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A practical guide to insect cell cultures: establishment and maintenance of primary cell cultures

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Abstract

In the last three decades several insect species have been studied *in vitro* using cell culture revealing fundamental information about their biology. However, numerous important species have never been investigated at a cellular level so that insect cell culture is still in an early stage of its potential development. In the present review, we summarized the main steps involved in the establishment of primary cell cultures to serve as a practical guide for current and future entomologists to leverage the power of cell cultures. This approach has the potential to generate valuable results and suggestions about insect metabolism, vectorial capacity and adaptation to different stresses and challenges. Although most published papers discussed immortalized cell lines, we focussed our review on primary cell culture since they can give precise data in different research fields.

Key words: *primary cell culture, cell maintenance, in vitro analyses, immunocytes, insects.*

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Introduction

The establishment of insect cell cultures played an important role in different research fields, such as insect pathology, toxicology, insecticide screening and activity assay (Lynn, 1999; Monti *et al*., 2014).

The main breakthrough occurred five decades ago when Grace and Gao established long-term cultures of insect cells (Grace, 1962; Vlak, 2007). Since then, more than 500 cell lines have been established from more than 100 insect species representing every economically important insect order (Hink, 1972, 1980; Hink and Hall, 1989; Lynn, 1996, 2002). These cell lines have been used in diverse research fields and for instance, plant and vertebrate pathologists isolated and maintained cells capable of replicating viruses transmitted by insects (for a variety of papers on vertebrate, invertebrate and plant viruses in insect cell cultures see Mitsuhashi, 1989).

Most published papers focus their attention on established cell lines. Actually, primary cell cultures can furnish pertinent results for several types of experiments, since they tend to retain *in vivo* characteristics to a greater extent than continuous cell lines. As a consequence, the development of a continuous cell line is not necessarily the only means of performing *in vitro* assays for entomologists. For instance, in order to perform RNA interference experiments it can be sufficient to maintain healthy cells only for few days. Primary cell lines could be therefore a potential source of material for entomologists to use in a wide range of studies.

Primary cell culture refers to the initial stage of the culture after cells are isolated from the tissue. At this stage, if the cells proliferate, they can be subcultured (i.e., passaged) by transferring them to a new flask/dish with fresh medium. If the cell culture continues to grow and it is passaged at least ten times, it is considered a continuously replicating cell line. As

these cultures are passaged, cells with the highest growth capacity predominate resulting in a degree of genotypic and phenotypic uniformity in the population. If a subpopulation of a cell line is positively selected from the culture by cloning or some other method, this cell line becomes a cell strain.

Few insect cell lines are commercially available from suppliers or culture collections (such as the *American Type Culture Collection*, ATCC, www.atcc.org), but most insect cell culturists make their lines freely available to other scientists for research purposes. If no cell lines exist for a specific application, it is up to the researchers to develop their own insect cell cultures.

In this paper, we provide a brief overview of how new primary cell cultures can be established from adult insects. In particular, we focussed our attention on immunocytes that are involved in the interaction with both pathogen and symbiotic bacteria making them useful in different research projects (Pandey and Tiwari, 2012; Monti *et al*., 2014).

Equipment

The isolation and maintenance of cell cultures can be performed in a small cell laboratory equipped with few instruments (Figure 1). Minimal equipment consists of a laminar flow hood (or biological safety cabinet) to manage cells and sterile reagents, an inverted phase contrast microscope to check cells with 10X (or 20X) and 40X phase contrast objectives, a mechanical pipetting device to dispense reagents, medium and cells, a refrigerated incubator to maintain cells at 24-28°C, glass bead sterilizers for forceps and a stereo microscope for insect dissection. In the absence of a sterilizer, autoclaving utensils or sterilizing them in low percent bleach and then in 70% ethanol also works well.

The presence of a room ultraviolet air purifier can greatly improve the quality of the work since UV light can penetrate cell walls of bacterium, fungi and mold resulting in their death and safer working conditions. However, UV light can also be dangerous for researchers, so that its safe use has to be carefully planned.

Selection of the proper medium

Insect cell culture media contain the same basal ingredients as mammalian cell culture media (carbohydrates, amino acids, and salts), but at concentrations adapted to insect cell metabolism (Lynn 2002, 2007). Furthermore, insect media are generally more acidic (ranging in pH from 6.2 to 6.9) and buffered with sodium phosphate so that a CO_2 incubator is not required for insect cell culture. A further difference is that the osmotic pressure is generally more than twice as high in insect cell culture media as the osmolarity typical of mammalian ones.

The most important point to consider in attempting to develop a new cell culture is the medium (Lynn, 1996, 2002). While perhaps the easiest way to do this is with a shotgun approach in which every commercially available medium is tried, a certain amount of thought can go into selecting the order in which these have been tried. Many commercial media, for instance, have been developed and sold for Lepidoptera (such as the EX-CELL 405 and the SF-900), whereas other commonly available media are for dipteran cell lines, such as Schneider's *Drosophila* medium. The main points you should consider in selecting a medium for insects other than Diptera and Lepidoptera are the pH, the osmolarity, the amount and ratio of inorganic salts. A never failing reference is still the paper published by Altman (1961) that gives information about the concentration of inorganic salts and amino acids and pH of haemolymph of many insects. Stating from Altman's paper, it is possible to compare values of these factors with the published formulation of commercial media to select the most appropriate for your insect and make the necessary modifications.

As reported in literature (Lynn, 1996, 1999), some media can be used in different species even if they do not belong to the same order. For instance, Grace's insect medium is popular for insect cell culture (Mather and Roberts, 1998) and it has been used with different dipteran

Figure 1. Minimal equipment needed consists of a table or bench that can be cleaned with ethanol solutions for the performance of dissections at the stereomicroscope (a), a laminar flow hood (or biological safety cabinet) to manage cells and sterile reagents (b), a refrigerated incubator to maintain cell flasks/dishes at 24-28°C (c, d) and an inverted phase contrast microscope to observe cells (with a 10X or 20X, and 40X phase contrast objectives) (e).

species, even though it was originally formulated for Lepidoptera (Grace, 1962). Schneider's *Drosophila* medium was originally developed for *Drosophila* cell culture, but it can be also used for other dipteran cell lines. EX-CELL 420 medium has been used for different Diptera and Lepidoptera and more recently for the red flour beetle, *Tribolium castaneum* (Goodman *et al*., 2012). Good results for Coleoptera have also been reported with the Schneider's medium (supplemented with 15% fetal bovine serum) and the Kimura's medium (Kimura, 1984) suggesting that Coleopteran cells can also be maintained and cultured in different media (Zhang *et al*., 2014).

In some cases, common cell media seem to be inadequate and specific media or combinations of commercial media have to be tested. For instance, the Kimura's medium (Kimura, 1984) has been developed for hemipteran species, but it resulted inadequate for the hemipteran *Cacopsylla pyri*, *Cacopsylla melanoneura* and *Cacopsylla crataegi* cell cultures, so that we recently compared the maintenance of psyllid cells in three media (EX-CELL ® 405, Sf-900™III and the psyllid Hert-Hunter medium) (Monti *et al*., 2014). The Sf-900™III medium did not support psyllid cells, which shrivelled and died in the first two days post culture. Better results were obtained with EX-CELL ® 405 medium, since cells remained viable for more than one month, though with a low growth rate. On the contrary, extremely positive results were obtained with HH70 medium, which kept cells alive for more than sixty days, according to Marutani-Hert *et al.* (2009). Interestingly, the HH70 medium is a combination of four media (Schneider's

medium, Sf900 III, Medium 199 and CMRL) supplemented with heat-inactivated fetal bovine serum (Marutani-Hert *et al*., 2009).

A comprehensive listing of most serum-free media currently available for insect cell culture has been compiled by Agathos (2007).

Preparation of hood and dishes/plates

At the beginning of an experimental trial to isolate insect cells, it could be useful to work with small medium volume so that, in place of the commonly used 12.5 or 25-cm^2 tissue culture flasks, it can be more suitable to start the work using 35mm x 10 mm polystyrene cell culture dishes (with 1 ml of medium) or use 24 well tissue culture plates (with 1 ml of medium in each well). Polystyrene cell culture dishes are less expensive, but they can be difficult to manage as they have to be sealed with Parafilm® to maintain sterility and to facilitate their examination with the inverted microscope.

We generally prefer to work with tissue culture plates since they can be managed more quickly, they do not need to be sealed and information can be written on their lids (such as number of dissected specimens, date, medium volume and type, etc.).

As described in detail by Lynn (2002, 2007), before starting to prepare plates, wipe down the working surface of the hood with 70% ethanol (keep a 100–200-ml squeeze bottle of ethanol next to the hood for this purpose) and sterilize the hood for at least 40 minute with the UV lamp. After sterilization, turn on the air movement of the hood for a few minutes before starting your work to ensure the moving air is clean. Then remove a bottle of fresh medium from the refrigerator and place it in the hood.

It is best to use single-use disposable serological pipets to manage media and cells. Pay attention that the major source of microbial contamination in cell cultures is not the laboratory room, but the laboratory workers, so that it is important to wash hands and wear clean gloves to minimize contamination.

One of the most practical advantages of working with insect cells is that many of the contaminants that vertebrate cell culturists have to contend with are not an issue with insects (Lynn, 2007). For example, mycoplasma (which is the major source of problem of cell workers) is adapted to grow at 37°C and the temperatures at which insect cells grow (typically 25-28°C) are not conducive to the effective growth of this organism (Lynn, 2007). In many cases, a further source of contamination is related to viruses due to contaminations of the bovine foetal serum, but generally they do not replicate in insect specific media.

Media supplements

In order to have the best growth conditions for cells, insect media are generally supplemented with 200 mM L-Glutamine solution.

If you plan to use the medium for primary cell cultures, it is essential to add antibiotics, in particular gentamicin (at a final concentration of 50 µg/ml) and penicillin/streptomycin (at a final concentration of 50U/ml and 50 µg/ml, respectively). Lastly, taking into account that the main problem with cell culture contamination is not related to bacteria but to mold, the antimycotic agents nystatin (100 U/ml) or amphotericin B (0.5 ug/ml) can also be added to the medium.

When reagents are ready to use, loosen the caps on the medium, carefully insert the pipet only as far as necessary to reach the fluid and draw the different reagents into the pipet and then transfer them into the bottle containing your medium for supplementation. Serological pipettes for cell culture and liquid management and the pipettor should be the only item in the hood. Do not use the hood as a storage area for buffers, tips, or other equipment since these will interfere with the airflow in the hood and can lead to contamination. When the supplemented medium is ready, pipette 1 ml of medium into a well in your plate (or in the Petri dish if you plan to use it) always being careful to work inside the sterilized hood.

Depending on incubation conditions, small volumes of the cell culture medium may evaporate quickly, especially during long-term experiments. In order to reduce this effect, pipette sterile water into wells near where you plan to put cells. If you plan to use dishes, they need to be placed in a tightly sealed plastic (or glass) container with a small beaker of sterile distilled water and the entire container placed within the incubator to discourage dehydration.

How to dissect insects

Two factors make primary tissue culture of insects particularly arduous (as reported in Lynn, 1996). The first is, their generally small size, whereas the second problem is that insects often live in dirty environments. The former problem can be overcome by setting up primary cultures in small volumes, whereas the latter issue can be dealt with by using antibiotics. It is generally not a good idea to use antibiotics in continuous cell lines (it could favour the occurrence of resistant bacteria), nevertheless they are beneficial in initiating a new primary cell culture.

The general procedures we currently use for isolating cells are shown in figure 2. We normally disinfect insects by submerging them for 1 minute in a series of dishes respectively containing 2 ml of 70% ethanol, 0.115% sodium hypochlorite, sterile water, 70% ethanol and sterile water before dissection. After drying on a filter paper for a couple of seconds, insects can be moved into a tissue culture dish or a multi-well plate (we generally prefer multi-well plates) where they can be dissected using sterilized forceps.

Different approaches can be used for the development of primary cell culture and may involve the use of adults, eggs or specific organs (Freshney, 1987; Lynn, 1996, 1999, 2001, 2002, 2007; Mitsuhashi, 2002). Embryos have been a common source of cells for the development of new cell lines since they can be frequently obtained in large quantities and the insect chorion is sufficiently impervious to simple disinfectants for effective decontamination. However, they are not always available and you may be interested in specific organs/cell types.

In order to isolate circulating immunocytes, it is sufficient to cut the insect body in half using forceps and shake the abdomen with a pair of sterile forceps favouring the release of immunocytes. Then, all the body fragments need to be removed using forceps and plates incubated at 24-28°C.

Observation of primary cell cultures with a microscope

After 16-24 hours (h), it is possible to start the observation of cells at the inverted microscope with a 10X (or 20X) phase contrast objective. The medium in the culture should be relatively clear (debris can be present due to dissection) and cells should be somewhat refractive under the microscope (Figures 3a, b).

A cloudy appearance, which makes it hard to see cells with the microscope, suggests a bacterial contamination; such cultures should be autoclaved and discarded (Figure 3a). A further unlucky event could be the presence of mould and also these plates should be autoclaved and discarded (Figure 3d).

As marvellously written by Lynn (1996): “Patience becomes the greatest virtues at this stage”, since little or no growth of cells can be seen for weeks. During this period, additional culture medium should be added to the cells. We generally add 0.2 ml of medium every 5-7 days to our 1 ml cell culture, whereas the observation of cell cultures and the evaluation of the cell growth are carried out daily using an inverted light microscope. After 7 days, 700 µl of spent culture medium is removed and replaced by fresh medium. We generally prefer to add room temperature medium, but several researchers add fresh medium at 4°C.

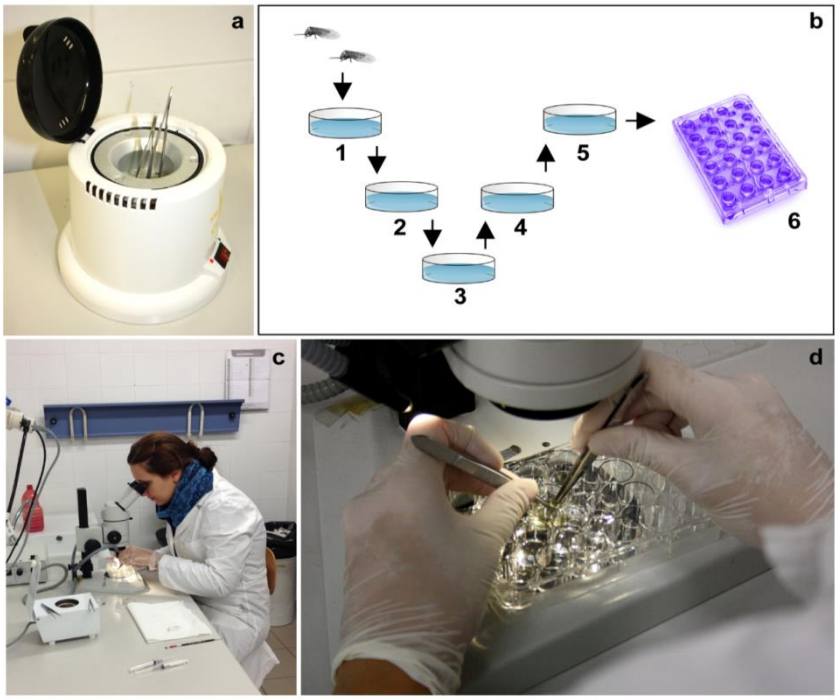

Figure 2. In order to have "clean" dissections it is important to use sterilized forceps to manage and dissect the insects (a). Before dissection, disinfect insects by submerging them for 1 minute in 75% ethanol (1), 0.115% sodium hypochlorite (2), sterile water (3), 75% ethanol (4) and sterile water (5), as summarized in panel b. After drying on a filter paper, insects are transferred into a multi-well plate and dissected using sterilized forceps (c-d).

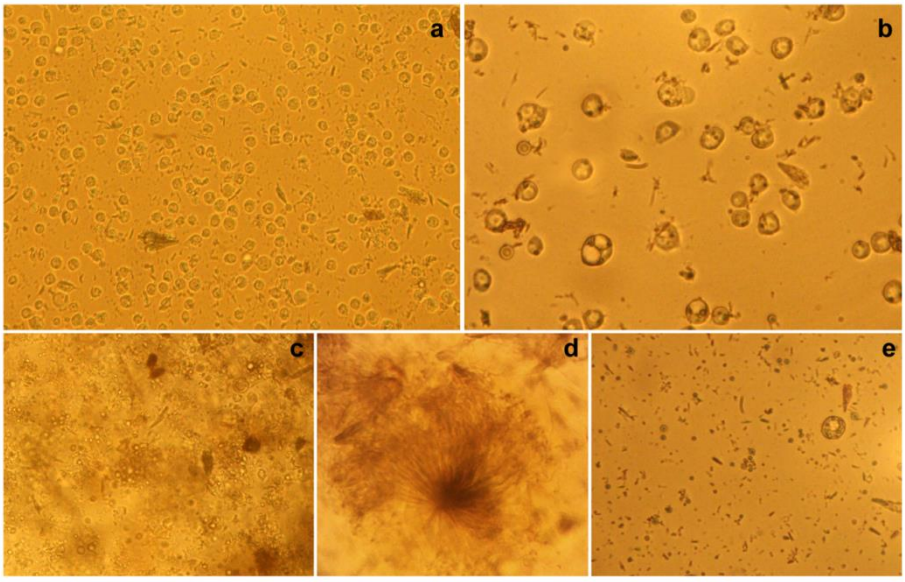

Figure 3. Observation of cells at the inverted microscope with a 20X (a, c) and 40X (b, e) phase contrast objective showing cell cultures in the absence of specific staining. A cloudy appearance is a direct evidence of bacterial contamination (c), whereas the presence of mold can be quickly evaluated (d).

Maintenance of primary cell cultures

Most primary cell cultures do not survive beyond 2 months, however this short period is sufficient for different type of analyses and work, such as the propagation of viruses in cultured cells and the study of immunocytes in mediating an immune response to different immunological challenges (for a review see Smagghe *et al*., 2009). The use of primary cell culture should be favoured for *ex vivo* analysis in respect to continuous cells, since stable cell lines assume morphological and physiological characteristics that can differ from the source of primary cultures. For instance established midgut cell lines bear little resemblance to midgut cells *in vivo* and their susceptibility to some stimuli (such as to toxins) is altered (Smagghe *et al*., 2009).

At 2–3 day intervals, it is necessary to examine the cell cultures using an inverted microscope and record what you observe/make in a record book. This information should include the date, the 'name' of the culture (cell line designation, dissection date, number of specimen dissected, etc.) and the type, amount and specific source of the used culture medium.

Even if it is true that microbial contamination is a relevant curse for cell culturists, well-maintained modern laminar flow hoods and proper aseptic techniques are sufficient to eliminate the need for some antibiotics and antimycotic reagents in the maintenance of the stock cultures. For this reason, after two weeks we start to use medium supplemented only with glutamine and penicillin/streptomycin, without any further use of gentamicin and antimycotic agents. Actually, to avoid the development of resistance to the antibiotic, all the antibiotics should be removed, but resistance occurs on rare occasions so that we use them and we immediately discard plates if a contamination does occur.

During the examination of cell cultures you can observe the number and the type of the cells that have been isolated during dissection, you can evaluate if their density increases week after week. In this regard, you may have to wait for more than a week before the observation of mitosis since cells must adapt to the new "environment".

A regular examination of the morphology of the cells in culture (i.e., their shape and appearance) is essential for successful cell culture experiments. In addition to confirm their healthy status, inspecting the cells by eye at a microscope each time they are handled will allow you to detect any sign of contamination early on and to contain it before it spreads to other cultures around the laboratory. Signs of deterioration of cells may include granularity around the nucleus, detachment of cells from the substrate and cytoplasmic vacuolation. Deterioration may be caused by a variety of reasons, including contamination of the culture, senescence of the cell line, the presence of toxic substances in the medium, or the need for a medium change.

Lastly, cell line cross-contamination is a serious concern because of the length of time it can go undetected so that it can be useful to avoid the simultaneous presence of different cell cultures in the hood.

Subculture of the cells

After an average of ten weeks (Goodman *et al*., 2001) you can start subculturing, also referred to as passaging, which consists of the removal of the spent medium, addition of fresh medium and the transfer of cells from the older vessel into a new vessel containing fresh growth medium. This procedure enables the further propagation of the cell line or cell strain. Indeed, when cells occupy all the available substrate or when cells in suspension cultures exceed the capacity of the medium to support further growth, cell proliferation is greatly reduced or ceases entirely. Additionally, to keep the culture at an optimal density for continued cell growth and to stimulate further proliferation, the culture needs to be supplied with fresh medium. If you observed an increase in the cell number you can decide to: *i*. increase the medium volume; *ii*. divide cells in two dishes.

It is important to pass your cells according to a strict schedule in order to

ensure a reproducible behaviour, which also allows you to monitor cell health on a consistent basis. Vary the seeding density of your cultures until you achieve consistent growth rate and yield appropriate for your cell type.

When cells are ready for passaging or you have to insert fresh medium, remove the spent medium with a sterile pipette and immediately insert fresh medium. If cells are not well attached to the dish, gently resuspend cells in the old medium, transfer them into a sterile tube and centrifuge the cell suspension at 100 × g for 3-5 minutes at room temperature. Discard the spent medium by pipetting it into a waste container and resuspend the cell pellet in fresh growth medium.

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

Several laboratories use trypan bleu staining to determine the number of viable cells (those not taking up the stain) (Lynn, 2002, 2007). We personally find this a time consuming step that does not greatly improve the probability of maintaining healthy cultures. We feel we can confidently recognize healthy cells just by examining them in the dishes with the inverted microscope. Beginners may want to use trypan blue staining until they gain confidence in their visual inspection of cells.

Immunocytes at work: an example of application of primary cell cultures

Immunocytes (frequently referred as hemocytes) play multiple functions in insect immunity, including nodule formation, phagocytosis, encapsulation and synthesis of antimicrobial peptides and other molecules, as recently revised by Pandey and Tiwari (2012). In view of these functions, primary cultures of immunocytes have been used for the characterization of the immune response in different insects, including the mosquitoes *Anopheles gambiae* and *Aedes aegypti* (e.g. Castillo *et al*., 2006), for studies of signalling mechanisms in immunity (Smagghe *et al*., 2009) and for the comprehension of the immunocyte migration at the infection site (Smagghe *et al*., 2009).

Recently, immunocytes have been shown to interact with symbionts and play a role in the vectorial capacity of insects (Mandrioli, 2009; Mandrioli *et al*., 2015), making their study an important challenge for the scientific community. There are therefore many scientific questions regarding the role of immunocytes in insect biology beyond their involvement in immunity, which also point towards the potential of future applications in entomological and biomedical fields.

Conclusions

The field of insect cell culture is facing a rapid expansion into different areas of biology, such as immunity, endocrinology, toxicology and biochemistry. We expect substantial advances to be made in the coming years in many different fields, including signal transduction, endocrinology, toxicology and in several other areas of insect cell biology. As reviewed by Smagghe *et al*. (2009), cell cultures will allow numerous advances in insect science that will become a part of the modern vanguard of agricultural science necessary for safer production of healthier foods to meet the demands of a rapidly increasing human population.

By paraphrasing Corrie S. Moreau (2014), in closing we hope that this practical guide may provide the foundation for future entomologists to leverage the power of cell cultures to address questions in insect metabolism, vectorial capacity and adaptation to different stresses and challenges.

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