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COMPUTER-AIDED MICROSPECTROPHOTOMETRY OF BIOLOGICAL SPECIMENS

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SUMMARY

A scanning microspectrophotometer controlled by a small digital computer is used to produce data which is analysed using a larger, remote digital computer. Programs have been written for the smaller machine to permit automatic scanning of a large number of preselected fields. Other programs, written for the larger machine, enable the experimenter to pick out cells in a scan field and measure their size and optical density, or automatically locate and measure cells within a scan field, and generate statistical analyses of size and optical density measures for a number of cells. The further implications of this work for cytochemistry are discussed.

Experiments in quantitative histo- and cytochemistry frequently require that accurate photometric measurements be made on objects of microscopic size. One method of microspectrophotometry [7, 12, 17] involves the insertion, into the light path of the microscope, of an aperture of regular shape, which is filled as nearly as possible by the image of the specimen under study, so that only the light passing through the aperture reaches the photometer in the image plane. Objects of interest to the histochemist are, more often than not, irregularly shaped, which introduces error into the measurement. In the scanning microspectrophotometer [4, 5, 6, 9, 10, 21, 25, 26], in which an aperture considerably smaller than the specimen image is used to make sequential measurements on a number of contiguous fields, and the total absorption of the specimen is determined by spatial integration, this error can be reduced, although the problem of defining the boundaries of irregularly shaped objects remains.

Attempts have been made to automate the techniques of microspectrophotometry as fully as possible [4, 5, 6, 9, 10, 14, 25, 27]. We have proceeded further in this direction by placing much of the measurement process under the control of a digital computer. This paper will described the use of computer technology to assist an experimenter in obtaining photometric information from arbitrarily shaped areas of previously scanned

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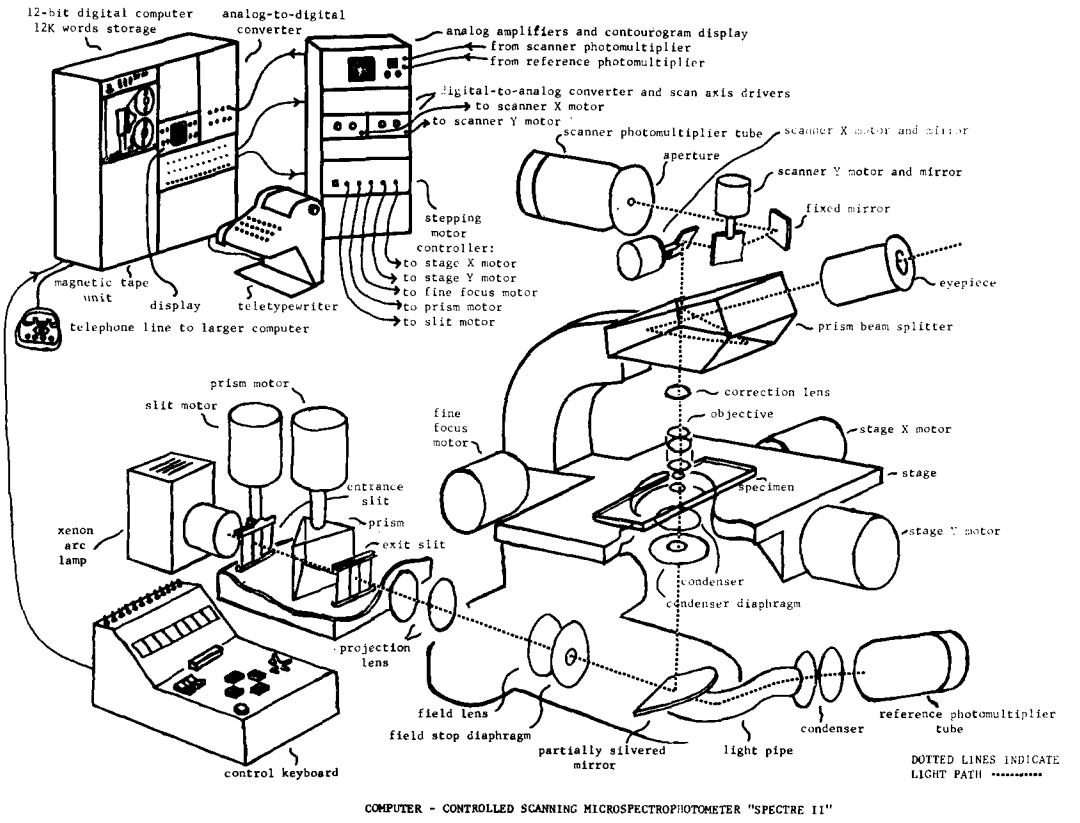

Fig. 1. The computer-controlled scanning microspectrophotometer.

fields, and the use of methods of image analysis to isolate and measure discrete objects in a field without any intervention by the experimenter.

MATERIALS, METHODS, AND RESULTS

A diagram of the apparatus used for scanning microspectrophotometry is shown in fig. 1. This system, previously described by Stein, Lipkin & Shapiro [25], consists of a laboratory microscope stand equipped with a motor-driven micrometer stage and fine focus mechanism. The light source is a 900 watt xenon arc lamp with a regulated power supply. The illumination wavelength and luminous flux are determined by an in-line prism monochromator with motor-driven prism position and entrance slit width controls. All of the above mentioned motors are of the incremental type (stepping motors), and are driven by pulses from a small digital computer. The microscope stage moves in steps of 0.6 μm, and the focus mechanism in steps of 0.2 μm.

A partially silvered mirror in the base of the microscope transmits some 2% of the incident flux, which then passes through a quartz fiber light pipe to the photocathode of the reference photomultiplier tube. About 95% of the incident flux is directed through the microscope condenser, specimen, and objective.

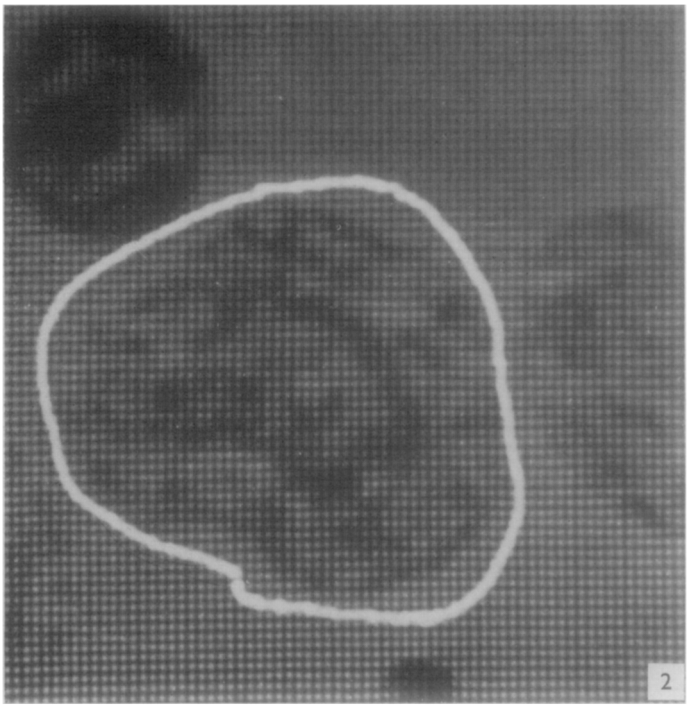

Fig. 2. Digital display (8 gray levels) of a scan field with the area selected for measurement outlined. Photos of the display lose clarity due to flicker.

The trinocular headpiece of the microscope contains a beam splitter, which diverts some 20% of the luminous flux reaching it to the eyepieces. Most of the remainder enters the scanner, which is built on the camera tube of the headpiece. The light is reflected from two mirrors, at right angles to each other, each of which is mounted on the shaft of a galvanometer motor. It is then reflected from a stationary mirror, and finally reaches a plate with a central circular aperture 10 μm in diameter, behind which is the photocathode of the scanner photomultiplier tube. The position of the scanned spot within the microscope field is changed by changing the current in each of the two scanner motors; this is done from the computer with the aid of 8-bit digital-to-analog converters, which allow sampling of a 256×256 raster pattern corresponding to an area 2 mm by 2 mm in the image plane.

The operator must mount the slide and set condenser focus, field stop and condenser diaphragm apertures. All further adjustments to the microscope by the operator are made through the use of a specially designed keyboard, and are mediated by the computer. Functions such as the speed and pattern of scanning and stage motion may be altered by changing parameters in the computer program, without any modification of the apparatus.

A control program was developed which enables the experimenter to move the stage, using the keyboard, until a cell on which he wishes to perform measurements is in focus and is centered in the microscope field, and to record the stage and focus stepping motor positions on a list which is stored in the control computer.

When a list of cell locations has been built up, the experimenter presses a single button, and the machine automatically returns to the selected position and focus

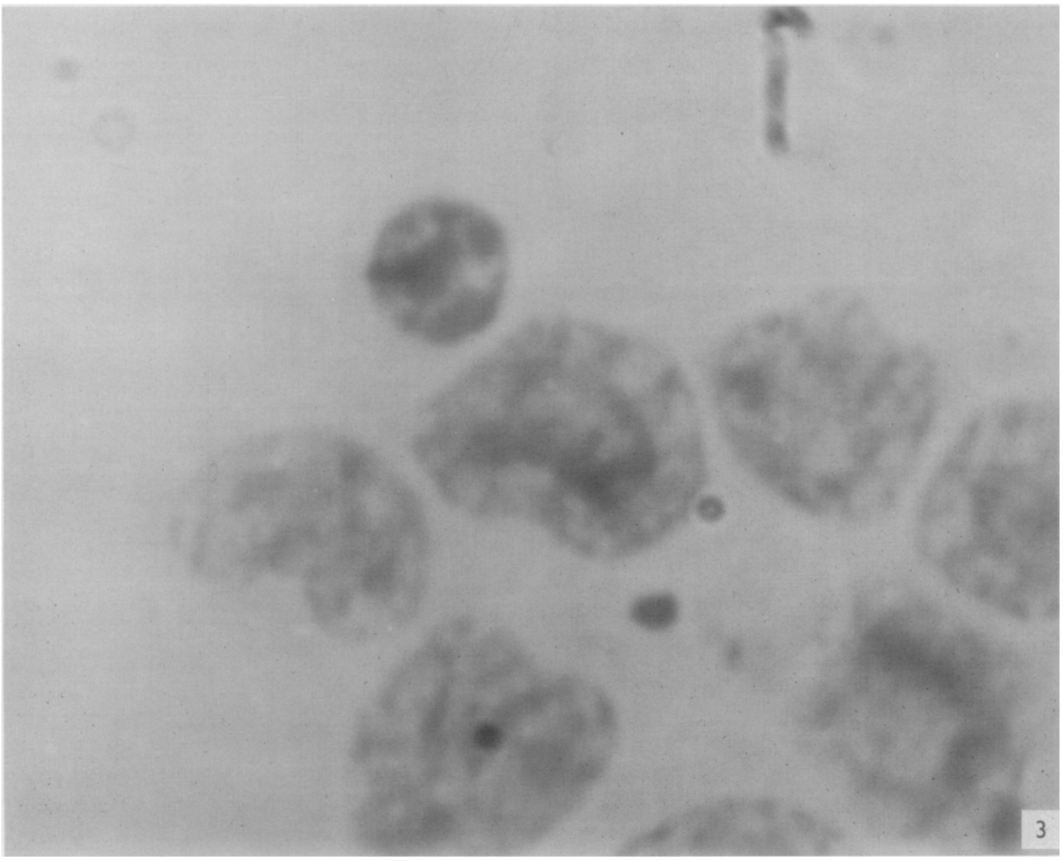

Fig. 3. Photomicrograph of the cells in the scan field of fig. 2, at a wavelength of 585±6 nm. This was not taken at the time of scanning.

settings, performs scans, and writes them on magnetic tape without further human intervention.

In the present experiments, the scanner was run as a single beam instrument, i.e., no use was made of the reference photomultiplier signal. The scanner photomultiplier signal was amplied and the video output converted to an 8-bit digital value in the computer. Gain and baseline controls were set manually to provide a digitized video signal value of 0 while scanning a clear area of the slide, and value of 255 while scanning the same area with a neutral density filter (nominal optical density = 0.28) in the light path.

Lymphoid cells from AKR mice were stained for DNA by the Feulgen method. The microscope was fitted with an achromatic-aplanatic condenser, N.A. 1.40, and a 70× fluorite objective, N.A. 1.25. Objective and condenser were immersed in oil. One hundred cells were scanned at a wavelength and bandwidth of 585 nm±6 nm, using a a program which scanned a square area approx. 37 by 37 nm, in the center of the microscope field, in a raster pattern of 256× 256 points. Each scan required about 45 sec. Ten scans were recorded on each of ten 9-track digital magnetic tapes. Scan tapes were processed on a large digital computer which has among its peripheral devices a

cathode ray tube display unit with eight gray levels and a capacitance-switch tablet for graphics input.

Two programs were developed to process photometric data. The first reads scan images one at a time from tape, and displays them on the cathode ray tube. The experimenter then uses the graphics input tablet to circumscribe an area on the scan field for which an optical density value is desired. The boundary of this area is displayed on the screen superimposed on the scan field as shown in fig. 2. This figure may be compared with the photomicrograph of the field in fig. 3. The selected area is measured by counting the number of scan points within the boundary, and a measure of relative optical density is obtained by taking the sum of the natural logarithms of the positive digitized video signal values at all points within the boundary. The program allows the experimenter to vary the threshold (i.e., subtract a positive number from the signal value at each point), and to redraw the boundary of an area of interest until it is satisfactory. Finally, it computes the means and variances of the area measure and optical density measure for all cells on the tape.

The second processing program also reads single scans sequentially from 9-track magnetic tape. It then identifies all "blobs" in the scan field, where a blob is defined as consisting of two or more contiguous scan points with video signal values above a specified threshold value, and computes a list of their areas and optical density values.

The scan field covers approx. 2% of the microscope field. While it was not always possible to position a slide so that only one cell of interest appears in the field, we could, under the conditions of our experiments, center the cell of interest in the scan field in such a way that it was the largest blob in the scan. The processing program could then search its list of blobs and extract the largest area measure, that of the cell, and its corresponding optical density measure. This program also computed means and variances of these parameters for all cells on the scan tape.

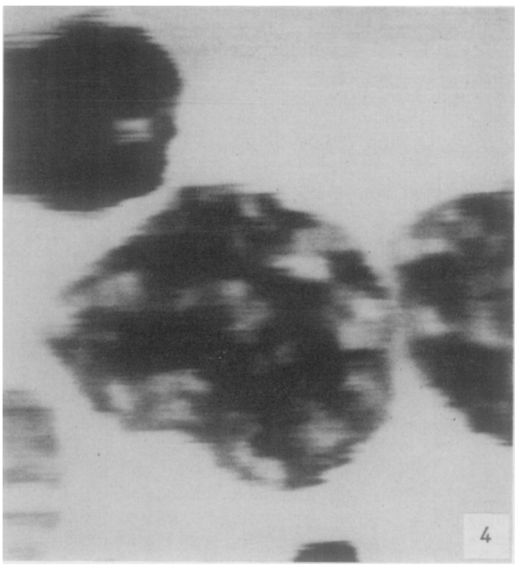

Fig. 4. Analog scan display of the cells in the scan field of fig. 2.

Repeated processing of a single scan field by the interactive program (i.e., using the graphics terminal) produced area and optical density values which agreed to within five percent, and were within five percent of the value obtained by the automatic processing program. The latter program produced identical sets of values for repeated passes over a single scan field.

Area and optical density values derived from several repeated scans of a single cell by the automatic processing program agreed to within two percent. The area values could be checked independently by measuring the cell on the analog video display (fig. 4). This is produced at the time of scanning by making a time exposure of the screen of an oscilloscope, the X and Y axes of which

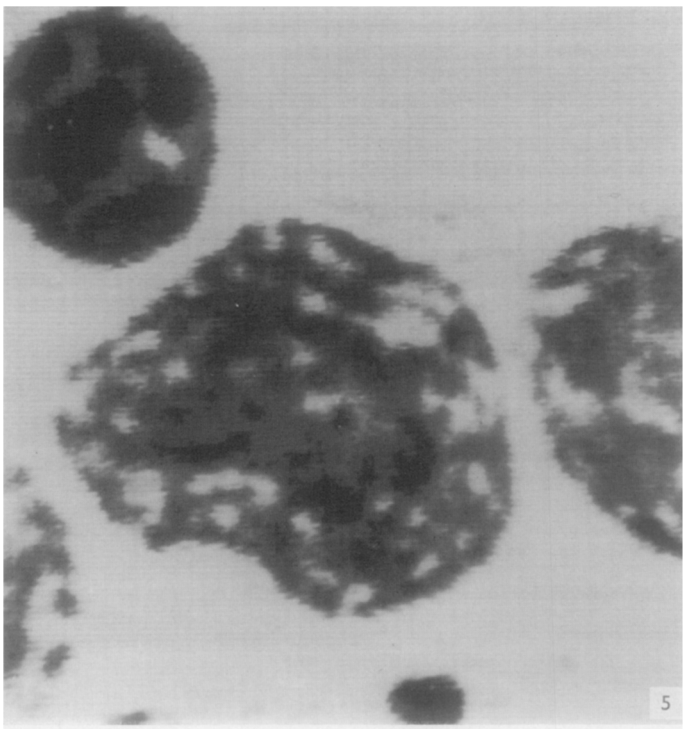

Fig. 5. Digital display of the scan field prior to automatic processing.

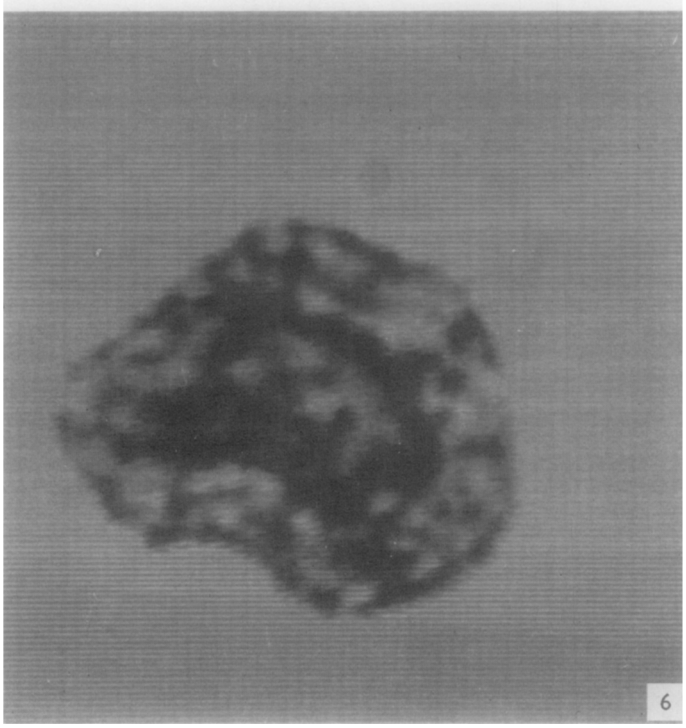

Fig. 6. Digital display of the scan field after the largest cell has been isolated by the automatic processing program.

are driven by waveforms derived from the scan driver circuits, with the beam intensity modulated by the video signal. The measures of cell area obtained with both processing programs correspond well to those made on the analog displays and on photomicrographs. The photomicrograph (fig. 3) can be superimposed on pictures of the displayed scan field (fig. 5), and of the cell extracted from the scan field by the automatic processing program (fig. 6).

DISCUSSION

The scanning system we have used was originally developed for use in image-processing experiments [22, 23] rather than as a microspectrophotometer; however, its accuracy compares favorably with that of, for example, the double-beam scanning photometric apparatus described by Engle & Freed [9], which also permits the operator to define an arbitrarily-shaped field of measrement.

Our spectrophotometer can be made to read in units of optical density by using the reference photomultiplier signal as a measure of the incident flux, I_0, and the scanner photomultiplier signal as a measure of the plux, I, transmitted through the specimen. Analog and/or digital techniques may be used to obtain $\log_e (I/I_0)$. This mode of operation should be preferable to the single-beam mode used in our experiments, although the reproducibility within two per cent of measurements from repeated scans of a single field speaks well for the accuracy of our instrument in the latter configuration. This figure compares favorably with that obtained by Engle & Freed [9]. The variation in luminous flux from our xenon arc source has been measured, and is about 5% peak and 2% R.M.S.; use of the reference signal should increase the reproducibility of our data. The current practice of digitizing the video signal to 8 bits is dictated by the computer hardware; on the basis of the measured noise level, only 5 or 6 bits are significant. The presence of glass elements in the scanner head restricts the present range of operation to the visible spectrum.

A scanning-stage microspectrophotometer of the type developed by Caspersson [4] has been interfaced to a digital computer, very similar to our control computer, by Wied et al. [27]. This system, called TICAS, has, like ours, been used for both image processing and spectrophotometry in the ultraviolet and visible portions of the spectrum. However, in the TICAS apparatus, the stepping motor-driven stage is used as the scanning mechanism, which, for the present, appears to preclude the addition of automatic specimen transport, a feature which we consider vital in an instrument to be used to scan large numbers of cells. The TICAS control computer, although, like ours, linked to a larger digital computer, is used for a great deal of the image processing work, whereas all of our processing is done on the larger machine [1, 2, 3, 25, 27, 28, 29, 30, 31].

At present, we are able to select a program option which enables any system function which could be initiated from the special microscope control keyboard to be performed instead in response to a command from a local or remote data terminal. Two such terminals, a teletypewriter and a cathode ray tube display type, are present in the laboratory; either terminal may be used as an input device to either the control computer or the remote processing computer. In addition, the two computers are connected by a 1200 baud telephone line, so that each may appear to the other as a high-speed data terminal. The control computer alone can be used to move the stage, to acquire scan data and write it on tape, and to write, edit, assemble, and test new control programs.

The latter functions are accomplished in about one-tenth the time if the remote computer, which, incidentally, is a timesharing system, is utilized for editing and assembly, and the assembled binary program is transmitted to the smaller machine; this is the method we currently use.

The existing hardware configuration also will permit a completely automatic closed-loop mode of operation in which a processing program in the larger computer requests data from the smaller machine, analyzes it, and then decides what the small computer should do next. All the apparatus required to do this type of work has been built. It will be more difficult to write the programs to control the interaction of the two computers; perhaps the most difficult aspect of this work will be the development of criteria and programs for automatic focusing, particularly in work with thick sections.

The programs described for microspectrophotometry have already been used for experimental work with good results. It was possible to use relatively simple logic in the automatic processing program by insuring, at the time of scanning, that the cell of interest was the largest object in the field. There exist computer techniques for image analysis [1, 2, 13, 18, 19, 20, 23, 24], which make it possible to identify and classify cells and other biological entities on the basis of more generally acceptable criteria.

The scanned image of one field of a tissue section, for example, can be ultimately broken down into some finite number of discrete elements, each of determinate size, optical density, and shape, and each corresponding to some component of the tissue. If the correspondences are known, a program can be written to derive measurements for the entire field of any subset of its components. The logic of such a program may be very intricate. Parameters such as the size, shape, and staining properties of a nucleolus might be insufficient to identify it, but the additional requirement that it lies within a nucleus, which itself can be identified by size, shape, and staining characteristics, might provide a sufficient criterion for positive identification. As the processing program becomes more involved, it is desirable to increase the degree of interaction between the processing computer and the scanner control computer.

The microspectrophotometer can be used to detect small differences in absorption maxima as well as changes in optical density not discernible to the human eye, in a display of the scanned image, or of a synthetic image made by combining scans at more than one wavelength. Such differences can be made apparent to the observer by manipulating the numerical relation between the gray levels of the data and the gray levels of the display. Far more can be accomplished in this area by digital processing of the data than can be done with analog circuitry, image intensifiers [8], and television-based display systems [32]. As the staining techniques of classical histology were developed to intensify differences between cell and tissue components as seen by the eye, it may be expected that the availability of computer techniques for image enhancement will result in the development of new stains, less useful for visual observation but better suited for quantitative cytochemistry. These, in turn, should help to provide a more objective basis for the automatic identification of cells, chromosomes, and other biological elements from their appearance under the microscope.

The principal advantage of the type of computer-controlled scanning system we have described appears to us to be that the apparatus can be used at the present time, to perform spectrophotometric measurements on biological materials with accuracy comparable to

that of other available equipment, and, in the future, with the addition of programs alone and not hardware, to perform a variety of complex operations, of which other systems are not capable due to their lack of sufficiently flexible mechanisms for scanning, illumination, or specimen transport.

CONCLUSIONS

The digital computer, now widely used to perform a variety of intricate procedures under automatic control with and without human supervision, can be employed by the cell biologist to automate a variety of experimental techniques.

We have used a small computer to control a scanning microspectrophotometer, and a larger one to analyse the data from experiments in quantitative cytochemistry. The machines simplify tedious tasks which could be done without them, and enable us to perform some biologically relevant experiments which otherwise could not. We expect that computers will play an increasingly important role in biological microspectrophotometry and in cell biology in general.

Keywords: cytochemistry, histochemistry, image processing, laboratory automation, microscopy, spectrophotometry

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