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Lepidopteran Insect Cell Line Isolation From from Insect Tissue

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Summary

This chapter describes procedures for initiating new cell lines from lepidopteran larval tissues. The internal morphology is described along with methods for treating excised tissues and the primary cultures. Advice on culture medium and the tissues that will provide the best chance for new cell lines is discussed.

Key Words: Cell line establishment; primary cultures; internal morphology.

1. Introduction

As shown in Chapter 6, established cell lines from Lepidoptera are widely available, with over 260 lines from more than 60 species. With such a diversity of material already available, most researchers working with insect viruses will not need to develop their own cell lines. On the other hand, many of the baculoviruses that have been discovered have never been grown in cell culture, so the possibility exists that efforts on a new virus may also require development of a new cell line. The methods described here are a collection of procedures that other researchers and the author has found effective for initiating new cell lines. The author has previously described methods for initiating cultures from embryos ***(1)*** but will primarily concentrate on larval tissue in this chapter with some notes describing other sources for insect cells.

2. Materials

2.1. Solutions

1. Commercial insect cell culture medium (*see* **Note 1**) supplemented with 5–10% (v/v) fetal bovine serum and 5 μg/mL gentamicin sulfate (*see* **Note 2**).

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2. Divalent ion-free phosphate buffered saline for enzyme dissociation: 800 mg NaCl, 20 mg KH_2PO_4, 20 mg KCl, 150 mg $Na_2HPO_4 \cdot 7H_2O$, 23 mg Na_2EDTA in demineralized water to 100 mL.
3. VMF Trypsin (virus and mycoplasma-free; cell culture tested).
4. 70% Ethanol.
5. Sterile (autoclaved) demineralized water.

2.2. Tissue Culture Labware

1. 35-mm Tissue culture Petri dishes.
2. 12.5- or 25-cm^2 Tissue culture flasks.

2.3. Equipment

1. Laminar flow clean bench (*see* **Note 3**).
2. Dissecting tools, including two or more pairs of jeweler's forceps, microscalpel, microscissors (*see* **Note 4**).
3. Insect pins.
4. Small jar with a layer of cotton in the bottom containing 95% ethanol (*see* **Note 5**).
5. Alcohol lamp with wind screen (*see* **Note 6**).
6. Sterile (autoclaved) glass Petri dish containing 4–5 mm paraffin (= wax dish).
7. Stereo dissecting microscope and light source.
8. 1-mL Sterile pipets.
9. 250-µL Pipettor.
10. Sterile pipet tips.
11. Plastic box, approx $5 \times 6 \times 2$ in. with air-tightairtight lid.

3. Methods

3.1. Disinfection

Most insects can withstand a short period in disinfecting solutions without significant harm. The author's preferred method is as follows:

1. Submerge the insect in 70% ethanol for 5–20 min. The shorter time should be used with particularly fragile (thin-cuticled) insects and the longer time if the insect's typical environment is particularly dirty (*see* **Note 7**).
2. Rinse in at least two changes of sterile demineralized water. Several insects can be disinfected simultaneously and then held for 30–60 min in the final water rinse until needed. Keeping them submersed in this manner will result in a buildup of CO_2 from their natural respiration and this will act as an anesthetic to keep them immobile during the dissection.

3.2. Dissection

1. Place a dissecting microscope, alcohol lamp and jar, and dissecting tools in the clean bench (**Fig. 1**) and turn on the airflow.
2. Wipe down the surface of the microscope with 70% ethanol and place the dissecting tools in the alcohol jar for at least 10 min, then burn off the alcohol by

Fig. 1. Typical arrangement of equipment in the clean bench for dissections. The jar in the center contains ethanol and a small layer of cotton in the bottom. The dissecting tools are placed in the jar for at least 5 min, after which the alcohol is ignited by passing the tip over the alcohol lamp's flame (*see* **Note 9**). They can then be positioned on a small tray (in this case, it was fashioned from the container in which the microscalpel was purchased) until needed for the dissection.

briefly holding them over the lit alcohol lamp (*see* **Note 8**). Place them on the holding tray until needed.

3. Position the disinfected larva, dorsal side up, in a sterile wax dish. Insert an insect pin through the head and last abdominal segment of the larva and into the wax in the bottom of the dish (**Fig. 2A**, *see* **Note 9**).
4. Aseptically add enough culture media to the dish to completely cover the insect.
5. Pinch the insect's cuticle with forceps and use the disinfected scissors to cut a small hole on the dorsal side of the next to last abdominal segment only deep enough to penetrate to the hemocoel.
6. Keeping the scissor blade parallel to the insect's body,body cut the cuticle from tail to the first thoracic segment, taking care to not pierce the gut.
7. Use the forceps to grasp the cut edge and use either the scissors or another forceps to cut/tear the tracheoles that are connected to the gut. Once this is accomplished, more insect pins can be used on each side to hold open the incision (**Fig. 2B**).

3.2.1. Internal Morphology

The internal morphology of lepidopteran larvae can be somewhat daunting to the uninitiated, but is actually fairly simple.

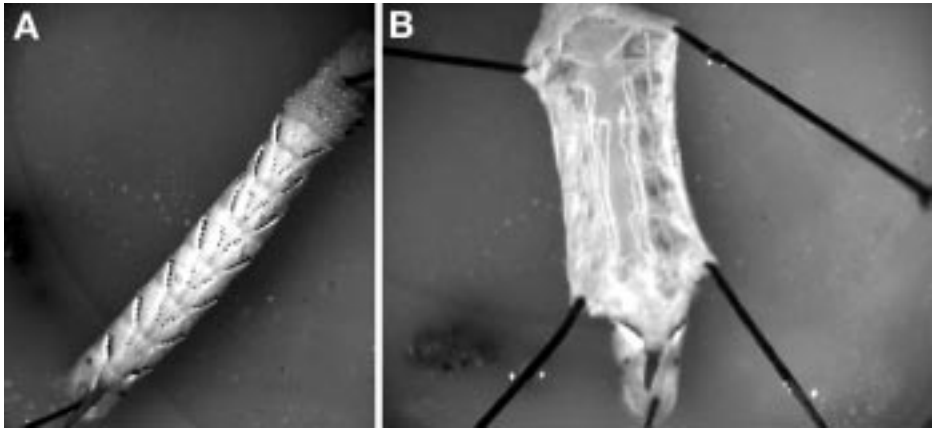

Fig. 2. A fourth instar *Manduca sexta* larva pinned in a wax dish containing tissue culture medium. **(A)** Larva before first incision. **(B)** Larva with cuticle pinned to each side to expose internal organs and tissues.

3.2.1.1. Digestive Tract

Because larval Lepidoptera are essentially eating machines, the most obvious structure is the digestive tract (**Figs. 3–5**, FG and MG), which is divided into three morphologically and physiologically distinct sections—the foregut (FG), midgut (MG), and hindgut. Unless you specifically want to set up cultures from gut cells ***(2)*** (*see* **Note 10**), you should be especially cautious to avoid rupturing it.

3.2.1.2. Malphigian Tubules

Loosely connected to the midgut are the Malphigian tubules (**Figs. 3–5**, MT), white tubes running along the digestive tract parallel to the body and often looped near the thorax and continuing back toward the posterior. These are the kidneys of the insect, responsible for removing nitrogenous wastes from the blood that are excreted into the digestive tract at the interface of the mid- and hindguts. The uric acid crystals formed from the insect's nitrogenous waste creates their bright, refractive nature.

3.2.1.3. Tracheals/Tracheoles

The tracheals/tracheoles are also quite apparent (**Figs. 3–** and **4**, T). This is the respiratory system for insects and is a series of branching tubes that supply air to each tissue (*see* **Note 11**). They are typically very obvious, appearing somewhat silvery owing to the refraction of the air they contain.

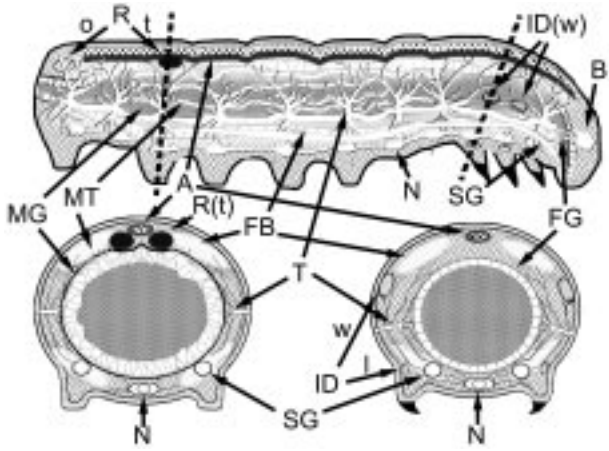

Fig. 3. Diagrams of lepidopteran larva showing the locations of many tissues. **(Top)** Lateral view; **(Lower right)** cross section through thorax; **(Lower left)** cross section through abdomen. R, reproductive tissue (o, ovaries; t, testis); ID, imaginal discs (w, wings; l, legs); B, brain; MG, midgut, MT, Malphigian tubules; A, dorsal aorta; N, ventral nerve cord; SG, salivary (silk) glands; FG, foregut; FB, fat body; T, tracheoles.

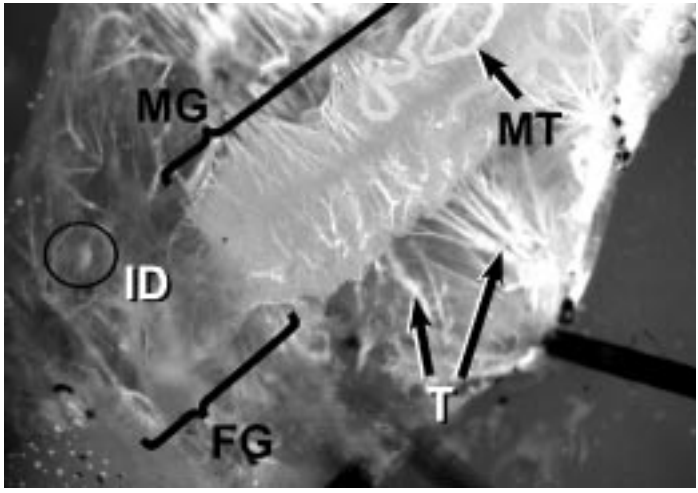

Fig. 4. Thoracic and anterior abdomen of *Manduca sexta* larva. ID, imaginal discs; MG, midgut, MT, Malphigian tubules; FG, foregut; T, tracheoles. The circle is the location of one of the imaginal discs. At this stage in the dissection, the lateral muscles largely obscure it, but the faint white mass in the middle of the circle is the mass of tracheoles that eventually become the wing venation in the adult.

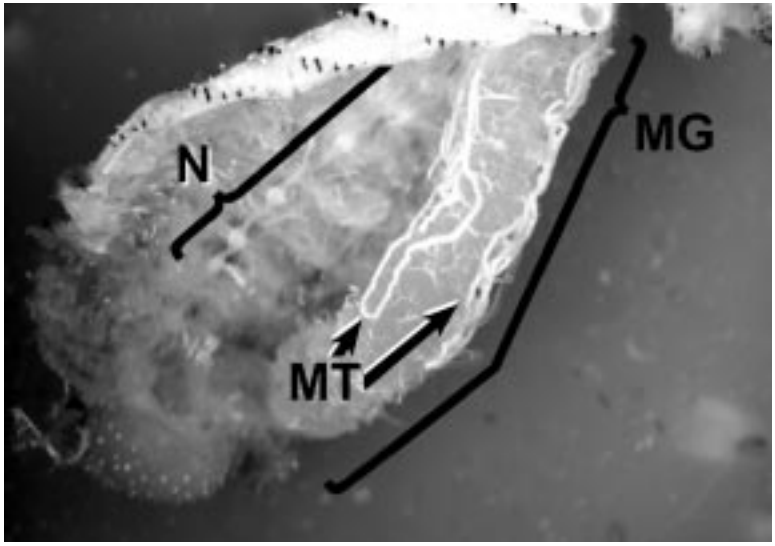

Fig. 5. Thoracic and anterior abdomen of *Manduca sexta* larva showing a large portion of the abdomen with the midgut moved to the side revealing the ventral nerve cord (N). MG, midgut; MT, malphigian tubules.

3.2.1.4. Fat Body

In the later stages of larval development, the fat body (physiologically equivalent to the mammalian liver and fat cells) becomes a prominent cell type. In most lepidopteran larvae, these occur in four bands of a relatively loose tissue (**Fig. 3**, FB), generally appearing bright white because of the large amount of lipids they contain.

3.2.1.5. Nerve Cord

After severing the tracheoles connected to the digestive tract, the gut can be gently stretched and moved to the side of the body. Doing so will reveal the ventral nerve cord (**Figs. 3** and **5**, N). This tissue, a white/light cream-colored structure, runs the length of the ventral side of the insect with enlarged areas (ganglia) in each segment.

3.2.1.6. Salivary Glands

The salivary glands are a pair of translucent tubes running from the head for about half to three-quarters the length of the larva. These are usually on the ventral side of the digestive tract and slightly to each side, somewhat (albeit loosely) connected to the digestive tract. (**Figs. 3** and **5**, SG).

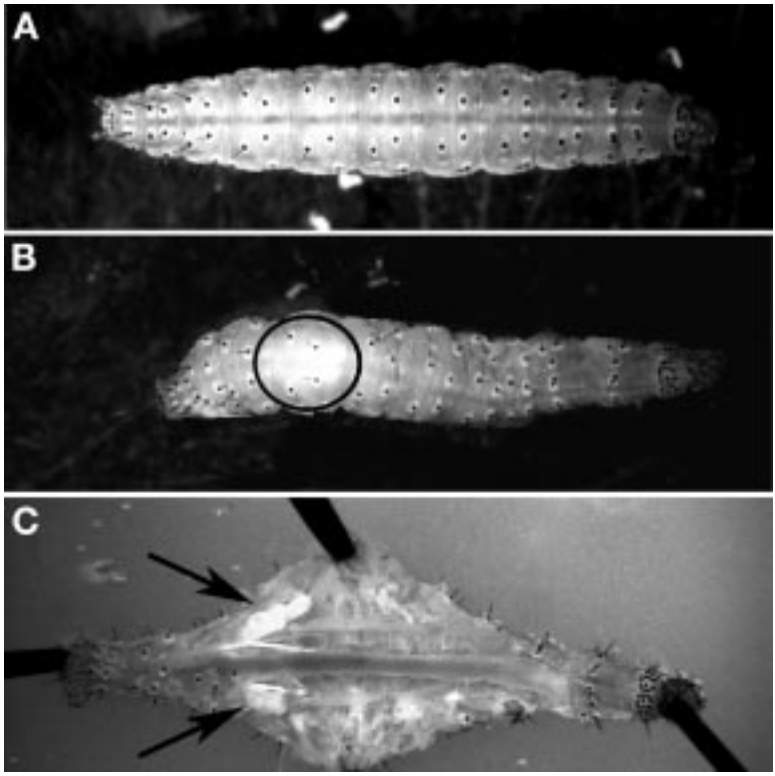

Fig. 6. *Plutella xylostella* larvae. **(A)** Female last instar, **(B)** male last instar. The circle indicates the location of the testes, **(C)** dissected male larva, arrows indicate testes.

3.2.1.7. Dorsal Aorta

Less apparent, because it typically is the same color as the cuticle and connective tissue, is the dorsal aorta, the insect's heart (**Fig. 3A**). It is tightly connected to the insect cuticle and, depending on how near the midline you made your incision, you may have cut across this organ. However, it can often be identified by the regular muscle contractions.

3.2.1.8. Reproductive Organs

The reproductive organs will be in the abdomen, generally dorsal to the hindgut (ovaries) or midgut (testes). Males typically have two ovoid testes (**Fig. 3**, R[t], **Fig. 6C**) that fuse into a single organ late in the last larval instar in some species. These can be brightly colored organs and, with species that have a

lightly colored cuticle, can occasionally be seen through the cuticle as a means of identifying males from females (**Figs. 6A**,**B**). The ovaries in female larvae are typically a pair of cream-colored structures connected to a common oviduct and are smaller versions of the organs in the adults. These can be more difficult to identify in the larval stages because they typically remain quite small (contrary to the testes) until the pupal stage.

3.2.1.9. Imaginal Discs

More challenging than the organs/tissues already mentioned are the imaginal discs (**Figs. 3** and **4**, ID, and **Fig. 7**). These are the tissues in immature insects that are destined to become adult structures. Their name derives from the Latin "imago" in the sense that these structures are the likeness of the adult in the larvae, but they can be so difficult to find that one might think the term derives from "imaginary." Actually, this is more accurate with Lepidoptera than in some other insects. In *Drosophila* larvae, imaginal discs are loosely connected to the cuticle and can be isolated in mass ***(3)***, but the tissues are more tightly connected in lepidopteran larvae. Still, they may be worth the effort to locate because, as undifferentiated cells, they can be effective sources for development of cell lines ***(4**,**5)***. Imaginal disc tissues that are destined to become eyes, antennas, legs, and wings have been identified, but the wing discs are the easiest to locate and excise in Lepidoptera. Because moths and butterflies have two pairs of wings, they also have two pairs of wing imaginal discs, located in the second (meso-) and third (meta-) thoracic segments, attached to the cuticle near the lateral midline. The discs are nearly transparent, but they are each supplied with a large number of tracheoles that are necessary to supply oxygen during the rapid cell growth that occurs late in the last larval instar and pupal stages. As noted earlier, tracheoles are generally highly visible owing to the refraction of the air they contain and these can be used to help locate the discs (**Figs. 4** and **7**).

3.2.2. Initiation of Primary Cultures

1. When the tissue of choice has been identified (*see* **Note 12**), it should be carefully excised from the larva and transferred to a 35-mm tissue culture Petri dish containing 1.0 mL culture medium.
2. Hold the tissue in this dish while additional larvae are dissected. Depending on the size of the insect and specific tissue of interest, extracts from several individuals may be necessary. The same dissecting dish can be used for subsequent larvae with the understanding that this also increases the possibility of contaminating the primary culture. A better course of action is to use a separate dish for each dissection, pooling the tissues after completing all the extractions (*see* **Note 13**).

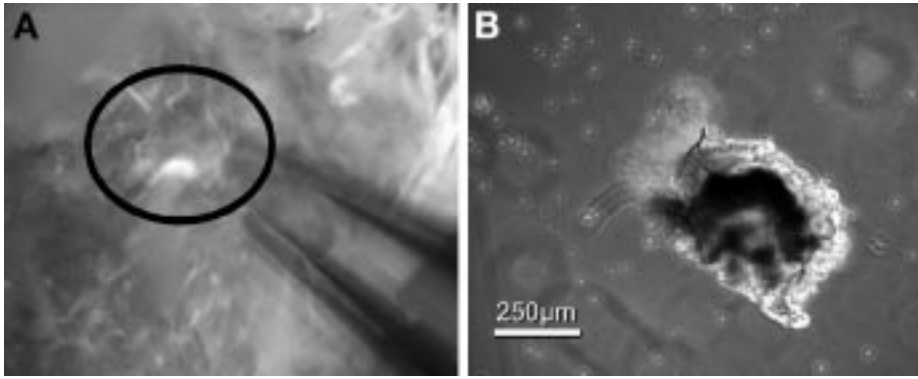

Fig. 7. Wing imaginal discs of *Manduca sexta.* **(A)** Disc (circled) being slightly lifted from the cuticle with forceps. The white spot is the tracheoles. **(B)** Wing imaginal disc in culture. The tracheoles, which are white in the other micrographs with reflective light appear as a dark mass under the transmitted light.

3. Once the tissue extractions are complete, transfer them to a new culture dish containing a standing drop (100–150 µL) of medium or enzyme buffer. At this point, the tissues will each have a distinctive appearance as seen in **Figs. 7–9**.
4. Use a microscalpel to cut the tissue into small fragments, or enzymatically disassociate the tissues with trypsin or another enzyme (*see* **Note 14**).
5. As an alternative to trypsinizing the tissues immediately after dissection, the wounding method can be used and if cells do not begin migrating from the cut tissues, then enzymatic dissociation can be used a few days later.
6. After completing the manipulations, the dish should be sealed by stretching a thin strip of Parafilm® (~(approx 8 × 100 mm) around its edge and then placed with other cultures into a small tightly sealed box kept humidified by a small beaker of water or a dampened paper towel.
7. Place the box into a 26–28°C incubator.

3.3. Initial Maintenance

1. One to 2 d after initiation, the primary cultures should be examined with an inverted microscope. Any contaminated cultures should be autoclaved and discarded and an additional 1.0 mL medium should be added to all remaining cultures.
2. Reseal the dish with a new piece of Parafilm, replace it into the humidified box and return it to the incubator.
3. Examine the cultures with the inverted microscope at 7- to 10-d intervals, adding 0.5 mL fresh medium to all healthy cultures. This routine should be continued until the dish contains sufficient cells for subcultivation.

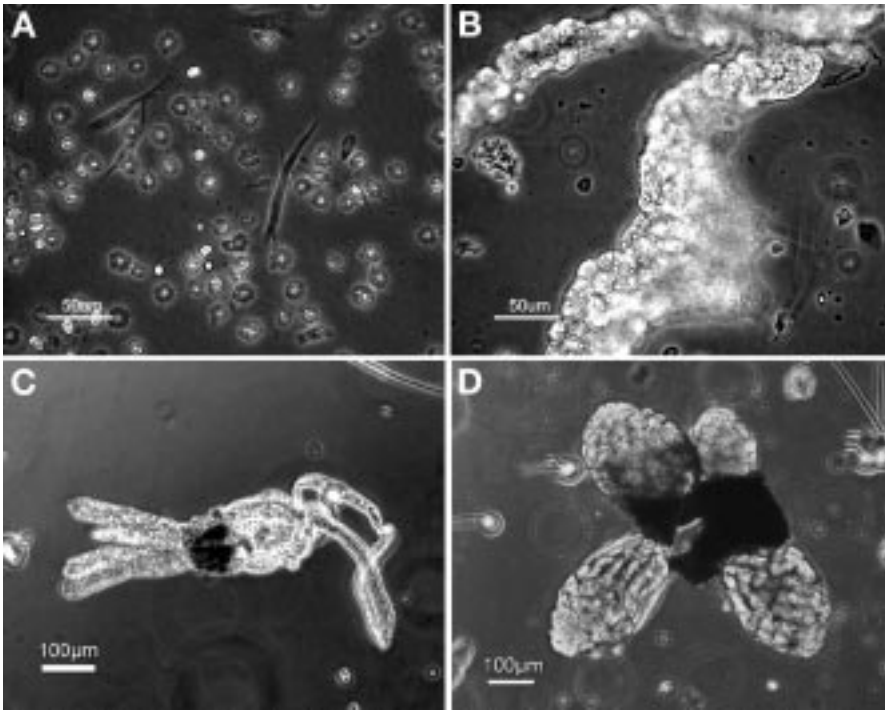

Fig. 8. Larval cells and tissues in primary cultures. Each of these tissues has been used for establishing cell lines. **(A)** Hemocytes in a dissecting dish (*see* **Note 14**), **(B)** fat body, **(C)** ovaries, **(D)** testis (the sheath has been ruptured showing four follicular bundles).

4. If the volume of medium reaches approx 3.5 mL before there are sufficient cells, all but about 0.5 mL should be transferred to a sterile centrifuge tube, the cells pelleted at 50*g* for 5 min and the pellet resuspended into 0.5 mL fresh medium and returned to the culture dish. The supernatant from this centrifugation can be transferred to a small tissue culture flask and incubated with the primary culture. It is not uncommon to have the low number of cells that are not pelleted at the low speed begin to grow because the medium is somewhat conditioned by the larger tissues. These "pour off cultures" can result in cell strains that are morphologically and functionally distinct from the main cultures. If these cultures appear to contain a significant number of healthy cells, then they should be regularly observed and some of the medium replaced as with the primary cultures.

3.4. First Subcultivation

1. When the primary (or pour off) cultures contain a substantial number of cells (*see* **Note 15**), they can be split into a new culture dish or flask. The author prefers to

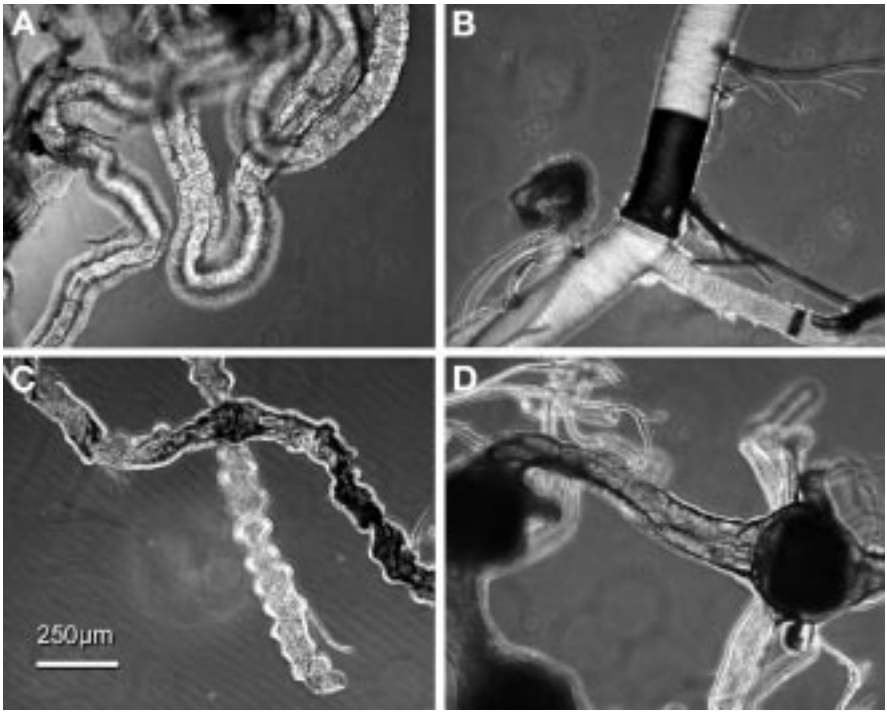

Fig. 9. Additional larval tissues in primary cultures. The tissues shown in this figure have not been used for establishing cell lines, although some have been used in primary tissue and cell culture studies. **(A)** Salivary gland, **(B)** tracheoles, **(C)** malphigian tubules, **(D)** ventral nerve cord.

use a small flask (some manufacturers make a 12.5-cm^2 version) for the first daughter cultures because these can be tightly capped to reduce the chance of contamination and dehydration.

2. Typically, a gentle rinse of the culture surface can be used for performing this first subculture and the entire medium is transferred from the culture into the new container; 1.0 mL fresh medium is added back to the original dish.

3.5. Additional Subcultivations

If there are a substantial number of attached cells remaining, then another subculture may be performed within 1 or 2 wk. For the second split, a more vigorous method is typically used for detaching the cells.

3.5.1. Cultures in a Flask

1. Chill the culture for 20 min at 4°C.
2. Use a pipet to flush the growth surface with medium.

3. Transfer contents to a new culture flask with a volume of fresh culture medium equivalent to 50–100% of the medium from the primary culture.
4. Add fresh medium back to the original culture.

3.5.2. For Cultures in a Petri Dish

1. Remove the medium by pipet (this can be added to the "pour off" culture or to the suspended strain created with the first split).
2. Add 1.0 mL enzyme buffer to rinse the culture surface.
3. Discard the rinse (or again pool it with the suspended cell culture).
4. Add 0.5 mL trypsin. Different cell strains can respond differently to trypsin, so it is recommended that the culture be checked with an inverted scope at 2- to 3-min intervals until the cells start to detach.
5. When cells become detached, add 1.0 mL fresh medium and suspend cells by gently drawing in and releasing the medium from the pipet.
6. Transfer the contents to a 12.5- or 25-cm^2 flask and rinse the primary dish culture with another 1.0 mL medium, also adding it to the daughter culture. The final volume should be approx 2.0 mL in a 12.5-cm^2 or 4.0 mL in a 25-cm^2 flask.
7. Add 1.0 mL fresh medium back to the original primary culture and reseal with Parafilm.

   These various subculturing methods typically lead to different cell strains that will have different properties such as susceptibilities to or productivities of viruses. Even if these features are not different, the cultures can be used for different purposes (plaque assaying/cloning with the attached strain, large scale suspension cultures with the unattached strain).

3.6. Strain Selection

3.6.1. Growth Rate

As soon as cells begin growing in a primary culture, a Darwinian natural selection process begins based on a number of characteristics. The most obvious of these is growth rates. If two cells exist in a culture, one of which completes the cell cycle in half the time of the other, the slower cell type will be outnumbered 1000 to 1 within 10 of its divisions and 1 million to 1 within 20. Every time you split such a culture, the proportion of faster growing cells becomes greater until none of the slow growing cells are transferred to the new culture.

3.6.2. Attachment

Other features besides growth rates can influence the distribution of cell types in the culture, as well. For example, suppose in the previous example the faster growing cells were much more adherent to the culture flask so that every time you split cells using the flushing method (described in Chapter 9), half of them were either mortally damaged or remained attached to the old culture. In

this situation, if you split the cultures at a 1:2 ratio at each passage, the fast and slow growing cells would exist at an equal level in each new culture. Alternatively, if the normal trypsin method was used for subculturing (also described in Chapter 9), then loosely and nonattached cells are removed from the culture prior to the enzyme treatment and thus those types of cells would be depleted from the cell line very quickly. Because of these and other factors affecting the cell population in cultures, more than one subculture method is often used, especially on early passage cultures, so as to maintain the widest diversity of cell types until some indication of which cell type is useful in the specific application for which it is needed. As an alternative to varied subculture methods, cells could be cloned at an early passage.

4. Notes

1. Several manufacturers now supply insect cell culture media and many of these are specifically designed for lepidopteran cultures. Previously, the author's medium of choice was TC-100 (originally described as BML-TC/10) ***(6)*** with some additional supplements (peptones, additional vitamins, and trace minerals as described previously ***[1]*** but in recent efforts, both the author ***[7]*** and other researchers ***[8]*** have used one of the serum-free formulations such as the Ex-cell 400 series of media [JRH Biologicals, Lenexa, KS], Sf-900II [Invitrogen/GIBCO, Carlsbad, CA], or Insect Express [HyClone, Logan, UT]). These can support growth of established insect cell lines without any additional supplements, but the addition of a small quantity, typically 5–10% (v/v) of fetal bovine serum (FBS) is recommended when using them in attempts to establish new lines. FBS is known to provide growth factors, typically small proteins that stimulate cell division in vertebrate cells. Little is known about these factors in insects although gene homologs have been found in some insects. Still, even if the mammalian factors are different, some positive effects are observed with FBS in insect cell cultures making it an effective supplement for primary culture work.
2. In Chapter 9, the author expounds on the virtues of not using antibiotics in continuous cell lines. Unfortunately, it is a fantasy to expect primary cultures from insect tissue to remain uncontaminated without the use of an antibiotic. Gentamycin is a broad-spectrum antibiotic that has relatively mild or no toxicity to eukaryotic cells. Researchers also have successfully used a penicillin-streptomycin combination (typically used at 100 U penicillin and 0.1 mg/mL streptomycin culture medium) to reduce contamination in primary insect cultures. Antifungal agents are particularly toxic to insect cells ***(9)***, probably because the biochemical pathways they target also occur in insects and thus the author does not recommend their use in efforts to establish new cell lines.
3. Most researchers working with cell cultures and viruses will find a biological safety cabinet a better choice for routine culture work. Unfortunately, most of these have a glass front that makes it impossible to use a microscope in the hood. A clean bench is much more appropriate for performing dissections while using a

microscope but, unless you are performing a lot of dissections, it may not be worth the extra expense of having both types of hood. If your facility has many other researchers, you may want to see if you can use someone else's hood for these procedures. Alternatively, the dissection can be performed without a hood. In this case, you should select a small room that can be kept closed during the dissection to minimize airflow across the field.

4. These are available from suppliers specializing in surgical equipment because they are also used in procedures such as eye surgery and other delicate operations.
5. Placing a layer of cotton in the bottom of the jar serves two purposes. First, the delicate tools can be placed in the alcohol with less chance of damaging the tips and, second, the cotton can be used to wipe off tissue fragments that adhere to the instruments during the dissections.
6. The wind screen is helpful in keeping the flame steady while working in the laminar flow hood's air current. The author has made one from a one-pound coffee can **(Fig. 1)**. A similar structure can be constructed from a heavy gauge aluminum foil.
7. Most lepidopteran larvae live in relatively clean environments. If you are using a laboratory colony on artificial diet, you may want to consider adding some antibiotics to the diet for the insects you use for setting up cultures. If initial efforts with a short disinfection time results in many contaminated cultures, a longer disinfection with 70% ethanol can be used or 0.05% $HgCl_2$ in 70% ethanol can be used instead. If the tissues of interest are embryos, the eggs can be pretreated for 1–2 min in 2.6% sodium hypochlorite (=50% [v/v] household bleach in distilled water), which will soften the chorion making removal of the embryos easier. The eggs are then rinsed at least three times with sterile distilled water prior to disinfection with ethanol (5 min should be sufficient because the sodium hypochlorite is also a disinfecting solution).
8. Care must be taken in flaming the instruments. Hold the instrument nearly parallel to the floor, with the tips just slightly lowered. Having the tips higher than your hand can result in the burning alcohol flowing down the instrument onto your hand, whereas holding them with the tips directly below your hand will result in the heat of the flame being directly below your fingers. Because the laminar flow clean bench is also blowing air toward you, the flaming instruments should be held on an angle so that your hand is not directly behind the flame.
9. Pinning the insect in this way serves a couple of functions. Most importantly, it immobilizes the larva so that the dissection can be performed more easily. In addition, many lepidopteran larvae regurgitate or defecate when they are disturbed. Although the anesthetic effect of the submersion in ethanol and water rinses should minimize this, inserting the pins as I have describe will effectively block these activities. Of course, the digestive tract is almost certainly pierced when you do this so the pins should not be removed until the dissection is complete and a new dish must be used for the next larva or there will be a greater risk of contamination from the gut contents.

10. If you frequently rupture the digestive tract during the dissection, then I suggest starving the insect for a few hours to reduce the amount of gut contents.
11. These have never been used for initiating cell lines from Lepidoptera but the results by Engelhard et al. ***(10)*** indicate they could be extremely useful as indicator cells because in some virus/insect systems, they are responsible for initially spreading the infection.
12. As discussed in Chapter 6, a wide variety of tissues have been used for establishing lepidopteran cell lines. Although not discussed in detail in this chapter, embryos are a common source of cell lines and one that the author has favored over the years. Although less precise than the larval tissues discussed in this chapter, progenitors for every cell type exists in the embryos and many of them are already in active states of cell division. The author's usual method for initiating primary cultures from embryos is to disinfect eggs (approximately half way through development to a first instar larva) in 70% ethanol followed by two sterile demineralized water rinses and then a transfer to culture medium containing antibiotics as described above. Squeezing one end with the microforceps ruptures the chorion or it can be cut with a microscalpel. Twenty to 50 embryos are then transferred to a standing drop (100–150 µL) of fresh medium with antibiotics and treated in the same manner as the specific tissues in **Subheadings 3.2.–3.4.**
13. Although I suggested using a wax dish and insect pins in the previous section to aid in the dissection, as you become accustomed to manipulating larvae and the dissecting instruments, you may be able to leave the insect unpinned and simply grasp it with a pair of forceps while cutting the cuticle and isolating the tissue. An advantage of this technique is that a tissue culture grade dish can be used in the initial dissection and, after the tissues of interest are removed, this dish will contain a substantial number of hemocytes and can become a primary culture itself **(Fig. 8A)**.
14. Many insect tissues have a wound response to cuts and will initiate cell division spontaneously. However, treatment with a mild concentration of trypsin, but not long enough to completely disassociate the tissue to single cells may improve migration and growth of cells from the tissue explants. Use 50 µg/mL trypsin in the divalent-free phosphate buffered saline. Treat the tissue for 5–20 min and then replace the trypsin solution with medium (100–150 µL in a standing drop) containing 50 µg/mL gentamicin. Two forceps are then used to tear it into smaller fragments.
15. The cell number necessary for successful subcultivation is not a precise quantity and is one of the decisions that become easier as you gain experience. If you let the cells become too dense, there is a potential that they will deplete the nutrients in the medium and stop growth. Alternatively, many cells produce autocrine growth factors that stimulate their own growth (as well as that of neighboring cells). If cultures are split too early, resulting in very low cell densities, these growth factors will disperse into the medium causing a reduced or even inhibition of cell growth.

Acknowledgments

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