



## Methods for Maintaining Insect Cell Cultures

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### Abstract

Insect cell cultures are now commonly used in insect physiology, developmental biology, pathology, and molecular biology. As the field has advanced from methods development to a standard procedure, so has the diversity of scientists using the technique. This paper describes methods that are effective for maintaining various insect cell lines. The procedures are differentiated between loosely or non-attached cell strains, attached cell strains, and strongly adherent cell strains.

*Keywords:* subculture procedures; tissue culture; cell lines; trypsinization; suspension; attachment

### Abbreviation:

|             |                               |
|-------------|-------------------------------|
| FBS         | fetal bovine serum            |
| PBS         | phosphate buffered saline     |
| VMF trypsin | virus/mycoplasma-free trypsin |

### Introduction

In the early part of the 20<sup>th</sup> century, entomologists in several fields of study had a dream of utilizing insect cells grown in vitro as a tool. For example, Goldschmidt (1915) placed spermatocysts from the *Cecropia* moth into culture to observe the development of the spermatozoa while Glaser and Chapman (1912) studied the progression of wilt disease (caused by what is now known as a nucleopolyhedrovirus in the family *Baculoviridae*) in cultured hemocytes. These early experiments used a simple saline solution or hemolymph as the culture medium and cultures could rarely be kept for more than a few days. A breakthrough occurred four decades ago when Grace (1962) successfully established long-term cultures of insect cells. Since then, over 500 continuous cell lines have been established from over 100 insect species (Lynn, 1999). Insect pathologists have cells capable of replicating dozens of insect-specific viruses (Granados and McKenna, 1995) while plant pathologists and vertebrate pathologists have cells capable of replicating viruses transmitted by insects (for a variety of papers on vertebrate, invertebrate and plant viruses in insect cell cultures, see Mitsunashi, 1989). Physiologists have fat body cultures (Mitsunashi, 1983; 1984; Mitsunashi and Inoue, 1988; Lynn, et al., 1988; Philippe, 1982) and other cell types, including cultures with neural properties (Sheppard and Lynn, 1996), for studying cell signaling pathways while developmental biologists have imaginal discs cell lines (Lynn et al., 1982; Lynn and Oberlander, 1983; Ui et al., 1987) and myoblasts (Lynn and Hung, 1991; Inoue et al., 1991) for studying

differentiation. Since the development of the baculovirus expression vector system (Smith et al., 1983), hundreds of proteins of interest in medicine and biology have been produced in insect cells. Virtually every cell line is a potential or actual source of material for molecular biologists to use in a wide range of studies.

With this diversity of use also comes diversity in the level of knowledge individual scientists have for keeping cells in vitro. Many researchers can find it difficult to maintain healthy cultures of cells, but without a good source of healthy cells, the results obtained in any experiments will be suspect. In the following sections, I provide detailed but straightforward procedures to follow in maintaining continuous cultures of insect cell lines. I provide a few examples of cell lines for which each procedure is appropriate, but researchers working with a cell line for the first time should ask the person providing the cells which method is appropriate for the specific cell culture of interest.

### Materials

Supplies: Mature (late log or stationary phase) cell culture<sup>1</sup>, 25-cm<sup>2</sup> tissue culture flasks<sup>2</sup>, cell culture medium, 1, 2 and 5-ml

<sup>1</sup> A few insect cell lines are available from commercial suppliers or culture collections (such as the ATCC <http://www.atcc.org/>) but most insect cell culturists make their lines freely available to other scientists for research purposes. I strongly recommend attempting to obtain cells from the originating laboratory. If no cell lines exist for your specific application, Lynn (1996) describes methods for developing new insect cell cultures.

sterile pipets, sterile 200  $\mu$ l pipet tips, trypsin diluent (divalent cation-free PBS), VMF trypsin (0.05 mg/ml in diluent), sterile 10-ml test tube, 70% ethanol, trypan blue solution (0.4%).

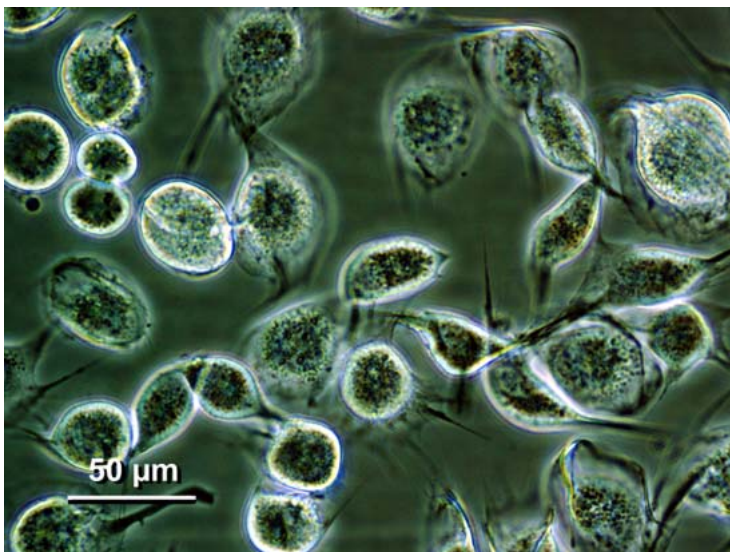
Equipment: Laminar flow hood or biological safety cabinet, inverted phase contrast microscope, mechanical pipetting device (either self-contained, battery powered or aquarium-pump type), refrigerated incubator, hemocytometer, compound microscope (optional).

## Methods

### Step 1 (for all cell types)

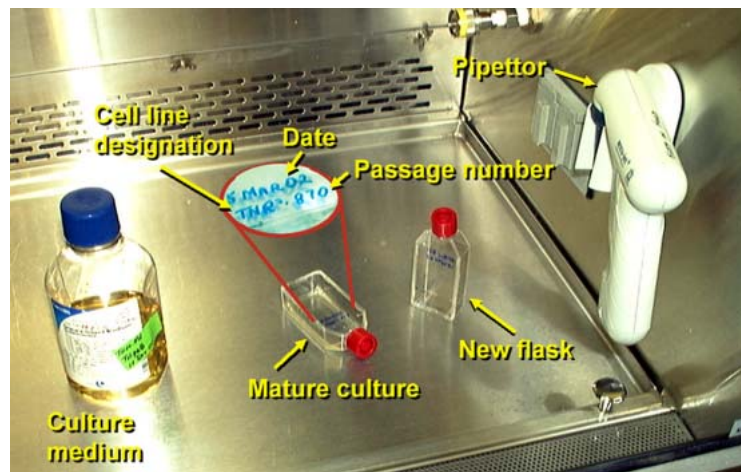
Preparation of hood, examination of culture and labeling of flask(s)

- A. Turn on laminar flow hood and wipe down working surface with ~2 ml 70% ethanol (keep a 100-200-ml squeeze bottle of ethanol next to the hood for this purpose).
- B. Remove mature cell culture from incubator and examine it with an inverted microscope with a 10 or 20X phase contrast objective. The medium in the culture should be relatively clear and cells should be somewhat refractive under the microscope (Fig. 1). (A very cloudy appearance, which makes it hard to see the cells with the microscope, suggests bacterial contamination. Such cultures should be autoclaved and discarded.)
- C. Record passage information in a record book. This information should include the date, the 'name' of the culture (cell line designation, passage level, culture ID), the amount to be transferred, and the type, amount and



**Figure 1.** *Trichoplusia ni* TN-368 cells; an example of a loosely attached cell line.

<sup>2</sup> A large number of suppliers now exist for tissue culture supplies and while there are certainly differences between different manufacturers, I have never seen growth effects that I could contribute to a brand of cultureware. Also, the procedures in this paper describe the use of 25-cm<sup>2</sup> culture flasks. The reader should be aware that other sizes exist, including smaller (12.5-cm<sup>2</sup>) and larger (75-cm<sup>2</sup>, 150-cm<sup>2</sup>, etc.) flasks which can be substituted, depending on the typical amounts of cells needed for the research program.



**Figure 2.** Contents of a biosafety cabinet during the subculture procedure. The only acceptable addition to the materials shown here are additional receptacles for additional cultures (i.e. more culture flasks, Petri dishes, or multi-well plates). The insert is a close-up of the culture flask showing the information that should be included on each new culture.

specific source of the culture medium to be used (that is, the individual bottle of medium should be identified in some way. I mark a bottle with the date when it is first opened and use this date as the identifier of the bottle). The amount of the old culture to be added to the new (the split ratio) varies with different cell lines. You should be given some indication of the proper split ratio by the source of the specific cell line but during the initial subcultures it is a good idea to use a range of split ratios.

- D. Label one or more new 25-cm<sup>2</sup> tissue culture flask(s) with the date, cell line designation, and passage number (Fig. 2). A fine tip permanent marker is useful for this purpose.

### Step 2. Transfer of cells

#### Method I. Nonattached and Loosely Attached Cell Types

(Fig. 1)

This procedure works well for loosely attached and nonattached cell cultures such as *Trichoplusia ni* TN-368, IAL-TND1, *Lymantria dispar* IPLB-LdFB, and *Mamestra brassica* IZD-MB0503.

- A. Place the bottle of fresh medium<sup>3</sup>, the mature culture and the labeled new culture flask(s) in the hood.<sup>4</sup> Loosen the caps on the medium and new culture flask(s). Take a new, sterile 5-ml pipet from the box. While holding it inside the hood, peel down the protective wrapper on the end containing the cotton plug 5 to 10 cm. Insert the plugged end into the mechanical pipettor and pull the wrapper the rest of

<sup>3</sup> Some researchers find it is beneficial to allow the culture medium to reach room temperature before using it for transferring cells. I personally have not found this to be an issue for the cultures I maintain and, in fact, I feel keeping the medium cold reduces degradation of components, but the reader should be aware of these different opinions. In the case of the procedure for "strongly attached" cells, since the cells themselves are chilled prior to subculturing, keeping the medium cold is probably advantageous.

the way off the pipet, being careful not to touch the pipet to anything.

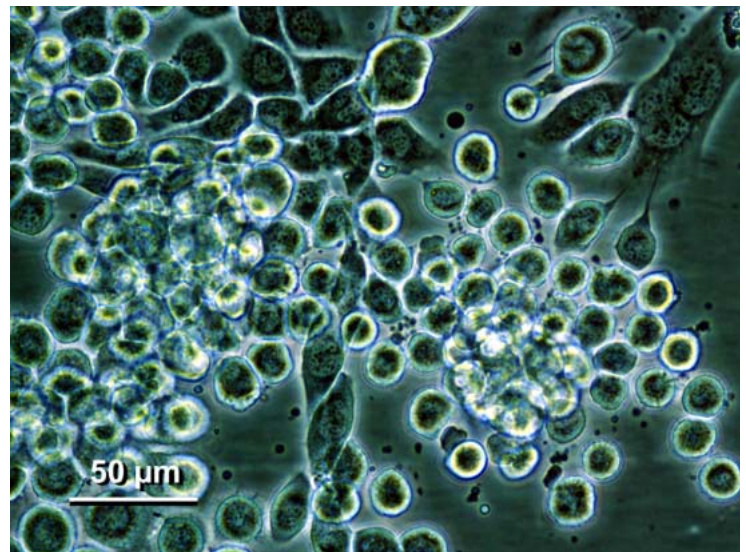
- B. Remove the cap from the fresh medium, carefully insert the pipet only as far as necessary to reach the fluid and draw in the appropriate amount of medium into the pipet. I normally use 4 ml of total volume in a 25-cm<sup>2</sup> flask. If you are performing a 1:10 split, this means you should measure out 3.6 ml fresh medium. Replace the cap on the medium and remove the cap from the new flask. Insert the pipet tip a couple of centimeters into the new flask and deliver the medium into the flask. Discard the pipet into a glass-safe trash receptacle<sup>5</sup>. If you need more than one new culture, repeat this procedure for the additional labeled flasks. While you can use a larger volume pipet to dispense aliquots into several flasks, to avoid potential contamination problems you should never reuse a pipet to make additional transfers from the bottle of medium.
- C. Gently swirl the mature culture to evenly disperse the cells. Stand the culture upright and loosen the cap. Using a new, sterile 1 ml pipet draw in the appropriate amount of the cell suspension from the mature culture into the pipet (in the above example, this would be 0.4 ml). Replace the cap on the mature culture and remove the cap from the new flask containing fresh medium. Dispense the cell suspension into the flask. Discard the pipet as above.
- D. Tighten the caps on the medium, old and new cultures and remove them from the hood. Place the new cultures in a 26-30 °C incubator and the medium back in a 4 °C refrigerator. Wipe down the working surface of the hood with 70% ethanol.

### Method II. Strongly Attached Cell Types

(Fig. 3)

This procedure works well for attached cell cultures such as *Spodoptera frugiperda* IPLB-SF21AE, Sf-9, *L. dispar* IPLB-LdEIta, *Anticarsa gemmatalis* UFL-AG286, *Plodia interpunctella* IAL-PID2, *Plutella xylostella* BCIRL-PxHNU3, *T. ni* BTI-TN5B1-4 (HiFive®), and *Manduca sexta* MRRL-CH1.

- A. Place the mature culture in a refrigerator (4 °C) for twenty minutes.
- B. Remove the mature culture and a bottle of fresh medium<sup>3</sup> from the refrigerator and place in the hood with the labeled new culture flask(s).<sup>4</sup>



**Figure 3.** *Spodoptera frugiperda* IPLB-Sf21 cells; an example of a strongly attached cell line.

- C. Hold the mature culture in one hand and strike it sharply on the side with the palm of the other hand two or three times to loosen the cells. Set it on end in the hood.<sup>6</sup>
- D. Loosen the caps on the medium and new culture flask(s). Take a new, sterile 5 ml pipet from the box. While holding it inside the hood, peel down the protective wrapper on the end containing the cotton plug 5 to 10 cm. Insert the plugged end into the mechanical pipettor and pull the wrapper the rest of the way off the pipet, being careful not to touch the pipet to anything.
- E. Remove the cap from the medium, carefully insert the pipet only as far as necessary to reach the fluid and draw in the appropriate amount of medium into the pipet. I normally use 4 ml of total volume in a 25-cm<sup>2</sup> flask. If you are performing a 1:10 split, this means you should measure out 3.6 ml fresh medium. Replace the cap on the medium and remove the cap from the new flask. Insert the pipet tip a couple of centimeters into the new flask and deliver the medium into the flask. Discard the pipet into a glass-safe trash receptacle.<sup>5</sup> If you need more than one new culture; repeat this procedure for the additional labeled flasks. While you can use a larger volume pipet to dispense aliquots into several flasks, to avoid potential contamination problems you should never reuse a pipet to make additional transfers from the bottle of medium.
- F. Loosen the cap on the mature culture. Using a new, sterile 1-ml pipet draw in the appropriate amount of the cell suspension from the mature culture into the pipet (in the above example, this would be 0.4 ml). Replace the cap on the mature culture and remove the

<sup>4</sup> These items and the pipettor should be the only items in the hood. Do not use the hood as a storage area for buffers, pipet tips, or other equipment since these will interfere with the airflow in the hood and can lead to contamination.

<sup>5</sup> I use a small trashcan lined with an autoclave bag for this purpose. When the bag is full, autoclave it and then seal it in a cardboard box prior to discarding in the trash. Unless you are working with known human or animal pathogens, insect cell cultures are not known to be hazardous. On the other hand, to the general public, tissue culture materials can look like medical wastes and it is prudent to put them through a decontamination process (such as autoclaving) for the peace of mind of refuse workers.

<sup>6</sup> Preliminary to this step, some cells benefit from having the entire medium replaced in the culture prior to suspending them into the medium. Adding this step helps maintain a firmly attached cell type by removing the most loosely attached cells and also provides the leftover (e.g. backup) cells with more nutrients.

cap from the new flask containing fresh medium. Dispense the cell suspension into the new flask. Discard the pipet as above.

- G. Tighten the caps on the medium, old and new cultures and remove them from the hood. Place the new cultures in a 26-30 °C incubator and the medium back in a 4 °C refrigerator. Wipe down the working surface of the hood with 70% ethanol.

### Method III. Very Strongly Attached Cell Types

(Fig. 4)

This procedure works well for firmly attached cell cultures such as the *Heliothis virescens* lines: IPLB-HvT1, IPLB-HvE1A, IPLB-HvE6A, and the *Diabrotica undecimpunctata* line IPLB-DU182A.

#### Trypsinization of cells

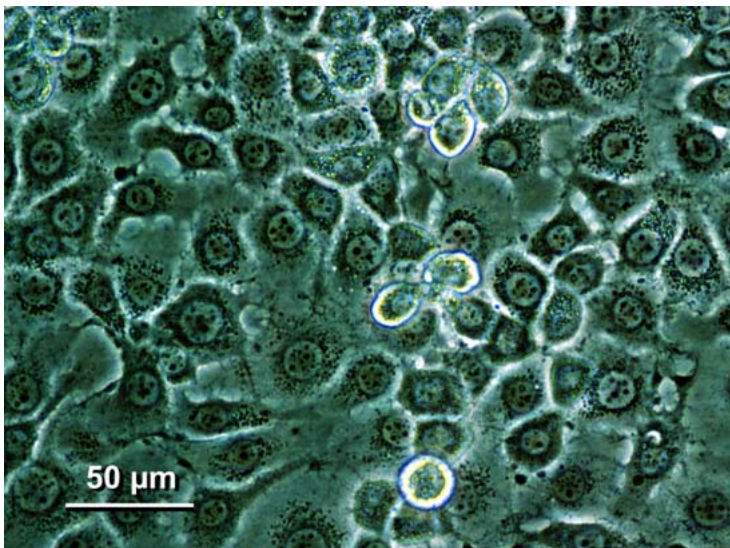
- A. Remove the trypsin solution and the diluent from the refrigerator and place them in the hood with the mature culture and a sterile 10-ml test tube.<sup>4</sup>
- B. Remove the cap from the test tube and the mature culture. Take a new, sterile 5 ml pipet from the box. While holding it inside the hood, peel down the protective wrapper on the end containing the cotton plug 5 to 10 cm. Insert the plugged end into the mechanical pipettor and pull the wrapper the rest of the way off the pipet, being careful not to touch the pipet to anything. Use the pipet to remove and transfer the medium from the culture to the tube. .
- C. Loosen the caps on the diluent and trypsin solutions. Remove the cap from the diluent and draw 2 ml of the solution into a new sterile 2 ml pipet. Replace the cap on the diluent and remove the cap from the culture. Slowly release the diluent solution from the pipet letting it wash across the cell monolayer. Draw the diluent solution back into the pipet and transfer it to the tube with the old culture medium (eventually this tube and its contents will be discarded). Some cell lines may

benefit from a second rinse with diluent.

- D. Take a new 1 ml pipet and transfer 1.0 ml from the trypsin solution to the culture. Replace the cap and tilt the culture flask back and forth to ensure the entire monolayer is wetted by the solution. Set the culture flat on the working surface of the hood and wait 2 to 5 minutes. Return the diluent and trypsin solutions to the refrigerator during this waiting period.
- E. Tilt the culture once more to ensure the surface is wet, then remove the cap and pipet out 0.7 ml of the contents into the test tube with the old medium and rinse solution. Replace and tighten the cap on the culture.
- F. Wait five more minutes. Tap the flask gently on the hood and hold the culture up to the light to see if the cells are loosened. It is quite apparent when the monolayer has become detached. If they have not, tilt the culture again to wet the cell monolayer, lay it flat and wait 5 more minutes. Repeat this process until the cells are loose.

#### Transfer of cells

- G. Remove a bottle of fresh medium from the refrigerator and place it and the labeled new culture flask(s) in the hood. Loosen the caps on the medium and new culture flask(s).
- H. Take a new 5 ml pipet from the box and open as described above. Remove the cap from the fresh medium, carefully insert the pipet only as far as necessary to reach the fluid and draw in the appropriate amount of medium into the pipet. I normally use 4 ml of total volume in a 25-cm<sup>2</sup> flask. If you are performing a 1:10 split, this means you should measure out 3.5 ml fresh medium. Replace the cap on the medium and remove the cap from the new flask. Insert the pipet tip a couple of centimeters into the new flask and deliver the medium into the flask. Discard the pipet into a glass-safe trash receptacle.<sup>5</sup> If you need more than one new culture, repeat this procedure for the additional labeled flasks. While you can use a larger volume pipet to dispense aliquots into several flasks, to avoid potential contamination problems you should never reuse a pipet to make additional transfers from the bottle of medium.
- I. Loosen the cap on the mature culture. Using a new, sterile 5 ml pipet draw in 5 ml fresh medium and dispense it across the cell surface in the trypsinized culture. Draw the medium back into the pipet and release it a few times to evenly disperse the cell suspension. While some foaming will occur in this process, care should be taken to minimize this since it can result in more damaged cells. Draw in the appropriate amount of the cell suspension (in the above example, this would be 0.5 ml). Replace the cap on the mature culture and remove the cap from the new flask containing fresh medium. Dispense the cell suspension into the new flask. Discard the pipet as above.
- Alternate procedure: The above procedure assumes



**Figure 4.** *Heliothis virescens* IPLB-HvT1 cells; an example of very strongly attached cells.

you are using a culture medium that contains FBS. FBS contains trypsin inhibitors, which stop the activity of the enzyme when the fresh medium is added. If you are maintaining the cells in a serum-free medium, you should add some serum-containing medium (or a some other trypsin-inhibiting solution) at this stage. If you want to maintain a serum-free culture, you can replace the medium in the new flask(s) with fresh serum-free medium after the cells have had a chance to attach (1 to 2 hours after the culture is initiated).

- J. Tighten the caps on the medium, old and new cultures and remove them from the hood. Place the new cultures in a 26-30 °C incubator and the medium back in a 4 °C refrigerator. Wipe down the working surface of the hood with 70% ethanol.

*Optional: Determination of Cell Viability (for all cell types):*

After the cells have been suspended into the medium, 0.2 ml of the cell suspension can be mixed with 0.3 ml PBS and 0.5 ml trypan blue (final concentration, 0.2% w/v) in a small test tube. An aliquot is then placed on a hemacytometer and counted with the compound microscope. The number of viable cells (those not taking up the stain) can be determined and used for initiating the new culture with a precise number of viable cells. I personally find this is a time consuming step that does not greatly improve the probability of maintaining healthy cultures but this is largely because I feel confident in recognizing healthy cells just by examining them in the flask with the inverted microscope. Beginners may want to include this step until they gain confidence in their visual inspection of cells.

## Discussion

If you have not used all the cells from the mature culture (for example, if you are making a 1:10 split but set up fewer than 10 new cultures), you can add some fresh medium into the old culture. This will keep the leftover cells healthy for some period if it is stored at room temperature or in a low temperature (17 °C) incubator.

At 2-3 day intervals, examine the new cultures with the inverted microscope. Most insect cell cultures can be maintained at 26-28 °C on a weekly subculture interval but this may require adjusting the split ratios (seeding density). If the lowest density culture you set up reaches confluency before a week has passed, subculture them early but use fewer cells for the next passage. Some researchers have also seen a benefit in growth rates when the medium is replaced two to three days after the cells are split. My feeling is that an appropriate split ratio can be established such that the cells will reach confluency without needing this additional procedure, but researchers should be flexible in their approach to culture procedures and develop a protocol that works best for them.

On the other hand, maintenance of healthy cell cultures depends on strict avoidance of certain practices. I mentioned above that you should never reuse a pipet (unless it has been thoroughly cleaned and resterilized. I find it is actually cheaper to use disposable sterile pipets than to hire someone to clean and sterilize pipets adequately for cell culture purposes). You should also never use the same bottle of medium for more than one cell line. You should never have more than one cell line in the hood at a time during routine

maintenance. All of these “never” statements are to avoid cross contaminating cultures, either with a different cell type or with a microbial contaminant that is present in one line.

Microbial contamination is the major curse of cell culturists. At first thought, antibiotics might seem a useful tool for avoiding such problems. In fact, this is a bad idea. Well maintained modern laminar flow hoods or biosafety cabinets and proper aseptic technique should eliminate the need for antibiotics in the maintenance of the stock cultures. On the rare occasion that contamination does occur, it is better to discover it quickly so a backup culture can be used to recover the cells rather than not finding out about the contamination until resistance to the antibiotic has developed at which time all of your backup cultures will also be contaminated.

Cross contamination of cell lines has been a significant problem since the early days of cell cultures. The most extreme example of this relates to human cell cultures in which scores of cells purported to be normal diploid or from specific tumors were, in fact, proven to be contaminants of the HeLa cell line (Nelson-Rees et al., 1981). This same report also mentioned that at least three insect cell lines believed to be of mosquito origin were actually Grace's *Antherea* cells and I know of at least three more recent examples of insect cell lines that are not from the species that they were originally claimed to be. The most likely scenario for these misidentifications is that a shared bottle of medium was accidentally contaminated with one cell line, which then out competed cells in the other (now misidentified) line. Use of a different bottle of medium for each cell line as described above will help avoid such a tragedy, but cell cultures should be checked regularly to confirm their identity. Isozyme analysis (Tabachnick and Knudson, 1980) is a simple, straightforward method and kits (Authentikit™, Innovative Chemistry, Marshfield, MA) are available for performing these assays. Alternatively, DNA fingerprinting is also a viable technique (McIntosh, et al., 1996).

Even when cells have not been contaminated, an issue of identity can exist. For example, in the introduction, I mention we have cells that have neural properties and others that are myoblasts. In the case of two of these lines developed in my laboratory, embryos were the original source of cells. Since theoretically, all cell types are represented in embryos, it is not necessarily surprising that cultures derived from embryos might have such properties but it is somewhat serendipitous that these were the types of cells that survived the process to become a continuous cell line. A number of factors contribute to this process, such as growth and survival rates and attachment characteristics. Perhaps it was even more fortunate in the case of the nerve and muscle cells that they were eventually identified as such (based on various biochemical and morphological properties). Many insect cell lines have never been characterized sufficiently to identify the specific cell type(s) in the culture.

On the other hand, some researchers have made concerted efforts to obtain specific cell types by using individual tissues or organs. However, even specific organs do not consist of a single cell type and some method needs to be employed to identify the resulting cells in culture. For the lines from imaginal discs I developed with Herb Oberlander (Lynn et al., 1982, Lynn and Oberlander, 1983), we used two-dimensional electrophoresis of proteins and the response of the cells to treatment with ecdysteroids

to confirm identity. Ultimately, the importance of identity will depend on the intended use of the cells.

Researchers familiar with vertebrate cell culture should be aware of a few differences that relate to the differences in the biology of insects as compared with mammals. First, most insect cell culture media utilize phosphate buffers rather than carbonate-based buffers commonly used for mammalian cells. This means a CO<sub>2</sub> atmosphere is not necessary and thus simplifies the incubators needed for insect cells. Also, since insects are 'cold-blooded,' the cells will grow at a much wider range of temperatures. As I mentioned above, leftover cells can be left at room temperature or at lower temperatures to extend their lifespan. In addition, I find a low temperature incubator (17 °C) a useful means for maintaining infrequently used cultures. Cells typically passaged weekly can be switched to a monthly interval.

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