Secondary cultures will grow just like a primary culture and the passing process can be performed repeatedly. Cells in culture behave much like cells in animals. As animals grow old, cells become senescent. It takes longer for wounds to heal and broken bones to mend. If one takes primary cultures from animals at different ages, such as a very old animal relative to a very young one, the cultured cells from the older animal divide less rapidly. In the same way, most secondary lines eventually become senescent. They may lose their ability to divide and eventually will die. On the other hand, as you go through the cycle of plating, digesting and replating, a small percentage of those secondary cell lines will eventually adapt themselves. Different genes are expressed and through selection, a small population of cells becomes established. These cells are called a *continuous* cell line. In essence, this means these cells become immortal, they are never going to die, and they can continue to be passed "forever." Some of the early cell lines that were established this way have now been passed in culture for over 35 years.

Here are a few more points about specific types of cultures. The process of establishing secondary cell lines is normally done with a large number of digested cells. This is referred to as *mass culture* because it is a mass of cells. Cloning is the process of taking a single cell or a small number of cells and allowing them to grow until they are numerous. One can also do this with established cell lines, and many cell lines may have some clones that have characteristics different than other clones.

Cell Characteristics and Selection

Now that I have established a rough idea of how cell culturing can be performed, let us run through some of the types of uses for cell culture. For the purpose of today's discussion, these uses have been broken into four categories: studies of cell growth and differentiation, cell metabolism, bioproduct production and bioassays.

Traditional or historical uses of cell culturing have involved the study of various aspects of cellular growth or cellular differentiation. Clearly, the big advantage of cell culturing over any other sort of technique is that we can take these cells out of the animal where the animal's regulatory systems are influencing metabolite or hormone levels. With cultured cells, metabolites or hormones can easily be manipulated to determine what effects they have on cellular growth and differentiation. The fact that the cellular environment can be readily controlled in culture also makes cell culturing useful for studying cell metabolism. For instance, it is hard to study steroid metabolism in adipose tissue because only certain cell types in adipose tissue synthesize steroids. In culture, these cells can be isolated and studied.

Another broader category for uses for cell culturing may be called bioproduction. These are not recombinant techniques where bacterial cultures are used, but could be hybridoma cultures which produce antibodies or other by-products. For example, the Buffalo rat liver (BRL) cell line produces a rat somatomedin.

The fourth category involves the distinction between using cell culture and in vitro incubation of tissues as a bioassay. The distinctions are somewhat fuzzy. Cell culturing bioassays are performed just like any other bioassay, where one exposes cells to various compounds, hormones or nutrients to assay cellular responses. The only difference from using pieces of tissue, such as slices of cartilage, is that one is able to maintain cultured cells for a longer time. Additionally, culture conditions are more favorable for growth and division as opposed to just maintenance of static conditions.

Basic Equipment and Materials

Let's take a quick run through some of the types of equipment used in cell culturing. It is similar to equipment used in many other aspects of the biological sciences with a few special modifications here and there.

First of all, you need an incubator. An incubator is used to maintain physiological temperatures for the cells. Basically, it maintains the temperature at 37°C. For cell-dependent parasites, it would be lower. Most tissue culture incubators have two additional features. They usually have some capability for maintaining humidity. There may be a heating element that introduces steam into the chamber, or it could be a large pan of water. They also have a facility to maintain the appropriate gaseous environment. Anywhere from 2% to 10% carbon dioxide in air is used in most culture incubators. The reason for this is that the majority of tissue culture media contain a bicarbonate buffer system and the high level of carbon dioxide is necessary to maintain the pH of the media. There are some buffer systems which forego the need for CO_2.

Microscopes are also essential any time one is working with a cell or tissue culture, in order to observe what the cells are doing and to evaluate the system. Typically, in cell culturing, inverted microscopes (where the objective is on the bottom) are used. This facilitates the examination of the culture flask, petri plate or other culture vessel.

Autoclaves are a basic necessity to sterilize instruments, supplies and glassware. There are special autoclaves made for tissue culturing, primarily for those people doing secondary culturing where the cells are a little more sensitive to water contaminants. These autoclaves use distilled or purified water as a steam source, rather than using line steam. Most line steam has anticorrosive additives which are toxic to cells.

Laminar-flow hoods are not an absolute requirement. However, these hoods are helpful to keep a sterile environment to work in. There are two types of laminar-flow hoods: The vertical flow, where filtered air comes down from the top and is basically recirculated; and the horizontal hood where the sterile air is just blown over the work space out into the room. The difference is that many biological safety committees will no longer approve the horizontal-flow hood because if you use tumor cells, virus or bacterially transformed cells, the operator is not protected satisfactorily.

The next item on the list is disposable sterile supplies, and this covers a wide spectrum from pipettes to centrifuge tubes, all of which can be purchased in sterile form. Culture plates come in all varieties and sizes – small petri plates to gigantic petri plates, square petri plates made to take microscope cover glasses, and multi-well plates are also available. Plates made of plastics have one distinction from microbiological

plates in that they have been treated so the cells attach. Mammalian cells do not normally attach very well to plastic or glass, so the surface must be cleaned or treated with something, such as collagen or polylysine. In the case of plastic, it has been exposed to a static discharge.

The last item on the list of essential equipment and materials is tissue culture medium. Initially, I indicated that one of the advantages of tissue culture was that one can maintain cells in an environment that is constant, and then one can manipulate hormones or metabolites. Therefore, tissue culture medium is very important in these manipulations. Basically, medium components can be divided into three groups: basal medium, serum and antibiotics. Basal medium has been developed to provide physiological salts; usually sodium chloride, magnesium, calcium and the ions the cells need to survive and grow. Media also contain buffers, and as was mentioned for incubators, the classic buffer is the bicarbonate (CO_2) buffer system. This is not to say that it is the best one; in fact, it probably is not a very good one. There are a number of modern media designed to work in an air environment, and there is no physiological reason to expose cells to 5% carbon dioxide. If you had 5% carbon dioxide in your blood, you would be in pretty tough shape! Basal medium also provides amino acids. Depending on the medium, one can have the full spectrum of amino acids or simply the essential amino acids or somewhere in between. Basal media provide an energy source, typically glucose, and some have pyruvate or galactose. Obviously, for cells to grow, media must also provide some source of nucleic acids and vitamins to serve as cofactors. There are as many basal medium formulations as there are people in this room. Everyone who uses cultures has their own favorite formulation that works best for them. In addition to basal media, primary cultures almost invariably require serum in the medium. The earliest attempts at tissue culturing included only serum or clotted plasma as a culture medium. Cells were put into chilled plasma and allowed to clot. It was difficult to change media with the clotted plasma, but this is the way it was done in the 1890's.

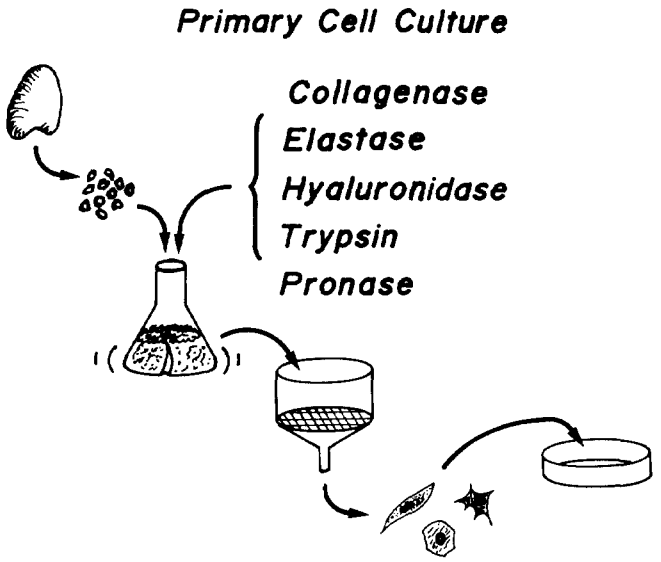
People have spent a lot of effort on evaluating what components serum contributes that have not been put into the basal medium. There are few answers as it is still seldom possible to grow a primary cell culture by replacing serum with any combination of components. Serum provides a number of things, such as various transfer proteins, i.e., transferrin for iron, etc., growth factors (FGF, EGF), insulin, T3, steroids and other hormones. One thing that serum appears to provide but that is not an essential component of serum activity is lipid. Most cells replicate faster if they have a lipid source to allow for membrane synthesis or for energy supply. Serum also provides attachment factors which allow cells to attach themselves to the culture dish, as well as some inhibitors of the proteolytic enzymes used when one has digested the cells in the first place.

There is another category not usually thought of as a medium component, but for people using primary cultures, antibiotics are important. Because most animals are heavily contaminated with microorganisms, it is difficult to grow primary cultures without antibiotics because one has to get tissue, cells or organs out of the animal and keep them in culture for a long period. All those good things in the media, such as buffers, amino acids, glucose and serum, make a terrific bacterial culture medium. If bacteria, mycoplasm or molds start to grow in the culture, they take over very fast. It is important to consider the antibiotics. Those traditionally used are penicillin and streptomyocin, but people are moving away from those as most of the bacteria these days are resistant to penicillin and streptomycin. Better choices include second or third generation β-lactamase resistant antibiotics or ones that may have some anti-mycoplasm activity, such as gentamicin.

Advantages and Disadvantages of Primary and Cell Line Cultures

Let us now look at some of the advantages and disadvantages of growing primary cultures, secondary cultures or established cell-line cultures. A primary cell culture is where one takes the tissue out and chops it up and digests it. This is illustrated in Figure 2. The digestion process is problematical, depending on what kind of cell is desired out of the tissue. Appropriate digestion conditions have to be established. The more commonly used enzymes are collagenase, elastase, hyaluronidase, trypsin, pronase and many others. Most of these are used in a physiological buffer such as Krebs' Ringer Bicarbonate, HEPES or PBS, to name the favorites. For most of these enzymes, a milligram or two per ml is used. Enzymes and tissue pieces are put in the buffer and incubated in a shaking water bath to digest the pieces. After digestion, there are dead cells, chunks of tissue that are not digested, and all these enzymes, as well as the cells of interest. In order to harvest the cells, some sort of screening and/or centrifugation is used, followed by media washes to remove the enzymes. Once the cells are obtained, there is still a big problem. There are not too many organs or tissues which have only one cell type. At the very least, with the exception of cartilage or eye lens, there will be some fibroblasts and connective tissue cells, smooth muscle cells from arterioles and venules and endothelial cells that line the capillaries, as well as the cells of primary interest. Therefore,

Figure 2.



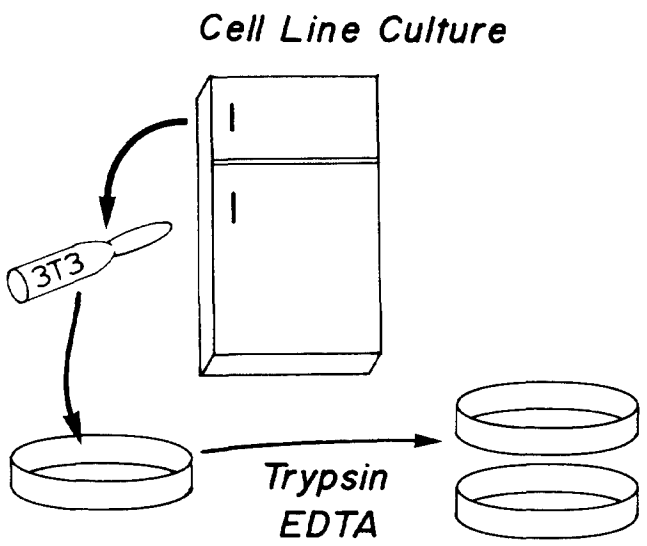
before the plating process, one has to do something to get rid of the unwanted cells. Getting rid of endothelial or smooth muscle cells, which don't grow very well, is no real problem. Fibroblasts create a real challenge, particularly if something like a preadipocyte that behaves similarly to a fibroblast is desired.

Table 1. Advantages and Disadvantages of Primary Cultures

Major Advantages	*Major Disadvantages*
Similar to tissues *in vivo*	Complicated preparation
	Heterogenous cells
Available from "any" source	Variation between batches
	Serum requirement
	Microbial contamination

The advantages and disadvantages of primary cultures are listed in Table 1. A major advantage of primary cultures is that since the cells came right out of the animal, one assumes these cells are very similar to cells in the animal. One assumes that the hormonal responsiveness and metabolic characteristics of these cells will be similar to those in the animal. There will not have been enough time for these cells to change or adapt. Similarly, since these cells are from an animal, cells are available from any place. You can make a western Kentucky gopher cheek-pouch cell culture if you want to. Counterbalancing these advantages, there are some serious disadvantages. As already indicated, preparation is quite complicated. If cells have a finite lifespan, primary cultures may grow for only a couple of weeks. Therefore, the preparation process must be carried out all the time. It is a big headache. A lot of enzymes are used and it gets to be expensive. By the same token, there is a heterogenous mix of cells. There will be the desired cell, as well as other cell types (garbage). One has to do something to get rid of the other cell types or get around the heterogeneity in some way. Because the cells are similar to tissue in the animal, one also has the same kind of variation you see between animals. Variations in cultures derived from different animals may be fairly large. This is the same type of variation we all see when we work with animals, and it does not go away by putting the cells in culture. Additionally, with few exceptions, primary cultures cannot be made without some serum. In using cultures as bioassays or where growth-promoting activity is being evaluated, addition of serum makes things a little hard because of all the strange things in it. It would be nice if every component in the medium was known. If so, there would not be the worry about all the strange proteins and growth factors and interactions that can occur with what you are interested in measuring. There is also great microbial contamination potential because one takes the cells out of the animal. Lab rats are famous for their mycoplasm infections that are difficult to get rid of.

Figure 3.



In contrast to primary cultures, cell line culture preparations are very easy. As illustrated in Figure 3, one can go to the freezer, take out a vial, thaw it, break the vial open and be ready to culture. If one is very slow, the whole process may take 10 minutes. To pass a cell line culture and make a number of dishes instead of one dish, a culture is digested with trypsin and/or EDTA, the cells come off and they can be replated in more flasks. Since the cells have been adapted to this enzymatic treatment during establishment in culture, they grow well under these conditions. Basically, in established cell lines, you have the big advantage (Table 2) of easy preparation. If you are growing a lot of cultures, this advantage can be enormous. Compare the 10 minutes here to the four hours required for establishing a primary adipocyte culture. The savings in time alone is tremendous. Because cell lines may be cloned, one has a very uniform cell population. All cells in the dish are similar. There is no longer the fibroblast and endothelial contamination problem. Similarly, a vial out of the freezer today is essentially the same as the vial taken out next week. Batch A is nearly identical to Batch B. Consequently, variation errors between assays are very small. There are a fair number of different cell lines that one can culture without serum. Typically, cells are passed into

Table 2. Advantages and Disadvantages of Established Cell Lines

Major Advantages	*Major Disadvantages*
Easy preparation	Artifacts caused by adaptation
Uniform population	Susceptible to genetic drift
Consistent batches	Behavior may not reflect *in vivo*
Serum free media	
Well characterized	

serum-containing media and then maintained in serum-free culture for several weeks. In growth and development studies, the use of serum-free medium has a distinct advantage.

Unfortunately, established cell lines have one big disadvantage. During the process of establishing these cells in culture, they adapt, possibly through gene mutation, and they may do some strange things. They may have some artifacts and the cells may not be like the cells they are supposed to reflect in the animal. A classic example is the 3T3 cell which is an adipocyte model. This was derived from a fibroblast clone. During the process of adaptation, the fibroblast developed a surface antigen that is a marker for endothelial cells. This fibroblast behaves a little bit like an endothelial cell. Things can get a bit confused, particularly for cells that have been transformed by a virus. They are not even normal cells any more, but tumorigenic cells in culture. By the same token, with some judicious care and selection, an established cell culture can be secured that will do what is desired.

Discussion

Question: Could you give me an example of an organ or tissue culture as illustrated in your first figure?

Novakofski: An example of an organ culture is cornea culture, but there are a number of others. The intact cornea of the eye can be placed in culture. An example of tissue culture would be skin culture. If you think of skin as being an organ covering the body, an organ culture for skin cannot be done. A piece of the skin can be cultured so that it maintains the same architecture that it has in the organ. In other words, that piece of skin in culture will maintain a skin structure.

Question: This may be a matter of semantics, but can you call this an isolated organ preparation?

Novakofski: Yes, you can. People call these all sorts of things. There is nothing binding in any journal publication. People typically use what they like. It doesn't really matter except what the function is, regardless of the semantic question. If you want to look at the entire organ, then you have to do an organ culture. If you want to look at some other behavior, it is just tissue.

Question: In my kind of business, we perfuse organs. Is this considered a tissue culture?

Novakofski: If it is a situation where conditions allow cells to divide, and cells are not losing proteins, than it would be a tissue culture. For example, in a heart preparation, if you could induce some fibroblast proliferation, and if the tissue is maintained in some positive nitrogen balance, it would be a tissue culture. However, if you are just maintaining cells at zero nitrogen balance or zero division potential, it would be called an incubation. There are a number of literature references that interchange terms. For instance, in adipose tissue cultures where cells are in culture for 12 hours, a more appropriate term is incubation. This is where the terms are difficult to categorize.

Question: What determines whether cells in secondary cultures become senescent or continue to grow in culture?

Novakofski: The answer is probably a random mutation. You can enhance the process by putting in carcinogens or mutagens – anything which will make the cells mutate and adapt. By the same token, if you want to establish a culture, a handy way of starting out to make an established culture line is to initiate the primary culture from a tumor. A large number of pituitary cell lines which secrete growth hormone and respond to GHRF or TRH were established from either naturally occurring or induced pituitary tumors.

Question: Did you say how you got rid of enzymes?

Novakofski: There are two ways to get rid of them. The easiest way is to add serum because it contains serine protease inhibitors which stop enzyme activity. Another way is to centrifuge the cells down, and remove the medium supernatant which contains the enzymes, then resuspend the cells in fresh medium. This does not always work because some cells do not go to the bottom of the tube. For example, adipocytes go to the top and you can never get rid of all the enzyme mixture. Serum must be added in this case.

Question: Is cell culture limited to eukaryotes?

Novakofski: I am not sure. It is the way I use the term. I would call cell culture of a prokaryote a bacterial culture but this may be an elitist attitude.

Response: I object! A prokaryote is a cell that does not exhibit a classical mitosis, therefore virus and bacteria are prokaryotic. Eukaryotes are cells that do exhibit mitosis and they are the cells of plants and animals. Most of the cells that are carried in tissue culture are cancer cells that are nucleated and reproduced. They obviously were derived from eukaryotic sources.

Question: I never understand the nomenclature of these cells. I see papers in journals and they all have fancy names with numbers and symbols and initials and Greek letters and Roman letters and I am confused by all this. Would you explain?

Answer: There is no rational basis for all of these names. Nothing is quite so handy as the Latin names for bacteria. What it boils down to is that there is no standardization. If I were to go out and make a cell line such as the gopher cheek-pouch cell line, I might call that the REA cell line in honor of Ron Allen or the REA-CP cells for Ron Allen cheek-pouch cells. This would be just fine. If I started using these cells and used this nomenclature, that is what they would be called. Some people have used some rationale. For example, the 3T3 cells were named for the way the cell line was established. Cells were split in a 1 to 3 ratio every third day. Unfortunately, most cells are not named this way.

Response: It took me several years to learn what Hela cells were.

Novakofski: Hela cells stand for Helen Lane.

Response: Her cervix is immortal because it is a cancer cell.

Comment: Not all these cells are tumor cells. There are a large number of lines that are not. The difference is typically what is referred to as transformed- or nontransformed-cells. The distinction in culture is that the transformed-cell line may be tumorgenic in animals and it does not usually exhibit the density-dependent inhibition of growth or the contact requirement for growth. The cell may grow in a suspension culture. 3T3 cells and a large number of others are normal cells in that one can place them in nude mice and they will not form