samples. One can visually determine when the specimens have attained dryness. The stage is next warmed slowly (10 to 12 minutes) to room temperature, and the specimens are removed. The aluminum specimen holders are then attached with Duco cement to the specimen stubs of the scanning electron microscope.

The specimen stubs are placed in a vacuum evaporator, and a thin film of equal parts of gold and palladium is evaporated at a high vacuum (5×10^{-6} torr) onto the specimens rotated 50 to 100 times per minute so that the metal coats all parts of the specimens. Evaporation is stopped when sufficient metal has been deposited to produce a slight metallic sheen (probably about 100 Å thick). Specimens are then stored in vacuum or chemical desiccators until examination. After storage they can be used repeatedly in the scanning electron microscope.

Pseudopods of sarcodinids are well preserved in the freshwater *Amoeba proteus* (Fig. 2). The myriad patterns and arrays of cilia so diagnostic for ciliate systematics are preserved, and when coupled with the great depth of field and excellent resolution of the scanning electron microscope, they can be seen as never before. Holotrichous ciliary patterns (Fig. 3 of *Nyctotherus* and Fig. 4 of *Paramecium*) are readily observable as are the ciliary pectinelles of *Didinium* (cover photo and Fig. 1), the cirri of *Uronychia* (Fig. 5), and the adoral zone of membranelles of *Tetrahymena* (Fig. 6) and others (Figs. 3 and 5). Loss of cilia may result from the unequal distribution of chemical fixative into the microdrop precisely when the microdrop is dropped into the fixative, or from an extant physiological condition at the instant of fixation (Fig. 7). Transmission electron-microscope investigation of the feeding of *Didinium* (*8*) indicates that, at the time of ingestion of *Paramecium*, the cilia of the prey are lysed as it enters the cytopharynx of the predator (cover).

Newly emergent cilia appear in cells fixed at appropriate times during the division process. The stepwise formation of neocilia in dividing *Didinium* has been observed (Fig. 1). Between the metachronous waves of the parental-ciliary girdle in the upper hemisphere, emergent cilia of different lengths appear, none of which are as long as the cilia of similar parental structures. In the posterior hemisphere, a light band is observable—presumably the anlage of the posterior ciliary girdle.

Although the examples presented here are protozoa, the fixation-sublimation technique can be used with other biological materials of small volume. Because the scanning electron microscope provides information on spatial relations, the biologist is freed from the restriction imposed by microtome sections, and many problems can be solved by more direct, simpler means.

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27 December 1968

Automatic Identification and Measurement of Cells by Computer

Abstract. *A visual-input computer system was used to automatically locate and perform measurements with high accuracy on single cells from microscopic slides. The nuclear radius, irregularity, area, and density of normal and malignant cells were automatically measured. These studies agreed with hand measurements and showed differences between normal and malignant cervical cells on Papanicolaou smears.*

We have used visual-input computer systems to locate and perform quantitative measurements on cells on microscopic slides. The purpose of our study was to develop methods for the automatic machine differentiation of normal and malignant cells exfoliated from the exocervix and to serve as the basis for an automated screening procedure for Papanicolaou smears. As a result, pattern-recognition programming techniques were developed that may be of value in the automated analysis of other cytologic material.

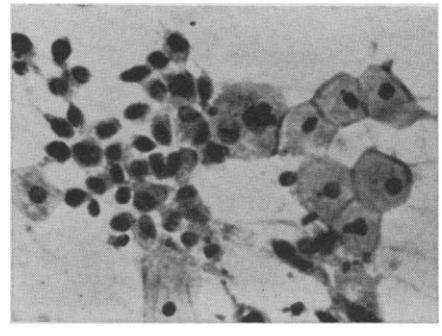

Fig. 1. Photograph of a microscopic field from a Papanicolaou smear of the cervix showing malignant cells (left) and normal squamous cells (right).

The cells were obtained by gently scraping the uterine cervix with a spatula. The material was placed on slides and fixed and stained according to the method of Papanicolaou (*1*). Both normal and malignant epithelial cells were present on the slides (Fig. 1). The smears were photographed at magnifications of ×25 to ×400 through a Zeiss microscope on Kodak Panatomic-X film. Although we have used film, the principles involved in direct examination of the slide are similar but require some equipment modification.

The photographs were scanned with a high-resolution programmable film reader (PFR-3, III) under the control

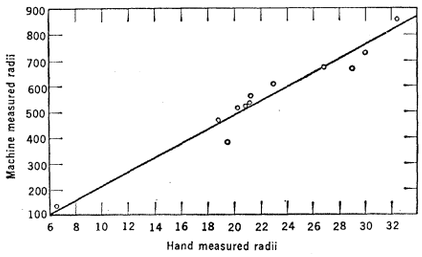

Fig. 2. Test of the accuracy of the computer system measurement of nuclear radius. Twelve cells were measured by both human and machine. The correlation between these measurements is high (correlation coefficient = .98).

of a high-speed digital computer (PDP-9, DEC). The film reader determines the density at a point on the film whose coordinates are specified by the computer and transmits this information to the computer; the computer uses the information to determine the next point to be measured by the film reader, and the process is repeated. In this way, either the entire photograph or only significant portions of it can be analyzed. The equipment and the computer programs have been described (*2*).

For the automated analysis of cells on smears, two programs were developed. The first program identifies cells on the slide, distinguishing single cells from overlapping cells, dirt, clumps, or other artifacts. The second program performs measurements on the identified cells.

To identify cells a random search of the photograph is performed for areas of specific density which could subsequently be examined for size and shape criteria specified for single cells (*2*). To test the ability of the program to locate single cells, photographs of the slides were automatically scanned by the computer system and the radii of all suspect cells were automatically measured. A human observer then examined the photographs and selected single cells, and the machine was used to measure the radii of these cells. The distribution of the radii obtained by machine-selected cells and by human-selected cells was virtually identical. Direct observation of the screening system on a video-display system also demonstrated that the computer rarely mistook artifacts for single cells.

Each time the computer system located a cell, its exact X and Y coordinates were determined. Repeated measurements of the location of a single cell revealed that this location could be determined to within 0.1 percent.

For our initial studies, measurements were made on each cell by the computer system. These were the average nuclear radius, the average deviation from the average nuclear radius, the nuclear area, and the average nuclear density.

The average radius was determined by locating the center of mass of the nucleus and measuring the distance to the nuclear boundary in 64 different directions from the center. This measurement was done 20 times for each cell to minimize error. The average radius thus obtained is a measure of nuclear size. The average deviation of these radii from the average radius is a measurement of nuclear irregularity. The nuclear area was measured by summing the calculated areas of each of the 64 sectors between pairs of adjacent radii. The nuclear density was determined by measuring the density on a scale of 1 to 64 of over 1000 points within the nucleus. These four measurements on each cell were performed in less than 1 second.

The accuracy of these computer measurements was tested by comparing the results of the machine with those of human observers. The average of the radii of 12 selected cell nuclei was measured by the computer as described. Enlarged photographs of these same cells were obtained by hand measurement of over 15 radii per cell. The computer measurements were plotted with the human measurements for each of the 12 cells (Fig. 2). The average nuclear radius obtained by the machine from measurements on over 100 normal cervical cells was 3.38 ± 0.70 μ. Reagan (*3*), measuring normal cervical cells by hand, reported an average nuclear radius of 3.37 ± 0.60 μ. The agreement between these figures is additional evidence for the accuracy of the machine measurements.

The computer system was then used to measure over 100 malignant squamous cells from the cervix and over 100 normal cervical squamous cells (Fig. 3). The normal and malignant populations differ in average nuclear radius, average deviation of the nuclear radii, nuclear area, and, to a lesser extent, nuclear density.

These criteria have not yet been combined as a screening technique. Many other kinds of measurements on cells can be used to distinguish among types of cells in this computer system. Thus, this computer system accurately can locate and precisely measure cells automatically, and is an aid in gathering quantitative cytologic information.

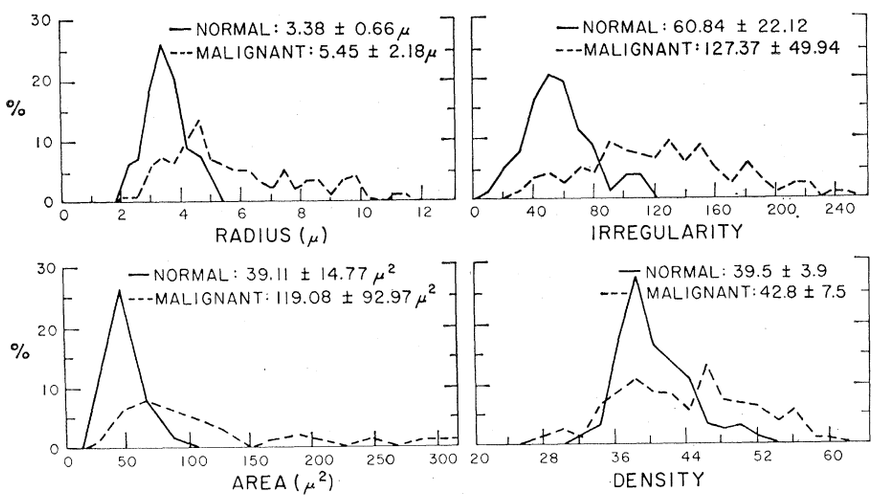

Fig. 3. Automatic computer measurement of over 100 normal and 100 malignant cervical cells. The distribution of several different geometric parameters of the cell nucleus are shown. (Upper left) Average radius. The distribution of this parameter of the two cell types is clearly different. The average radius represents the average of 1280 measurements on each cell. (Upper right) Irregularity. The average deviation of the 1280 radii measured on each cell nucleus is a measure of nuclear irregularity. Malignant nuclei are seen to be more irregular than normal nuclei. (Lower left) Area. Malignant nuclei are on the average larger than normal nuclei. (Lower right) Density. Only small differences in average density are seen between the two cell types.

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Ethylene: A Factor in Defoliation Induced by Auxins

Abstract. *Aerial sprays of synthetic auxins defoliate many species of tropical trees. Treatment of* Euonymus japonica *leaves with the* n*-butyl ester of 2,4-dichlorophenoxyacetic acid causes premature senescence and leaf fall and stimulates ethylene production by the blade 5- to 25-fold. Exposure to ethylene alone similarly accelerates senescence and leaf fall. Evidence indicates that the defoliant action of auxin is mediated through the enhanced amounts of ethylene in the blade.*

The esters of synthetic auxins have been used as defoliants of tropical trees and shrubs for the past 20 years (*1*). Foliage sprayed with preparations of the *n*-butyl ester of 2,4,-dichlorophenoxyacetic acid (2,4-D) or of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) becomes pale green or yellow within a few days, and the leaves of susceptible species may be shed a week later.

Under natural conditions a leaf undergoes abscission only when the blade and petiole are yellowing or senescent, or when the leaf is subjected to environmental stresses which lead to changes in the synthetic activity of the blade, similar to those that occur during normal senescence. A young green leaf which is synthetically active does not abscind (*2*). Young leaves have a relatively high auxin content; during aging and senescence the amount of auxin decreases. If auxin is applied to a debladed petiole stump or to the distal end of an isolated abscission zone (explant), abscission is retarded. Auxin can therefore substitute for a young leaf blade in preventing abscission, and much evidence supports the view that as long as the auxin content of a leaf blade is high, yellowing and senescence of the blade and petiole do not occur and the leaf does not fall (*2, 3*). How then does the addition of auxin to a leaf blade lead to premature abscission?

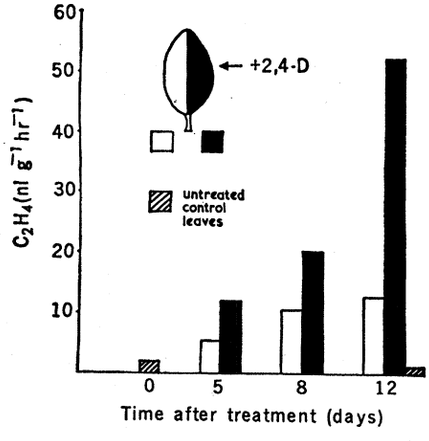

Fig. 1. Branches of *E. japonica* were maintained under greenhouse conditions. On day 0, the right half of the upper surfaces of about 600 2nd-year leaves were painted with the *n*-butyl ester of 2,4-D in methanol (6.67 mg/ml); control branches were untreated; 5, 8, and 12 days later leaves were removed and divided down the main vein. The half leaves were packed loosely in the dark glass jars and a current of moist ethylene-free air was passed through them for 18 to 24 hours at 26°C. The ethylene was collected in 0.25*M* mercuric perchlorate in 2*M* $HClO_4$ and was subsequently analyzed by gas chromatography. On day 0 and day 12 ethylene was also collected from control leaves from untreated branches. Values at day 0 represent ethylene production from 2nd-year (green) or 3rd-year (yellow) leaves. Values for control leaves on day 12 refer to 2nd-year leaves.

We have investigated this problem in the temperate evergreen shrub *Euonymus japonica.* Second-year leaves are induced to abscind within 10 to 14 days by application of droplets of the *n*-butyl esters of either 2,4-D or 2,4,5-T in methanol to the upper surface of the blade, and the pattern of events leading to leaf fall seems identical with that occurring in the tropical species examined earlier (*4*). The treated part remains green, but by day 4 after treatment, yellowing begins at the periphery of the treated area and subsequently extends outward. Abscission takes place when the petiole and major part of the blade are yellowing. After abscission, the treated area remains as an island of green tissue in the yellow blade (*5*). Experiments with 2,4-D labeled with ^{14}C have shown that the label (2,4-D as such) is restricted to the areas which remained green (*6*).

The areas of the leaf to which auxin is applied (and within which it remains) are characterized by an enhanced rate of metabolism. Protein and RNA synthesis and respiration are increased, and both carbon- and nitrogen-containing compounds move into the treated area (*4*). In contrast, in the untreated parts of the leaf, chlorophyll is lost progressively, total protein and RNA decrease, and the incorporation of precursors into both protein and RNA is reduced (*6*). Initially we suggested (*4*) that the enhanced metabolism in the parts of the blade treated with auxin leads to a withdrawal of metabolites from the untreated regions (depletion occurring first around the perimeter of the treated area), giving rise to premature yellowing and senescence. Although this situation must contribute to the premature senescence of the blade, we now propose that the senescence and subsequent abscission of leaves treated with 2,4-D is due to the increased synthesis of ethylene that occurs in the regions treated with auxin (*2, 6*).

Treatment with auxins promotes ethylene production in a range of plant tissues (*7*), and ethylene itself induces premature senescence of leaves and leaf fall in many species of plants (*8*). Painting half the surface of leaves of *E. japonica* with 2,4-D increases the production of ethylene by the treated halves nearly 25-fold in 12 days (Fig. 1). Yellowing of the leaf at the periphery of the treated half was visible by the 4th day, and leaves began to fall on the 11th day. Since there is no movement of auxin from the treated area, and since the amount of ethylene collected from naturally yellowing

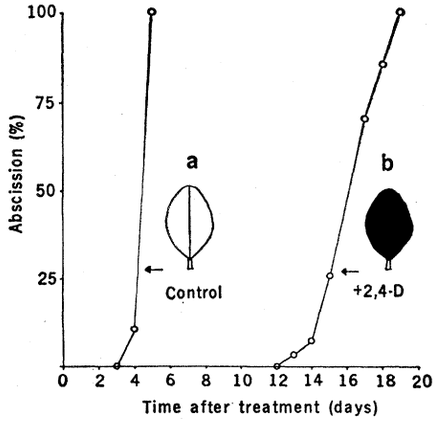

Fig. 2. Effect of ethylene (500 ppm) on abscission of 2nd-year leaves; (a) untreated leaves; (b) leaves treated with 2,4-D ester over entire upper surface. Branches were enclosed in glass containers that were maintained under greenhouse conditions; gas phase was renewed approximately every 3 days.