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Electron Microscopy of Living Insects

Abstract. *Electron micrographs of living specimens of the various developmental stages of the insect* Tribolium confusum *have been obtained with a scanning electron microscope. In most cases the specimens resumed their normal activity after being examined with the electron microscope and underwent metamorphosis into the next stage.*

Successful electron microscopy of living material must overcome the effects of the vacuum and of the electron beam on the specimen. Most studies have concentrated on mitigating the effect of vacuum (*1*). We report some results obtained using a scanning electron microscope (*2*), in which the electron current is very much lower than that in the conventional electron microscope (*3*).

Table 1. Survival of *Tribolium* after exposure to a pressure of 10^{-3} torr for 30 minutes.

Developmental stage	Specimens (No.)	Survived (No.)
Eggs	78	56*
Larvae	30	30
Pupae	30	30
Adults	30	30

* Represents the approximate hatch rate of control eggs.

Table 2. Survival of *Tribolium* after exposure in the scanning electron microscope.

Developmental stage	Specimens (No.)	Survived (No.)
Eggs	6	3*
Larvae	2	2
Pupae	4	4
Adults	2	1†

* Represents the approximate hatch rate of control eggs. † The adult that died suffered prolonged (50 minutes) exposure to the electron beam. Leg motion was observed in this animal after exposure. Although exposure to scanning electron microscopy did not prevent the insects from passing through successive stages of development, latest observations on these animals two months after exposure suggest that adult lifetime is significantly shortened.

The initial search was for specimens capable of surviving a vacuum; a previous report that a beetle had apparently survived being placed in a vacuum did not identify the species (*4*). The criterion for "survival" was that the specimen be able to pass into the next stage of development with normal appearance and activity.

We used *Tribolium confusum*, a small beetle that normally lives in a dry environment and whose behavior and appearance are well documented. Radiobiological studies of this particular insect have been carried out for some years at this laboratory (*5*). Samples of the eggs, larvae, pupae, and adults were kept for 30 minutes in a chamber evacuated to a pressure of 10^{-3} torr (Table 1). All developmental stages of *Tribolium* can survive this vacuum.

Samples of eggs, larvae, pupae, and adults of *Tribolium* were viewed with the scanning electron microscope. The periods of exposure to the vacuum varied from 2 minutes to 1 hour; the periods of exposure to the electron beam were of the same order. The electron current varied from 2×10^{-11} to 2×10^{-10} amp, and the electron energy was 25 kev. Living *Tribolium* in all four stages can be observed in the scanning electron microscope (Table 2).

Although the highest magnification shown in the micrographs is only 670 times, there is no reason why the full resolution of the scanning electron microscope should not be used if necessary—that is, a useful magnification of 20,000 times (*3*). In our experiments, the specimen was placed farther from the final lens so that an area large enough to show the whole insect could be scanned; this resulted in poorer resolution. Even so, the depth of focus shown in these micrographs is greater than that which could be attained in a light microscope.

Consider the density of radiation at the surface of a specimen when irradiated by a stationary electron beam with a current of 10^{-11} amp, energy of 25 kev, and a small diameter. The range in carbon of electrons having this energy is about 10^{-3} cm (*6*), and x-ray production is negligible. One can calculate the energy dissipated per unit volume assuming that the energy is dissipated uniformly in a hemisphere with a radius of 10^{-3} cm (that is, with a volume of 2×10^{-9} cm^3); hence the power dissipated is about 100 watts cm^{-3}. This represents a dose rate of 10^7 rad/sec or about 1 percent of the dose estimated for a specimen in the standard, transmission, electron microscope (*7*).

If the beam scans an area of 1 mm^2 (corresponding to a magnification of 100 times), then the power per cubic centimeter is reduced to

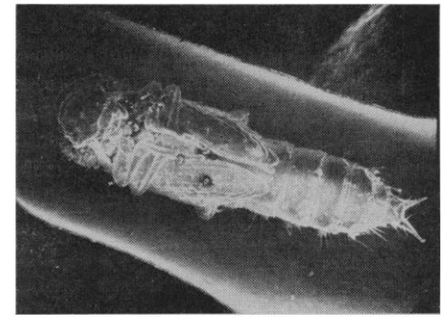

Fig. 1. Scanning electron micrograph of entire living pupa of *Tribolium confusum* (× 15). This picture demonstrates the very large field of view which can be studied with the scanning electron microscope.

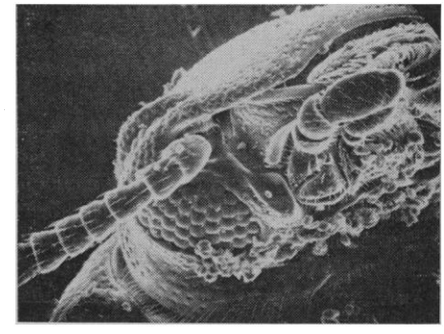

Fig. 2. Scanning electron micrograph of the head of a living adult *Tribolium confusum* (× 109). One antenna, one eye, mouth parts, and many other details are clearly visible owing to the large depth of field.

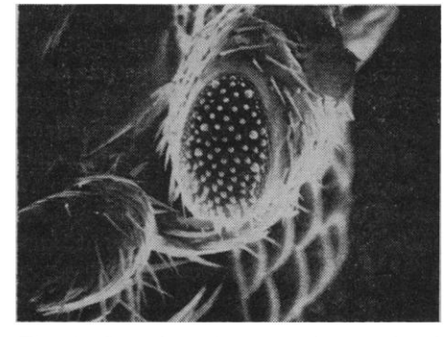

Fig. 3. Scanning electron micrograph of the end of the mandible of a living adult *Tribolium confusum* (× 436). The "papillae" are clearly visible; the eye is partly visible in the background.

about 2.5 × 10^{-2} watts cm^{-3}. If the magnification is raised to 10,000 times, then the area scanned is about 10^{-6} cm^2, and the value of power per unit volume increases to approximately that for a stationary beam. Although these dose rates are quite large, only the outer 10^{-3} cm of the specimen is significantly irradiated.

Consider now the thermal effect of an electron beam current (energy of 25 kev) scanning an area of 10^{-4} cm^2; if this area is (for mathematical convenience) in the form of a concave hemisphere of radius 5 × 10^{-3} cm, one can calculate the steady state temperature rise θ at this surface. If the heat loss is solely by uniform thermal conduction through the tissue, then θ can be expressed as

$$\theta = \frac{P}{\sigma}\int_{\infty}^{5 \times 10^{-3}} \frac{dx}{2\pi x^2}$$

where P is the beam power, σ is the thermal conductivity of the tissue, and x is the distance into the tissue. In our case P is equal to 25 × 10^{-8} watt and σ is equal to 20 × 10^{-4} watt cm^{-1} $°C^{-1}$ [this is the value quoted for wood (*8*); no figures were available for insect tissue]. Hence θ is equal to 0.005°C. This increase should not be troublesome; the fact that the increase is so small may be one reason why scanning electron-micrographs can be taken of living specimens, apparently without serious effects caused by electron irradiation.

The current or the energy of the electron beam can be raised, and hence it should be possible to study the biological effects of irradiation on selected areas as small as 10^{-6} cm^2 in living specimens of the various developmental stages of *Tribolium*.

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Establishment of Four Functional, Clonal Strains of Animal Cells in Culture

Abstract. *The single-cell plating technique was used to develop four clonal cell lines that perform organ-specific functions after being serially cultured for prolonged periods. These strains include steroid-secreting Leydig cells, melanoma cells that form pigment, and two strains from a hormone-secreting rat pituitary tumor. One of the cell lines from the pituitary tumor secretes growth hormone, while another line derived from the same tumor secretes a substance similar to adrenocorticotropic hormone.*

The loss of organ-specific function is commonly observed in cultured animal cells during serial propagation (*1*). This loss has been ascribed to (i) selective overgrowth by connective tissue cells, (ii) a phenotypic change in the cultured cells, or (iii) inadequate or harmful environmental conditions (*1*). By using the single-cell plating technique to eliminate the possibility of selective overgrowth, we have established four new clonal cell strains. Even after having been continuously cultured serially for a period of up to 1 year, each cell line performs at least one of the major organ-specific functions of the tissue from which it was derived.

Cultures were established by the method of alternate culture and animal passage (*2*). Primary cultures were made from three transplantable animal tumors that retain differentiated function after serial transfer in animals: (i) a rat pituitary gland tumor, MtT/W5 (*3*); (ii) a tumor of Leydig cells from mouse testicle (*4*); and (iii) a mouse melanoma (*5*). After short periods (3 to 10 days) in culture, the surviving cells from each type of tumor were harvested and injected into appropriate host animals. In this way new tumors were obtained, and these, in turn, were placed in culture. This process was repeated from three to six times with each tumor. As Buonassisi *et al.* had previously shown for adrenal tumor cells (*2*), these new culture-derived tumors also had an enhanced ability to survive and grow in vitro. The rationale for this approach is that the initial culture periods select for cells that can withstand the conditions of culture. A further advantage of this method is that it does not select for supporting tissue cells, which usually overgrow the desired cell type. Since the connective tissue cells are not malignant, they would not be expected to survive grafting and would not contribute to a new tumor. Cultures were grown in plastic petri dishes (60 by 15 mm) and incubated at 37°C in a humidified atmosphere of 5 percent CO_2 and 95 percent air. Synthetic medium F10 (*6*) was supplemented with 15 percent horse serum and 2.5 percent fetal calf serum. The cells were recovered for subculture (at 2-week intervals) by being incubated in 0.1 percent Viokase solution in phosphate-buffered saline for 5 to 10 minutes at 37°C (*7*). Single-cell platings, without feeder layer, were made from each of the cultures by the method of Puck, Marcus, and Ciecura (*8*). Stainless steel cylinders were used for the isolation of individual colonies. The isolated colonies were detached and dispersed into single cells with Viokase solution. The dispersed cells were counted, serially diluted, and distributed in individual petri dishes. Plating efficiencies of 5 to 10 percent were regularly obtained. Original clonal strains were recloned, in some instances more than three times, and no morphological or functional differences between the parent and derived clonal strains were seen.

We determined differentiated function of each clonal cell line by examining the cells or the culture medium for a product characteristic of the tissue from which the cells were derived. We tested the medium from the cultures of pituitary cells for adreno-