

# **CELL CULTURE LABORATORY EXERCISES**

Thomas B. Shea  
Maya Dubey  
Valerie Graves

Center for Cellular Neurobiology and Neurodegeneration Research  
UMass • Lowell  
Lowell, MA



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## FOR THE INSTRUCTOR, BUT ALSO USEFUL FOR STUDENTS...

Once considered a somewhat esoteric, and by some, a useless approach that had no relevance to *in situ* physiological processes, cultivation of cells has pervaded cell biology, developmental biology, immunology, biochemistry, and molecular biology. Over a decade ago, we established a separate course in Cell Culture Techniques in order to prepare our students for the ever-expanding biotechnology industry. Industry response to this course has been universally enthusiastic, and our former students routinely report that their experience in this course helped them secure a position and/or prepared them well for their new duties.

The exercises are designed for a 4-hour laboratory session that meets once per week for a single semester. We have run up to three separate laboratory sections during the week. A lecture component accompanies this laboratory, and, unlike most advanced biology classes, the lecture really accompanies the laboratory, rather than the laboratory accompanying the lecture. We suggest using a basic cell culture textbook, such as *Culture of Animal Cells* (Freshney, 2005, published by Wiley & Sons; [www.mrw.interscience.wiley.com/freshney](http://www.mrw.interscience.wiley.com/freshney)), and we also utilize key readings from current journal articles to highlight each technique. The texts provide a good basic understanding of techniques, while recently-published articles demonstrate contemporary applications. We prompt students to critique the articles, devise additional controls, and suggest follow-up experiments, in an attempt to mimic what might occur during a weekly section meeting in a biotechnology company. The lecture as we present it is by no means meant to serve as a complete cell biology class; this is offered separately at our institution. However, in institutions where the curriculum is already compacted, certain, if not all, of these exercises could be utilized as part of a more general advanced cell biology laboratory.

Students are required to maintain a standard industry-grade laboratory notebook, which consists of a hand-written “real time” record of what was done in the laboratory during each session, signed and dated. Protocols, in the students’ own words, are prepared separately, prior to entering the laboratory (and are routinely examined by the laboratory instructor as a criterion for admission on a given day). The laboratory notebook is written in past-tense, as each operation is performed. Any alterations require a single line drawn through any “deleted” text, with annotations referred to and dated. The laboratory notebook is subject to examination at any time, and is collected twice during the semester – once for overall evaluation, with suggestions and an unofficial grade given at the midpoint, and on the last day of classes, with a final grade assigned. Reports are required for some, but not all, laboratories. We stress that reports differ dramatically from the notebook, and they are required to be presented in journal-article style.

Each exercise as presented herein lists what we have found is needed for students, followed by basic materials and hints that, while good for students to see, are important for the individual prepping the exercise. We of course suggest that you have extra materials on hand. Students are also expected to use the minimum amount of laboratory consumables, i.e., pipettes. Therefore, they are to determine (prior to the lab) how many pipettes must be used in order to carry out the experiment without causing contamination, but without wasting equipment.

The exercises in this manual are designed to build upon each previous exercise, with the cumulative result being that students generate their own cultures for subsequent experiments, and derive samples for various technical analyses by harvesting cultures from earlier laboratories. For example, the first laboratory simply introduces the basic aspects of judging confluency of a culture (how thick the cell layer is), generating subcultures (“splitting” or “passing” culture), and feeding (replacement of media). However, the students generate their own cultures for the next laboratory, in which they study differentiation and serum requirements. Students learn to maintain their own ongoing stocks of cells from week to week, which is an inherent part of working in a cell cul-

ture facility. (We always maintain some back-up stocks for accidents, but, by-and-large, students do learn to keep their own cultures going.) It should be noted that, since most, if not all, cultures require medium replacement every three days, participants will have to plan to stop by the laboratory at least two additional times per week to change their cultures' media and, for some exercises, carry out passage of cells. Accordingly, our institution gives two college credits for this laboratory.

We typically have students working in pairs, which promotes cooperation and lets us report to prospective employers that student "X" worked well with others. We do stress that partners cannot readily take turns judging confluency or counting cell numbers, for example, on alternate days, without first taking steps to confirm that their techniques yield similar values.

An important lesson that should be learned during this and other "upper-division" laboratories is that one's experiments do not always turn out exactly as intended. We have included anticipated results within various exercises. However, everyone's hands are different: two people preparing the identical recipe for a cake do not necessarily produce the identical cake. Cells are living entities, and if you pipette them gently, they may survive and attach better than if you pipette them harshly. When your data differs dramatically or even slightly from the anticipated results, do not immediately conclude that you have made an error and trash the experiment. Your data is your data, and it is important to determine why things came out the way they did. Review your protocol and methods, and discuss them with your laboratory instructor. This is why it is critical to record things as you do them in your laboratory notebook; if you changed something by mistake, it will much more likely be recorded than if you brought in a protocol sheet and never recorded what you were actually doing. Sometimes useful information is derived from an unintended alteration in a procedure. Your laboratory report should then include what was altered, including if in error, and how you think this influenced your results. Unfortunately, there is often no time to repeat a laboratory exercise, due to scheduling. With that in mind, a laboratory report with an alteration, well-recorded and presented with data, and a strong discussion of what influenced the alteration and the outcome derived from it can still receive an excellent grade in our hands.

Cell culture utilizes relatively sophisticated equipment for its day-to-day operation. We have been fortunate to receive a substantial equipment grant from the National Science Foundation, which allowed us to purchase multiple Class II laminar flow hoods and inverted microscopes; we encourage other institutions to investigate this grant program, which is specifically designed to upgrade laboratories. Prior to this time, however, we successfully ran this laboratory using inexpensive "dead boxes," which are simple plexiglass/plastic enclosures containing a UV light for overnight sterilization of the enclosure; these are available via standard laboratory supply houses, such as Fisher or VWR. The front panel area was wiped with 70% alcohol before opening, after which the inner area was similarly wiped. Slow movement, and use of an alcohol flame (from a free-standing burner) maintained sterility in most cases. While we consider it important for students to have familiarity with laminar flow hoods and inverted microscopes, by contrast, in all of our exercises, we stress the most fundamental approach, using appropriate, although minimal, equipment. For example, we recommend usage of a hemocytometer for cell counting throughout the entire semester's exercises. While "Coulter counters" will automatically quantify cell numbers, we maintain that this approach is NOT appropriate for teaching basic cell culture techniques. This is analogous to teaching someone who has never cooked how to make salads using a food processor; a knife and a cutting board are analogous to a hemocytometer, while a food processor is analogous to a Coulter counter. Not all academic laboratories have a Coulter counter, and, even if they did, work should not come to a halt should that particular piece of equipment break down.

The exercises and approaches presented herein have served us, and our students, well. We are confident that they will do the same for you, but encourage you to adapt things as you see fit.

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## INTRODUCTION TO CELL CULTURE

The distinct advantage of cell culture is that we can examine isolated cells, which are exposed to no additional influences.

The biggest drawback of cell culture is that we examine isolated cells, which are exposed to no additional influences.

Why is the same feature both good and bad? Certain eukaryotic pathways could never have been disclosed without culture of cells, but one must keep in mind that cells do not behave in culture as they do in an intact animal, and are deprived of signaling and attachment factors that they normally receive from other cell types. In this section, we discuss several aspects of cell biology/physiology that impact cell culture. Rather than insert references for the following topics, we suggest that the reader conduct a search of his or her own, which can be directed more specifically toward any area of interest. Moreover, new references appear every day, so any that we suggest can rapidly be supplanted by a more relevant contemporary reference.

Cells are cultured in a complex mixture of sugars, amino acids, and salts. There are many different media types, some of which are generic, and will support many cell lines. There are also “best fit” lines for some more “selective” cell types. One can always find the appropriate medium and supplements (as follows) in journal articles. Simply start with what others have used successfully.

Animals and plants are composed of cells, but not as an unorganized bag of cells. Neither are organs individual “collections” of particular cell types; they are comprised of tissues. What is essential for tissue structure is polarity of the individual cells that comprise them. Polarity refers to cells orienting themselves, with up/down and left/right axes. When cells are essentially “tossed” in isolation in a culture dish, and no longer have their tissue organization, they can only partially restore polarity. This severely compromises any specific actions of a given cell type, and in a way, causes most cells to “revert” to a sort of generic phenotype. What does happen is that the cell can partially orient itself, with the plate being recognized as the basal lamina and the media being recognized as the lumen. However, the integrins of cells must attach to specific molecules in the extracellular matrix to invoke many intracellular pathways, many of which are cell-type specific.

With this in mind, researchers often coat the plate with extracellular matrix molecules prior to seeding cells. These include collagen, fibronectin, laminin, or various mixtures of these. A wealth of information is available in the literature on the best-fit for each cell type, and to invoke particular functions of given cell types. Commercial mixtures are available that have been refined over several years-to-decades of research. Many cell types, notably, simply will not adhere to plastic, and require some sort of coating. A more generic coating is poly-L-lysine, which assists in attachment of certain cells, but does not necessarily induce cell function in the same manner as extracellular matrix components.

We also enrich the medium by adding serum (which is the fluid of blood). Serum contains growth factors, attachment factors, helps neutralize cell wastes, and provides a bit of “thickness” to the medium, therefore, acting as a bit of a physical buffer. Cell types vary in their serum requirements. Fetal serum is richer in the above factors than is neonatal, which in turn is richer than adult. Some cells strictly require fetal; others can tolerate adult. However, fetal is expensive, and is therefore a significant cost burden to a smaller laboratory. Mixtures of fetal and neonatal are sometimes used. Fetal bovine serum is particularly rich and common. Adult horse serum from donor herds sometimes supports cells that will not tolerate calf serum other than fetal calf. Serum concentrations also vary: 10% is a typical concentration to stimulate growth. Lowering the serum concentration

to 2% is sometimes invoked for a “maintenance” situation, in which cells will survive and continue specific functions, but the stimulation for division is lessened.

Use of serum unfortunately leaves us with an “undefined” medium. Many serum-free formulations have been developed. The major constituents of most of these include insulin, transferrin, and selenium, which are then supplemented with a host of factors. Use of these can simplify a study of the influence of factor “x” on a cell type, since one can eliminate the potential or known influence of the host of factors in serum. In this regard, many cells, once established, can tolerate serum-free medium for a relatively short period of time for such an experiment.

It should be noted that if the goal of your research is to produce a product/factor for human use, serum presents a major problem. The simplest problem is that the high protein/peptide content of serum can easily exceed that of any secreted product of interest, and necessitates a series of purification steps that would not be required in the simple absence of serum. A larger problem is that serum, being an animal product, can by definition contain viruses or prions, the possibility of which can preclude human usage of any product generated in the presence of serum.

Cells also like to form cell-cell lateral associations (another aspect of polarity), which are mediated by cadherins and catenins. Such “handshaking” can be essential for survival and/or be important for studying various intracellular functions. Unfortunately, this requires near 100% confluency. This is readily established with a contact-inhibited cell line (one that ceases division when touching another cell on all sides), but is difficult if not impossible in a line that does not exhibit contact inhibition (since it will keep dividing and rapidly exhaust the medium). An additional approach to help cells establish this additional aspect of polarity is by the use of “conditioned medium.” This refers to collecting some medium from an established culture (typically relatively dense), and mixing it in with fresh medium for another (typically newer or less confluent) culture. This can mimic paracrine secretion, by essentially tricking the cells into thinking many more of them are present, and can lessen or eliminate the stress of passing cells or passing them more sparsely than they can tolerate. Conditioned medium can be obtained with every change of medium (and stored in the refrigerator) and/or decanted just prior to passage of a dense culture, and can be returned to the same cells once re-plated. One can also pass conditioned medium from cell type A to a culture of cell type B, to invoke cell-cell signaling. This can be used both for survival of cells in culture and to induce particular differentiation pathways. Note that conditioned medium, while potentially rich in factors secreted into it by the established culture, may be deficient in one or more serum factors (since they may have been consumed by the established culture). Combining it 1:1 (or any appropriate ratio) with fresh medium will help, but in addition it may be necessary to “spike” it with the amount of serum originally in the conditioned fraction of the final mixture.

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## SOME BASIC METHODOLOGIES

### *Passage of Cultures*

In laboratory jargon, passing cells = splitting cells = making new plates. You are unlikely to come across this in publications, but this is how various people refer to it.

When cells are approaching confluency, they must be moved from one vessel to another, usually with a reduction in density.

This can be accomplished simply by scraping cells from the plate, dispersing them into more medium, and dispensing them into more plates; in fact, this is the historical method of generating new plates. This method, however, yields uneven results in terms of generating new plates, since some cells remain in clumps, *but it is actually utilized to harvest cells for biochemical analyses*.

Trypsin is a digestive protease. It cannot penetrate the cell membrane, but will readily cleave surface proteins, including those by which the cell attaches to the plate. Cells will therefore detach from their culture surface. This is why it is not an ideal method for subsequent biochemical analyses, since this method has proteolytically altered the cell. Moreover, since it is likely that one will homogenize or otherwise disrupt all cells for biochemical analyses, it doesn't matter if they were "clumpy" upon removal from the plate.

Trypsin is typically provided in a calcium- and magnesium-free solution. This is because these cations help cells adhere to the extracellular matrix, and they will be depleted from the matrix when cells are bathed in a solution lacking them. Simple rinsing in a calcium- and magnesium-free solution is enough to detach some cells.

#### Basic Trypsinization Method:

The following volumes are for small (35 mm<sup>2</sup> plates). For 100 cm<sup>2</sup> plates, use 3 mL volumes.

- Remove medium. Tip the plate (more medium will collect at the bottom), wait a few seconds with the plate tilted, and you will see additional medium collect at the bottom. Decant the rest of this medium.
- Rinse with 1 mL or so of SERUM-FREE medium.
- Remove by the above method.
- Add 1 mL trypsin, swirl, and return plate to incubator for approx 5 min.
- Examine your plates under the microscope. Tap the edges of plate gently. To do this, hold it on the microscope stage with one hand, tap with the other, NOT hard enough to shake the microscope or cause the medium to "wave." This will help dislodge cells, and/or clarify whether they all have detached.
- When *most* of your cells have detached (not all will), add 1 mL medium with serum (the serum neutralizes trypsin). Pipette the medium up and down a few times to loosen more cells and dissociate cell clumps.
- Bring up the volume into enough medium to generate your desired number of new plates. Rather than place fresh medium into plates, then aliquot a small volume of cells into these plates, some (including we) prefer, when possible, to generate a "final solution" of cells and medium that can be dispensed directly into plates at one time. In either event, you must swirl your cell suspension often. Cells are like sand in water, and they settle rapidly. Thus, the last plates could receive many more cells than the first, etc.

Note that we never removed the trypsin above. This procedure assumes that you will be adding a substantial amount of new medium to your resuspended cells (e.g., 10-fold or more versus the original resuspended 2 mL). Should you only be generating 2 or 3 new plates, you must centrifuge

your resuspended cells (this only requires 5 min. at approx 1,500 rpm in a “table-top” centrifuge), then resuspend them in fresh medium. This is because resuspending 1 plate in a volume sufficient for just 2 or 3 plates will not provide sufficient serum to neutralize the remaining trypsin (and the desired final medium and serum concentration will be substantially diluted).

Alternatively, when you become familiar with your cells, and have a good idea of how long it will take for trypsin to detach them, you can carefully decant the trypsin mixture from your plates just prior to large-scale cell detachment, and then add serum-free medium. Gentle pipetting up and down will then dislodge the cells. We recommend utilizing the above method for students in their initial attempts at trypsinizing in order to avoid losing the entire culture while attempting to decant the trypsin.

In serum-free situations *soybean trypsin inhibitor* is utilized once the cells are loosened.

### ***Generating New Plates***

Following is an easy method, which we have termed the “plate equivalency” method:

*First, some simple considerations...*

- A small plate holds 2 mLs of medium.
- To generate, for example, 10 small plates, you need 20 mLs of medium with the correct amount of cells. To generate 20 plates, you would need 40 mL, etc.

If we had a plate that was 100% confluent, we could, once the cells were resuspended, make 10 plates at 10% confluency. These plates could then be incubated for x days (a 1:10 passage), or 20 plates at 5% confluency (a 1:20 passage).

Let's trypsinize a single plate at 100% confluency with the desire to make 10 plates at 10% confluency. During the course of this, we resuspended the plate in a total of 2 mLs of volume. So this = “1 resuspended plate.” And to make my desired plates, we need only disperse these 2 mLs of medium in the total amount of medium needed to generate my desired number of plates.

We could make 10 plates at 10% confluency based on the total number of cells we have. So, we would disperse the 2 mL resuspended cells into 18 mL medium, mix well, and dispense 2 mL each into 10 new plates.

If we only needed 5 plates at 10% confluency, we could do the same as above, and only dispense 2 mL each into 5 plates. The rest could be diluted further for STOCK cultures (maybe 1:50 or 1:100 dilution).

Avoid waste: You could have dispersed just 1 mL of the resuspended mixture into 9 mL medium, then generate only 5 plates. This becomes very important when dealing with 100 plates, or roller bottles, for example.

*Sometimes you want to generate plates of multiple densities when passing cells. Here's an example:*

Using same 100% confluent plate, resuspended in 2 mL:

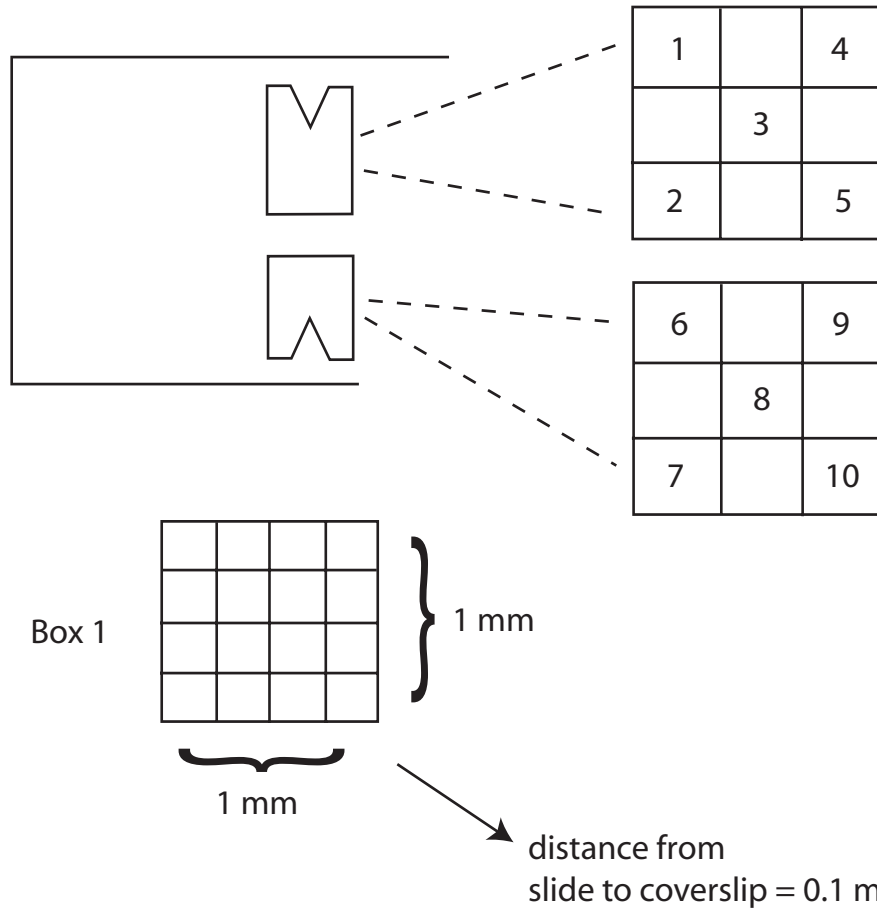
- Take 1 mL, dilute into 4 mL, dispense 2 mL each into 2 plates (a 1:4 passage).
- Take 1mL, dilute into 20 mL, dispense 2 mL each into 10 plates, then dilute the remaining 10 mL into 10 mL more medium, dispense 2 mL each into 20 plates (1:20 dilution).

### ***Counting of Cells via Hemacytometer***

Resuspend a plate as above, or disperse it. Take up about 0.3 mL of the mixture (swirl well first to evenly disperse cells).

Dispense one drop on each side of the hemacytometer at the edge of the coverslip.

Here's a diagram of a hemacytometer, with "higher mag" inserts. Compare it to an actual hemacytometer under the microscope for clarity.



The total volume occupied by each numbered box above is therefore  $0.1 \text{ mm}^3$ .

Count all 10 boxes from 1-10. *Do not be confused that each box is further subdivided: this is just to help you score cells.*

You *must* count between 30 and 300 cells for accuracy. So if you see wall-to-wall cells, make a 1:10 or 1:100 dilution of your original solution, and try again. If you only see a few cells, you have too little (sometimes this can't be avoided). The (SUM of all 10 boxes) x (any dilution factor) x 1000 = # cells/cm<sup>3</sup>, and since 1 cm<sup>3</sup>=1 mL, you now know the # cells/mL in your original sample.

You must perform this operation at least 2 times and average the SUMS of each trial, and 3 times is better than 2.

### ***When to Split and How to Seed***

The “% confluency” refers to how much of the visible area (under microscopic examination) is covered with cells versus how much is not.

“My cultures are 10% confluent” = only 10% of the available area has cells.

You can, and often will, work with confluency in general laboratory settings once you are familiar with the given cell type’s behavior. This supercedes the need for counting cells for every routine passage or on any given day. However, the surface area occupied by a cell often changes as the cultures become more dense. *Cells stretch out when sparse, pack in when dense, just like people on an elevator or subway car can pack in more densely when necessary.* Every cell type is different, and you will become used to the particulars of the cells you work with.

#### Considerations:

- 1) Each doubling increases the % confluency by a factor of 2. Once you know the growth rate of your cells, you can predict, just by confluency, when passage is desired, and when it is ESSENTIAL. Primary cells, or cell lines that have not transformed, will typically exhibit contact inhibition, while transformed cells will not. This dictates how often you MUST split your cells. Cells at 20% confluency, if they double in 24 hrs, will be 40% tomorrow, and 80% the next day. One more division will be greater than 100% (too many cells per plate; they will pile up on each other and exhaust the medium very quickly!).
- 2) Cells seeded too thin sometimes die, or exhibit a long lag time before they divide regularly. This is due to paracrine secretion (not always just among heterologous cell types). Use CONDITIONED MEDIUM from more dense cultures of the same cell type. Conditioned medium is medium that has been in contact with cells for a few days. You can dilute it 1:1 with fresh medium.
- 3) Note that routine passaging of cells too thin encourages the “drift” of cell lines, since it gives an advantage to variants that can divide faster, and these will, in time, overtake the total culture.

### ***Passage Number and Cryogenic Storage of Cells***

When you receive cells from another lab, or generate your own cell line, you must define passage numbers. “P0” is what you receive (or isolate from an animal). The next passage is P1. The next passage is P2, and so on. Get it?

Cells drift over time! PC12 cells were one of the first neuronal cell lines in the world. They were distributed widely; many labs kept their own stocks. MANY sublines evolved. This, in itself, is fine, as long as you are aware of it. But your laboratory’s results remain unapplicable to another laboratory if your line has drifted apart from theirs.

Cell lines also can die out, or a robust contamination can destroy your stocks. This is usually avoided by keeping stocks in cryogenic storage. This should be done as soon as possible. For this, cells are simply resuspended in medium containing 10% DMSO (which prevents ice crystal formation in membrane). Some laboratories use 100% serum mixed with DMSO; others use medium containing 10% serum.

The following is a brief scheme to allow long-term storage of a new cell line (which you start at P0) in your laboratory's cryogenic tank:

From your P0, make 10 P1 plates; grow to confluency.

- Freeze 8 of these.
- From the other 2 P1 plates; make 20 P2s; grow to confluency.
- Freeze 18 of these.
- From the other 2 P2 plates; make 20 P3s; grow to confluency.
- Freeze 18 of these.
- From the other 2 P3 plates; make 20 P4s; grow to confluency.
- Start your experiments; maintain some stocks at a lighter passage. Continue until you have reached around P10 or so, depending upon cell type.

#### *How Does This Pan Out?*

For example, the NB2a/d1 neuronal cell line “lasts” about 3 months when passed once per week at about 1:10 or 1:20. After this, it begins to spontaneously differentiate, rather than divide, and undergoes eventual senescence. Data obtained at these later times is spotty and ruins experiments on differentiation.

If I use a P4 plate, I can, based on the above observation, work for 3 months. Then, I need another P3 (which I can use to generate a bunch of P4s), and I can work for another 3 months. Therefore, 4 P3 plates will allow me to do constant work for 1 year.

I have 18 P3 plates in cryogenic storage. This means I can go for 3 x 18 months, which is a total of 54 months (4.5 years with no break). Then I need to take out one of my frozen P2 plates and start P3>P4 again.

I have 18 frozen P2 plates, which means I could work for 18 x 18 x 3 months (972 months, or 81 years). Then I need to take out one of the P1s....

In a way, I am the caretaker of the cells. This also allows me to share an early-passage stock (P2 or P3) readily with other laboratories, and in doing so, I advise them of the above scheme and discourage the generation of sublines. I can also share a P2 or P3 with other scientists. (Note that sharing of reagents, including cell lines, is a mandate of publishing in contemporary science.)

Along the lines of trying to avoid loss of cell lines, it is wise to store some vials in an alternate cryogenic tank, and some in an associate's laboratory.

Most cell lines are available from the ATCC, American Type Culture Collection, and if you generate a new one that ends up in demand, you should submit it to the ATCC, which will even provide you with another level of safety against cell loss.

#### A Few Thoughts about Transformed Cells

Transformed cells often remember “who they are.” Many stages accompany the change from a *totipotent stem cell* to a “committed” *stem cell*, to a *differentiated cell*. Errors in this process lead to unregulated tumors in situ, and to generation of a cell line in culture. This chain of events typically involves gene regulation (turning on and off sequential pathways), which is a major aspect of cell biological studies and forms the basis for much work in culture. For the most basic of cell culture considerations, however, transformed cell lines, like tumors, recall some aspect of their origin.

- *Myoblast* cell lines can be coaxed into expressing properties of mature muscle cells.
- *Neuroblasts* and *glioblasts* (or *neuroblastomas* and *glioblastomas*) can be coaxed into expressing properties of neurons and glia.
- *Hepatomas* can be coaxed into expressing some properties of mature liver cells.

Note that we state “some properties” since nothing in culture really represents truly mature cells. Nevertheless, you have the chance to cultivate millions of cells and induce them all to differentiate at once. This is very powerful for elucidation of biochemical pathways, such as transient aspects of signal transduction. *In situ*, it is unlikely that we can ever get this many cells to perform synchronously, and even if we could, we could not readily harvest them and detect transient molecules.