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Spectre II: General-Purpose Microscope Input for a Computer

Modular design and digital control facilitate optical measurements in biology.

Philip G. Stein, Lewis E. Lipkin, Howard M. Shapiro

A connection between a digital computer and an optical microscope is considered to be essential for the solution of several classes of problems in biology and medicine. Automatic control facilitates quantitative microspectrophotometric histochemical studies (*1*) for which the limitations of the human eye as a colorimeter have necessitated various electronic additions to the microscope itself. Also, the difficulty of training human observers in diagnostic cytology and hematology, and the amount of material to be observed, provide motivation for the development of automated image-processing systems to be used in clinical screening. Finally, techniques for automatic analysis of microscopic images and storage and retrieval of such images as data would significantly advance the study of the three-dimensional microarchitecture of tissues.

Several systems incorporating an interface between microscope and computer have been built and described (*2–5*). The design of most of these has been restricted by the particular application for which the system was intended. The apparatus described in this article is designed to be as flexible as possible so that, in addition to being employed as a research instrument in the above-mentioned areas, it can be used to evaluate optical, electronic, and computer system requirements for the solution of specific problems.

Microspectrophotometry

The microspectrophotometric analysis of biological specimens (*6, 7*) involves two dissimilar procedures. First, the area of the specimen to be analyzed must be placed in the light path of the photometer. This requires a complex and ill-defined series of decisions on the part of a trained biologist. The process becomes intuitive, and is rapidly performed. The second procedure, the actual measurement of light absorption, fluorescence, and so forth, at one or more wavelengths is operationally well defined, but it is time-consuming even for skilled experimenters. It therefore seemed logical to separate the two phases of microspectrophotometry in an initial attempt at automation. The first step in this direction was the development of a microscope stage with a "memory." This permits the biologist to control stage motion and focus controls, and to record the position coordinates of all areas of interest on a given specimen, for example, a stained slide. A list of objects to be measured and the measurements to be made on each is transmitted to the photometer controller. The controller, now unattended, makes a second pass over the slide. At this time the measurements requested are made automatically, with any change of wavelength accomplished by a motor-driven monochromator, on all areas of interest on the slide. A list of the measured values is returned to the experimenter. To our knowledge, no other apparatus currently used for biological microspectrophotometry is automated to even this extent.

Scanning Optical Microscopy

Two biological research programs led us to develop an instrument to scan microscopic fields and produce digitized images for analysis by computer. The first of these required a comparison of reflectance photometry (*8*), special-purpose image-analyzing devices (*9, 10*), and more general computer techniques for counting grains in blood cell autoradiographs. The second involved the creation of a formal system for the description and analysis of the structure of nervous tissues (*11*). Both of these projects, and particularly the latter, give rise to an unmanageable amount of stored data when handled by conventional scanning techniques.

Consider that the practical limit of lateral resolution in light microscopy is of the order of 0.25 micrometer. Although the limit of vertical resolution is less simply expressed, we can approximate a three-dimensional resolution element in the object plane by a cube 0.25 micrometer on a side. A

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typical stained section of tissue is 1 square centimeter by 10 micrometers thick and, therefore, contains more than 6×10^{10} resolution elements. If we assume 256 identifiable levels of optical density, that is, eight bits of information per resolution element, a complete scan of a typical slide produces 5×10^{11} bits of information. A computer tape is typically 2400 feet long (roughly 3×10^4 inches) and stores about 10^4 bits of information per inch. This means a scan of every point on a single slide at maximum resolution would fill 1700 reels of tape!

The apparatus originally developed by Caspersson (*6*) and used for image processing by Wied, Bartels, Bahr, and Oldfield (*5*), scans a raster pattern by moving a precision stage in increments of from 0.25 to 1.0 micrometer. Digital readout of extinction coefficients from 0.01 to 1.50 in steps of 0.01 is provided. The stepping speed is 60 per second. The illumination wavelength may be varied with a monochromator on the larger instruments and with interference filters on the smaller ones.

CYDAC, as described by Mendelsohn *et al.* (*3*), is a flying spot scanner with peak cathode-ray tube emission at 503 nanometers. An area, 50 by 50 micrometers, is scanned in a 192 by 192 point raster pattern, and a 256-level gray scale is used. Digital images are recorded on magnetic tape. The CYDAC system also has an image plane scanner with a Nipkow disk which enables operation at other wavelengths.

CELLSCAN I, used by Preston *et al.* (*4*), is an image plane scanner employing a vidicon tube to scan a field, 20 by 20 micrometers, in a 63 by 63 point raster pattern. Wired preprocessing logic is used and only two levels of gray (black and white) are recognized. The wavelength of illumination is variable.

An image plane scanner, the "computer eye," with a vidicon tube which is addressable by a general-purpose digital computer, has been used for chromosome analysis (*12*). Any point or pattern of points in a 4096 by 4096 point grid may be scanned, which offers a possible reduction of the volume of output data. A 64-level linear or logarithmic gray scale is employed and illumination wavelength is variable.

Prensky (*9*), Husain (*13*), and Mawdesley-Thomas and Healey (*14*) have described the use of the "Quantimet" image-analyzing computer for biological work. This is a special-purpose device which counts and sizes areas darker or lighter than a preset threshold level using wired logic. A vidicon in the image plane provides a 312-line scan with a 50-hertz power source and a 262-line scan with a 60-hertz power source. A stage, driven by stepping motors in increments as small as 50 micrometers, with an automatic programmer and sequencer, may be used with this system although there is no provision for closed-loop operation. The illuminating wavelength may be varied.

Subsystem for Automatic Microspectrophotometry

The hardware requirements for the simplest strategy of automatic microspectrophotometry are very demanding. We felt that a precision stage with reproducible positioning driven by stepping motors was needed. The optical and electronic components for a photometer were readily available. It also was feasible to mount stepping motors on a prism monochromator already in the laboratory to provide a light source of variable wavelength and flux.

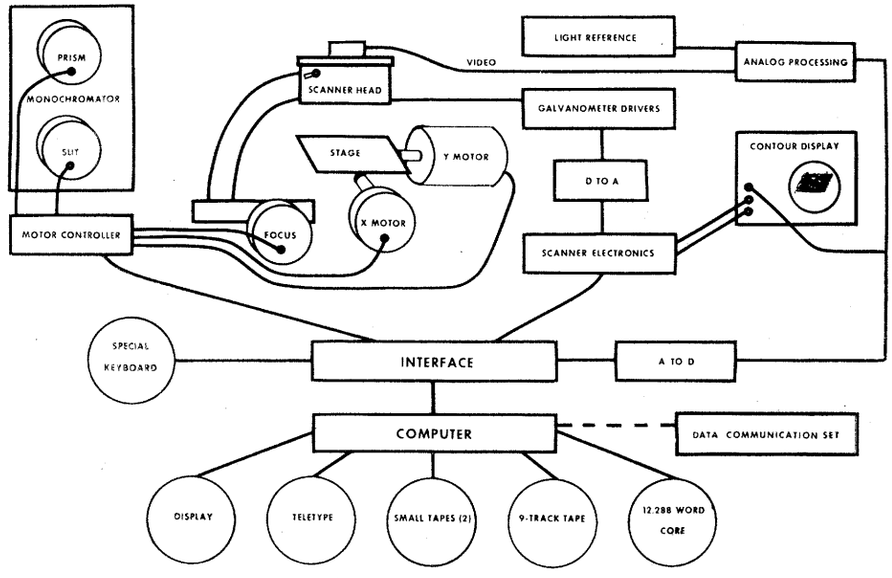

Fig. 1. The Spectre II system.

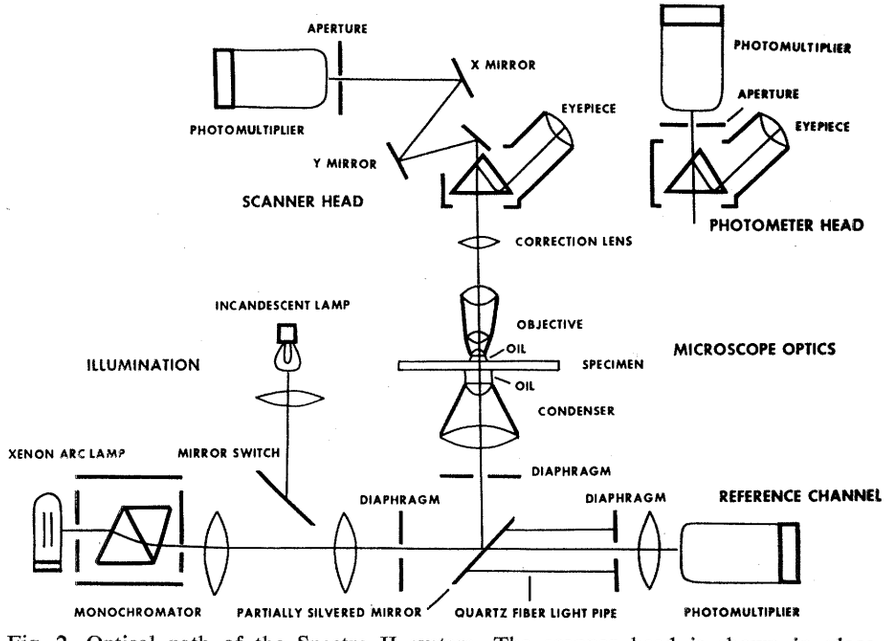

Fig. 2. Optical path of the Spectre II system. The scanner head is shown in place, with the monochromatic light source in use.

It was decided to use a general-purpose digital computer with a stored program to control the system. This permitted us to change the parameters of operation merely by changing the programming of the computer, eliminating most of the almost inevitable wiring changes associated with instrument prototypes. Finally, we constructed a manual control device with which the operator, via the computer, might run the stepping motors to change stage position, focus, wavelength, and luminous flux. This keyboard also enables the operator to record a list of object positions, initiate automatic measurements, and calibrate the instrument. The computer keeps track of and displays (on an associated cathode-ray tube) the settings of all motors. Photometric measurements, typically converted to relative optical density with analog circuitry, are digitized with an analog-to-digital converter in the control computer.

The system for biological microspectrophotometry described above fulfills a most important design criterion by leaving the complex heuristic operation to the human operator and the tedious but algorithmically well-defined measurement process to the machine. This is made possible largely by the presence of the computer-positioned microscope stage and monochromator. The applications of this component of the system, however, extend beyond microspectrophotometry.

Subsystem for Scanning Optical Microscopy

We have shown above that an attempt to describe the structure of even a small biological specimen in terms of microscopic resolution elements produces an unwieldy amount of data. It is, moreover, inappropriate to consider the resolution element in a formal system for such descriptions. An individual resolution element by itself is of even less significance as a structural component of an image than is a single alphanumeric character as a structural component of a word. This is so because there exists a known vocabulary and an implicit grammar for printed language text. We proposed to develop the equivalents of these, namely a picture grammar, to describe the structure of tissues (*11, 15*). The equivalents of words in this scheme would be elements of cellular structure; nucleus, cytoplasm, mitochondria, nucleolus, and so forth, rather than individual resolution elements. Since the storage requirement for the amount of information in even an extremely small volume of tissue is so immense, it would be necessary to use the microscope slide itself as the storage medium and to develop the means to selectively and repetitively gain access to the information. The computer-controlled stage would provide some of this capability.

A further reduction in the amount of information which would have to be stored for computer processing could be achieved by allowing the microscope scanner to operate in an addressable mode rather than a fixed rectangular raster pattern. This would permit selective scanning of designated objects of interest within a microscopic field.

Again, it seems best to have the scanner addressing under computer control. This would make it possible to change the scan pattern merely by changing the program. It also would enable fully closed-loop operation of the system through use of programs which compute the address of the next scan point as a variable dependent on the results of measurements at previous scan points. An example is the use of the scanner as an edge detector which follows a density gradient.

The scanner hardware should ideally be discrete from the programmable stage to allow greater flexibility. It would be advantageous, however, to use the same general-purpose computer to control both the scanner and the stage, as well as the illuminating system developed for the photometer. Although the small control computer is adequate for simple processing tasks such as photometry or contour scanning, the more elaborate programs necessary for autoradiographic grain counting, cell identification, or structural analysis of tissue must be run on a larger computer. Data transmission between computers will be accomplished by direct line which would permit fully closed-loop operation. Less complicated processing can be done if one uses magnetic tape as an intermediate medium for data exchange.

This enlarged system will enable us to simulate, in all but the time domain, the performance of various special-purpose image-analyzing and microspectrophotometric devices. By repeated simulations, it will be possible to optimize the design of instruments for specific applications. We are, therefore, willing to accept a slower system to achieve flexibility.

The scanner and photometer, with the stage, illumination sources, and control computer form a general-purpose microscope input for a large digital computer. We have built this system (Fig. 1).

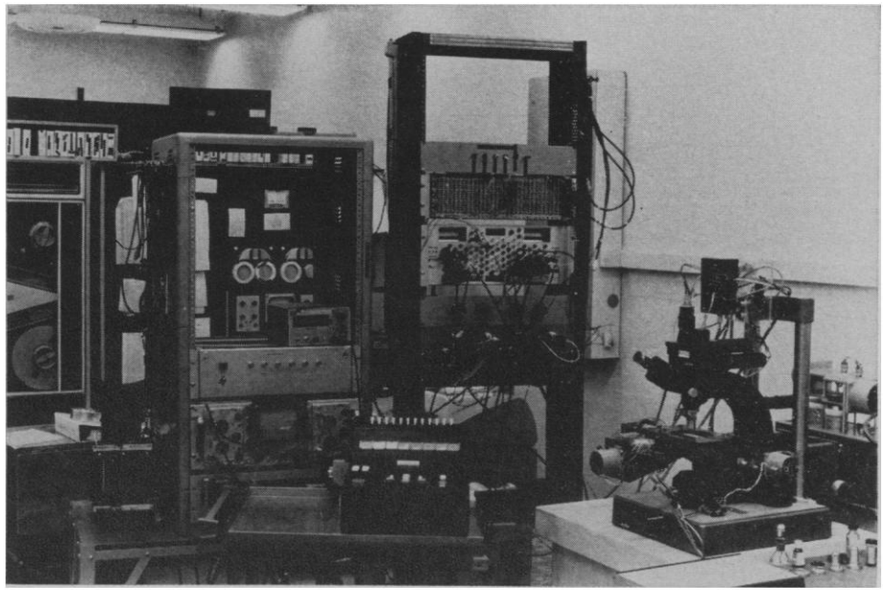

Fig. 3. The Spectre II system. The controlling computer is seen in the background. In the foreground are (from left to right) the rack housing the stepping motor controller and display oscilloscope, the operator's keyboard, the rack housing the scanner electronics, the microscope, and the monochromator. The scanner head is mounted on the microscope.

System Description

It is convenient to discuss the components in the optical path of the system (Fig. 2) separately from the elec-

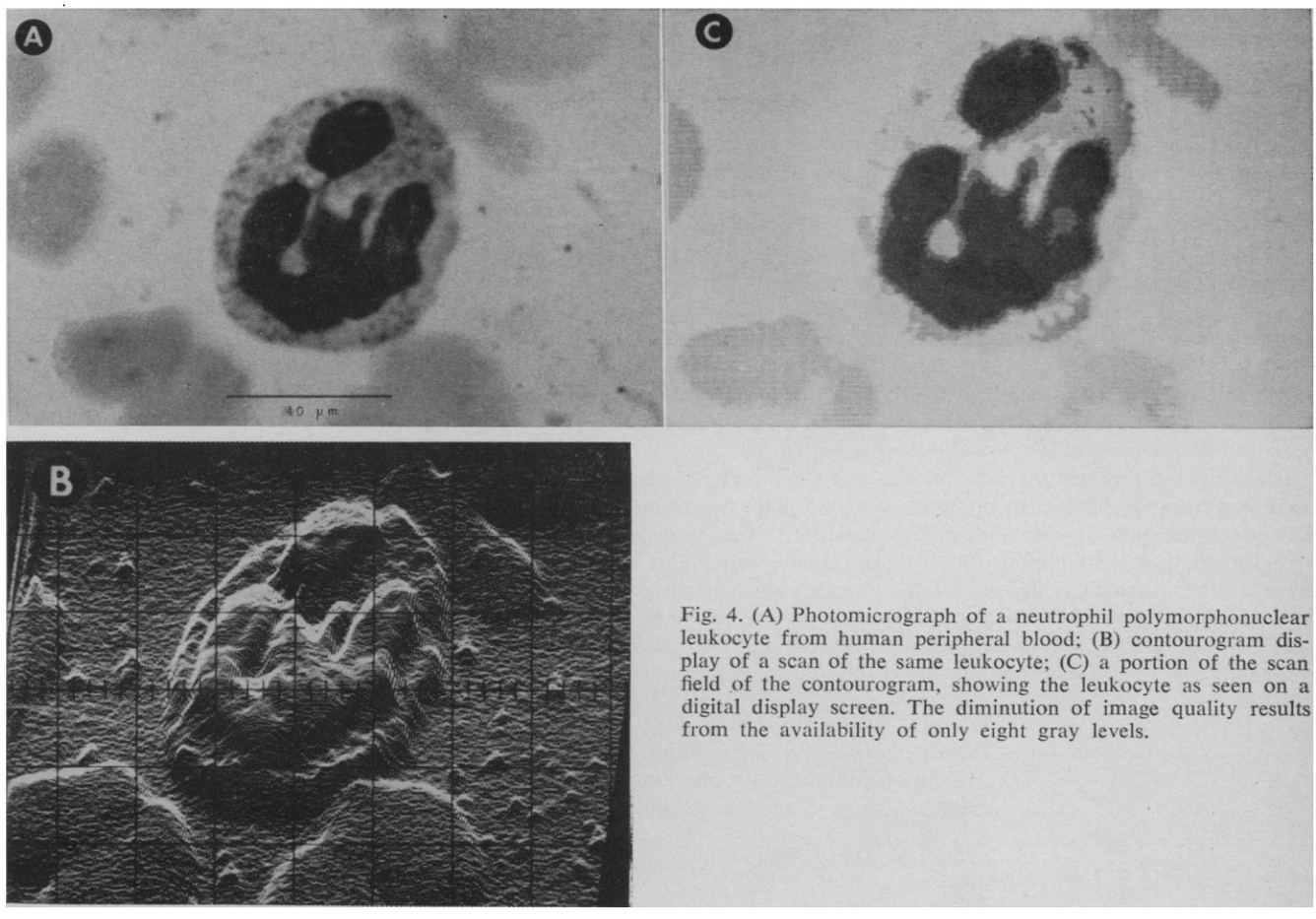

Fig. 4. (A) Photomicrograph of a neutrophil polymorphonuclear leukocyte from human peripheral blood; (B) contourogram display of a scan of the same leukocyte; (C) a portion of the scan field of the contourogram, showing the leukocyte as seen on a digital display screen. The diminution of image quality results from the availability of only eight gray levels.

tronics. The microscope is built on a commercially available research stand. The incandescent light source is a 6-volt, 5-ampere lamp with a regulated power supply. A mirror switch, manually operated, allows use of either incandescent lamplight or monochromatic light, the latter being furnished by an in-line prism monochromator equipped with a 150-watt xenon arc lamp. Light from the mirror switch passes through a field lens and a field stop diaphragm in the base of the microscope stand and strikes a partially silvered mirror. Two percent of the incident flux is transmitted through this mirror by way of a quartz light pipe to a condenser lens system and photomultiplier tube. The signal from this tube is used to provide a reference for the illumination.

The remaining portion of the illuminating beam not absorbed by the mirror is reflected upward from the partially silvered mirror through the condenser diaphragm and into the condenser lens system. We currently use an achromatic-aplanatic oil immersion condenser with a numerical aperture of 1.40. The condenser and coarse focus are manually adjusted to fulfill the conditions for Koehler illumination.

Light from the condenser passes through the specimen into the objective lens which is mounted in a manually adjustable centering clutch with a built-in tube length-correction lens. The image plane scanner and the microspectrophotometer are each mounted in the camera tube of an interchangeable prism headpiece, allowing only one of these to be used at any time.

The photometer head, made from standard commercial components, consists of a collimating chain, diaphragms, a set of interchangeable circular aperture stops on a centerable mount, and a photomultiplier to measure the light passing through the aperture. A prism and eyepiece permit visualization of the field measured. The smallest aperture corresponds to a circle 2 micrometers in diameter in the object plane.

In the scanner head the light, after passing through the objective and tube length-correction lens, is reflected by two mirrors at right angles to each other (*16*). Each of these mirrors is mounted on a galvanometer coil; the position in space of the center of the real image, formed by the objective and reflected by the mirrors, may be moved electrically by varying the current through the galvanometers. At the image plane is an aperture, 10 micrometers in diameter, behind which there is a photomultiplier tube.

The galvanometers are driven by digital signals from the computer through eight-bit digital-to-analog converters, which allows the central 2 by 2 millimeters of the image plane to be divided into a 256 by 256 grid, any element of which may be reflected through the aperture onto the photomultiplier. The distance between the scan points on the grid is currently set to be approximately 0.25 micrometer in the object plane.

The S-20 cathodes of the photomultipliers respond, though not uniformly, to all wavelengths in the visible spectrum. The tubes used in the reference circuit and in the transmission circuit of the photometer are matched for spectral response. The dynode voltages are derived from a highly regulated power supply to improve the stability of the photomultiplier amplification. The photomultiplier anode current goes directly to the summing node of an operational amplifier. Further signal-processing amplifiers scale and filter the

signals from both data and reference phototubes to remove unwanted frequency components. The output signal is converted to eight-bit digital form in the computer.

The scanner signal is normally amplified in a linear mode, and is not compared to the reference signal, but the scanner may also be set up with some reduction in signal-to-noise ratio to read in relative density, as does the photometer. This is done with a network of operational amplifiers and logarithmic transconductors which forms the logarithm of Φ_t/Φ_i, where Φ_t is a measurement of the luminous flux transmitted through the specimen as derived from the scanner or photometer photomultiplier signal, and Φ_i is a measurement of the luminous flux incident on the specimen as derived from the reference photomultiplier signal. The density is relative with the amplifier gains set so that a clear area of the slide reads as a density of zero. Neutral-density filters in the light path are used to establish a true logarithmic scale for higher density values. This calibration must be done at each wavelength of interest. The logarithmic calculations may also be done by the computer so that calibration at different wavelengths may be included automatically.

The optical resolution of the scanner is better than 2000 lines per millimeter; indeed, when using microscope objectives of high power and high numerical aperture, for example, 100 power and numerical aperture of 1.32, the 10-micrometer aperture referred back to the object plane corresponds to 0.1 micrometer. The scan rate and pattern are under computer control, and the rate is varied to allow a signal-to-noise ratio of 40 decibels. The minimum time for a corner-to-corner motion of the mirrors is 40 milliseconds. Traverse time between adjacent points is correspondingly faster, and so a full raster scan of 256 by 256 points takes about 120 seconds.

The mechanical stage, which was built at the National Bureau of Standards, is driven by orthogonal micrometer heads geared to splines on the shafts of stepping motors. The gear ratio gives a step size of 0.6 micrometer. Total stage travel is 2.5 centimeters in horizontal (x) and vertical (y) directions, and the stage may be returned, following an arbitrary excursion, to any point within plus or minus one-half step. The stage is moved with a jogging motion to avoid mechanical hysteresis (*17*).

Reproducible motion demands that a point be approached from the same direction each time. An incremental motor is also mounted on the microscope stand and geared to the fine focus control. It moves the stage

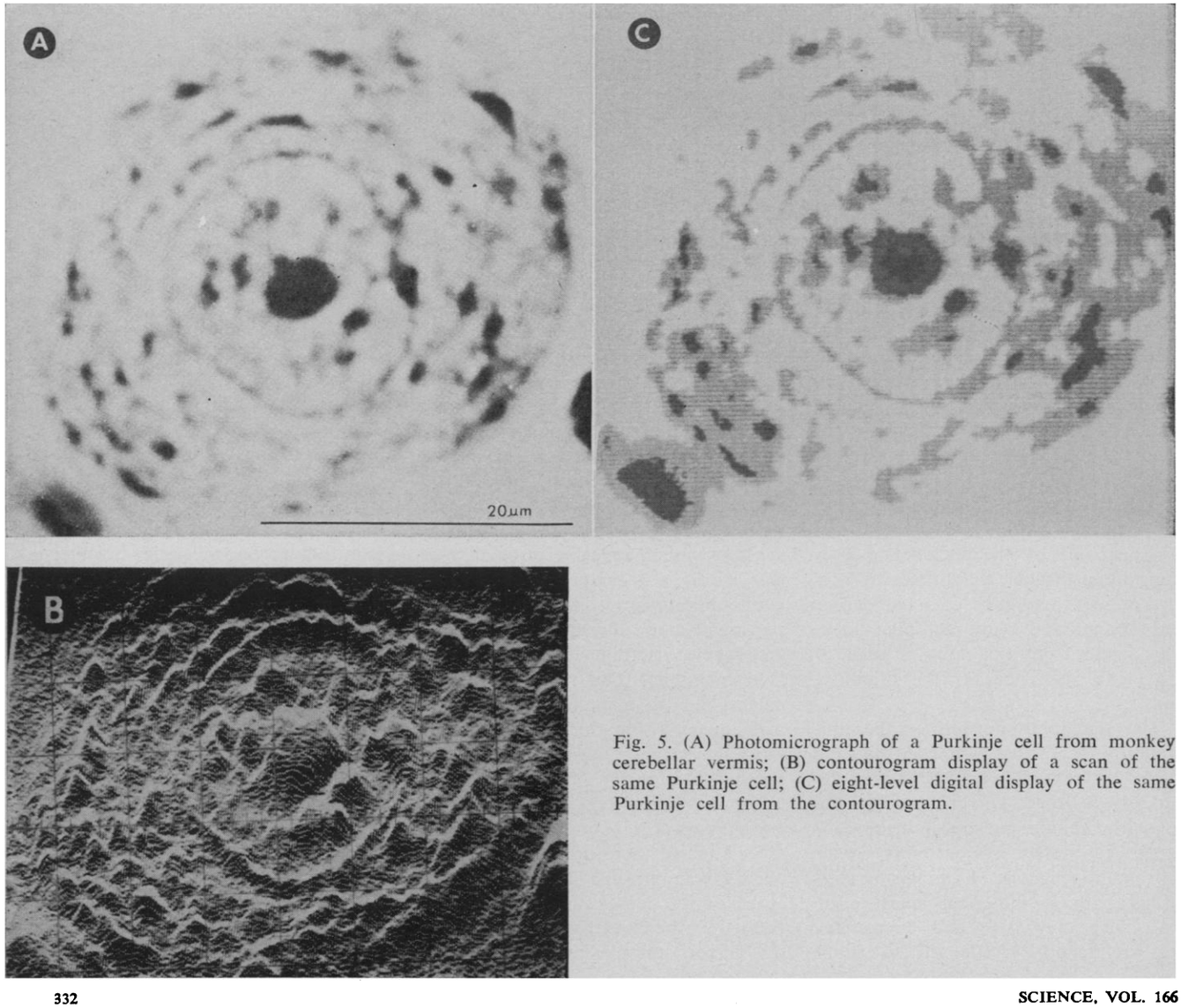

Fig. 5. (A) Photomicrograph of a Purkinje cell from monkey cerebellar vermis; (B) contourogram display of a scan of the same Purkinje cell; (C) eight-level digital display of the same Purkinje cell from the contourogram.

and condenser system over a range of 0.20 centimeter in 0.2-micrometer steps, repeatable to plus or minus one step over small excursions. Stepping motors are geared to the prism position and entrance slit width controls of the monochromator. The exit slit width is fixed to give a bandwidth which varies with prism position and is plus or minus 6 nanometers at a wavelength of 549 nanometers.

The incrementally controlled entrance slit width varies the radiant flux. The wavelength is variable in some 13,000 steps over the visible range from 400 to 700 nanometers. Quartz optics usable to 250 nanometers in the near ultraviolet are available for the photometer but not for the scanner.

The computer used as a controller is a 12-bit machine with 12,288 words of core storage, typewriter, cathode-ray tube display, two small magnetic tape units, a nine-track tape drive, and a 16-channel eight-bit analog-to-digital converter. The computer has been modified to provide a priority interrupt system and digital data transmission to the scanner, microscope stage, and monochromator.

The operator may exercise manual control over the microscope system by means of a special keyboard working through the interrupt system of the computer. Thus, the actual stage, monochromator, and scanner operations remain under computer control at all times. The keyboard allows fast and slow motion along the x, y, and z axes, and allows change of radiant flux and wavelength. In addition, a series of buttons can be activated to initiate operation of various computer programs. These perform such tasks as scanning a microscope field and writing a nine-track digital tape, measuring the absorption spectrum of a single field, recording a list of coordinates of points of interest on a slide, and returning automatically to these points.

Currently, the nine-track magnetic tape serves as a link to the larger digital computers used for image processing. The next phase of our project will implement a high-speed data link to one of these large machines. In addition, the optical system will be rebuilt on an optical bench with beam splitters to permit microscopy, photometry, scanning, photography, or closed circuit television observation. The television signal will also be available to the operator of the large computer to permit rapid comparison of raw and processed data.

Discussion

We feel that the Spectre II system (Fig. 3), as currently implemented, represents the most flexible interface yet developed between the biological microscope and digital computer. Perhaps the weakest part of Spectre II is our microspectrophotometer, which admittedly has not reached the degree of accuracy of double-beam or even the better single-beam instruments (*6*, *7*). On the other hand, the system compares quite favorably with a variety of other instruments recently used in microscopic image analysis with respect to both optics and electronics.

The vibrating mirror scanner which we use is slower than any of the cathode-ray tube scanners described above by a factor of about 100. It is comparable in speed to the moving stage apparatus. We find the lack of speed entirely tolerable in view of the primary use of the system as a research instrument.

The computer-controlled fine focus, enabling reproducible positioning along the z coordinate, is presently unique among microscope scanners. It permits us to use the technique of "optical serial sectioning"; that is, scanning at different focus settings with objectives of high numerical aperture and small depth of field to reconstruct three-dimensional structure in relatively thick sections of tissue. This is essential in our studies of nervous tissue, and is also useful in separating the cell plane from the film plane in autoradiographic grain counting.

As an example of the scanner's performance, compare the contourogram, an artificial three-dimensional display made by using the horizontal sweep signal of the scanner to which 10 percent of the vertical sweep signal has been added to drive the horizontal amplifier of an oscilloscope with the corresponding photomicrograph and computer-generated cathode-ray tube display of the subject (Fig. 4). The scanner vertical sweep signal, to which the video signal has been added, drives the oscilloscope's vertical amplifier. Darker areas in the specimen appear "higher" in the contourogram, which is a time exposure using the oscilloscope camera. Another photograph, contourogram, and partial digital representation (Fig. 5) are presented for comparison.

The modular structure of the Spectre II system is basic to its use as a design facility in developing specification for special-purpose image-processing systems. The operation of these systems would first be simulated on Spectre II, and the necessary components of Spectre II could be duplicated in the special-purpose machines, perhaps controlled by a smaller and less expensive digital computer.

Summary

We have developed a system of instruments used in biological and medical research for microspectrophotometry and computer analysis of microscopic images. The apparatus includes an optical microscope, spectrophotometer, a scanner, and a motor-driven stage and monochromator, all controlled by a small general-purpose computer. A keyboard permits manual operation of the equipment under computer control. The system was constructed with great flexibility to permit its use in the evaluation and design of a special-purpose apparatus for specific biomedical applications.

The extension of capabilities of Spectre II to other modalities, for example, phase contrast and polarized light microscopy, or microfluorimetry, will be necessary to take full advantage of the system in studies on living cells. Again, the modular design should make it easier to do this.

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