IN THE LABORATORY

A Staining Procedure for the Study of Insect Musculature[1]

SOL KRAMER

University of Illinois, Urbana

Microdissection and study of insect musculature is frequently a slow and tedious task. When adult insects are killed with no particular effort to assure differentiation and recognition of the muscles, the difficulties of locating their exact origins and insertions are considerably increased. It is, however, possible to obtain good preparations of adult insect muscles in many cases by the use of special killing and fixing agents. Chloral hydrate and several fixing fluids containing formaldehyde can be used to distinguish the muscles from other internal tissues.

With soft-bodied larval forms of insects such as the Diptera, which frequently have a large amount of adipose tissue running through the body cavity, the problem of differentiation becomes more acute. When killed and fixed, the muscles of such larvae are often the same milky white color as the fat body and other internal tissues, and the origins and insertions of the separate muscles are confused. In other cases the muscles remain completely transparent and unrecognizable.

In the hope of finding some method of obtaining good *in toto* muscle differentiation, a series of killing and staining procedures was undertaken. Reared, 4-day-old housefly larvae were used in these procedures. One of the attempts not only gave remarkable differentiation of the muscles, but made it possible to see almost every separate muscle quite clearly when viewed with a binocular microscope, directly through the body cuticle, thus eliminating the need for dissection. In addition, due to the peculiar optical properties which this technique brings about, it is possible to see first the outermost body muscles, then the intermediate muscles, and finally the innermost muscle bundles by rotating the specimens in the light. As any one layer of muscles is visible in a given position in the light, the other muscles are almost transparent.

The steps in this procedure are: (1) Bouin's fluid (30° C), 8–10 hrs; (2) 50% ethyl alcohol, 10 min; (3) 70% ethyl alcohol, 1 hr; (4) 95% ethyl alcohol, 10 min; (5) 0.5% eosin alcohol, 6–8 hrs; (6) return to 95% alcohol and add oil of wintergreen dropwise to larvae in 95% alcohol at hourly intervals over a period of 4–5 days; and (7) transfer larvae to oil of wintergreen.

The Bouin's fluid should be prepared with formalin solution which has been neutralized with magnesium sulfate. The larvae die slowly when dropped into Bouin's fluid, permitting the fixative to penetrate the body tissues. Most of the larvae are killed within an hour or two, but occasional larvae may stay alive in this fluid for 4 hrs. After being removed from the fixative and washed in 50% ethyl alcohol, the larvae are turgid but exceedingly soft and pliable. The outer cuticle has a glistening appearance and is entirely transparent, as are the muscles. The silvery luster of the main longitudinal tracheal trunks and their branches are distinctly visible through both the cuticle and body muscles. Also visible are the fat body, the ventral nerve branches, the alimentary canal, the cephalopharyngeal mouth hooks, and other internal organs.

As indicated above, the larvae are then transferred to 70% alcohol for 1 hr, but may be stored in 70% alcohol for several days or longer. They are then removed to 95% alcohol for 10 min prior to being placed in a 0.5% eosin solution (wt/vol) in 95% alcohol, where they are kept for 6–8 hrs, or until they take on a light pink color, after which they are returned to 95% alcohol.

The addition of oil of wintergreen, which will replace the 95% alcohol, is the most critical step in this procedure. If this is not carried out gradually, either the larvae will collapse and shrivel or the cuticle may split and the body organs be displaced. A dozen or more larvae are placed in a stendor dish, or other suitable vessel, with about 8 cc of 95% ethyl alcohol, which is sufficient to immerse them completely. Four to 6 drops of oil of wintergreen are added to the dish and swirled until uniformly miscible with the alcohol. This procedure is repeated at about hourly intervals during the working day over a period of 4–5 days. The stendor dish should be kept covered to prevent excess evaporation of the alcohol, as such evaporation will also cause collapse of the larvae. Occasional examination of the larvae should be made with a binocular microscope during the period of addition of the oil of wintergreen in order to detect possible collapse of the specimens. If the larvae show signs of flattening, the oil of wintergreen should be added at intervals of several hours.

With the addition of oil of wintergreen, the larvae become opaque as the muscles become visible. The larvae retain their pink color for about 4 days and then turn orange red. At this point the remaining alcohol may be evaporated from the solution by removing the lid of the stendor dish for several hours. The larvae are then transferred to oil of wintergreen.

When a spot of light is directed into any portion of a larva as it is studied beneath a binocular microscope, the larva takes on a yellow-green color. The green-tinted cuticle is transparent with distinctly visible opaque muscles beneath. The individual muscle fibers of each muscle band can be seen readily, and their exact insertions on the cuticle are visible. As a larva is moved in the light, the different muscles of the various muscle layers show up distinctly, and it is possible to obtain an accurate knowledge of the relationship of almost all somatic muscles in this way.

[1] Contribution No. 285, Department of Entomology, University of Illinois, Urbana.

This technique has given good results with other dipterous larvae such as the rat-tailed maggot (*Eristalis* sp.) and has rendered visible the muscles of the head of mosquito larvae through the head capsule. It has also proved applicable for the study of the relationship of some of the thoracic muscles of an adult homopteran insect (*Ceresa bubalus*) and was particularly useful for delineating the muscles of the small labium. It is possible that the staining and optical properties of this technique, with various modifications, may be useful for the study of the musculature of other insects and soft-bodied invertebrates.

References

1. GUYER, M. F. *Animal micrology.* (4th rev. ed.) Chicago: Univ. Chicago Press, 1936.
2. LEE, A. B. *The microtomist's vade-mecum.* (10th ed.) Philadelphia: Blakiston, 1937.

A Practical Method for the Illumination of Biological Material With Ultraviolet Rays

BARNEY V. PISHA

Madison Foundation for Biochemical Research, Inc., New York City

The study of self-fluorescence in tissues and vital staining with fluorescent dyes in the living body requires a source of ultraviolet light that is intense and at the same time constant. Until recently we had been unable to find a source of ultraviolet light embodying all these features. The arc light was usually used when a source of ultraviolet light was needed in a laboratory. However, it always had three major drawbacks: (1) it gave off an intense amount of heat which made the operator rather uncomfortable; (2) the automatic carbon feeder never worked properly; and (3) the carbons never lasted more than 30–45 min, usually extinguishing themselves just when something of interest was visualized. Then, also, there had to be a short delay for the lamp housing to cool off so that the operator could change the carbons. This caused the investigator who was studying the movement of fluorescent solutions in the living body to miss a part of the action taking place during this interval. After much trial and error it was found that certain microscope lamps available on the market could be easily converted into a very efficient source of constant and intense ultraviolet light.

For general all-round efficiency, the Bausch & Lomb spherical lamp housing was found to be the best in the low-priced field. The B & L research lamp housing can be used with one minor change. The Spencer lamp housing (#370-A) is also readily converted, but in our experience it was found to have some disadvantages.

In converting the B & L spherical lamp housing, we removed the bayonet type socket and substituted a porcelain-shelled admedium socket (GE #3280). To hold this socket in place and to enable regulation of its vertical movements, a special holder was constructed along the lines of the original holder for the bayonet type socket. This holder was made in the form of a ring to encircle the admedium socket, a lip being made on each end so that the ring could be tightened around the socket. On opposite sides of the ring were brazed two slotted upright pieces which would fit under the thumb screws of the lamp housing provided for this purpose. These thumb screws

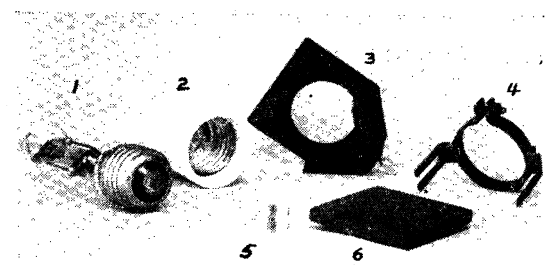

FIG. 1. Accessories: 1, AH-4 mercury vapor lamp; 2, admedium lamp socket; 3, filter holder; 4, socket holder; 5, slotted holder for filter holder; 6, ultraviolet filter.

hold the socket stationary after the vertical centering of the lamp is accomplished. To complete the lamp housing we used a B & L filter holder, which was placed in front of the iris diaphragm. To hold it in place a piece of metal was slotted on both ends and bent in such a manner that the opening of the filter holder was centered with respect to the condenser. One slotted end of the metal strip is slipped under the thumb screw holding the iris diaphragm in place, while the opposite end is used to hold the filter holder in place by its thumb screw. Fig. 1 shows the individual parts of the ultraviolet lamp and in Fig. 2 the completely assembled lamp is seen.

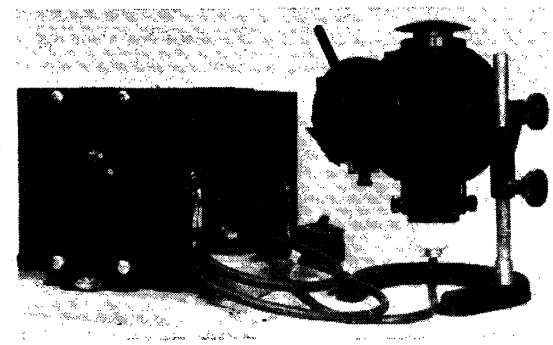

FIG. 2. Assembled ultraviolet lamp including transformer and lamp housing.

The constant and intense source of ultraviolet light we use is the General Electric AH-4 100-watt mercury vapor lamp. To operate this lamp a General Electric Autotransformer No. 59G22 is absolutely necessary.[1]

For filtering out the visible light a number of glass filters are available. We have found that those most suited for ultraviolet light are the Corning glass filters

[1] Our AH-4 mercury vapor lamp, porcelain admedium socket, and autotransformer were obtained from G. W. Gates & Co., Franklin Square, Long Island, New York.