A Microfluorometric Scanner for the Differential Detection of Cells: Application to Exfoliative Cytology

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THE PURPOSE OF THIS PAPER is to describe developmental work on a microfluorometric scanner designed for the automatic searching and detection of cancer cells in preparations (smears) of exfoliated cells, obtained from tissue secretions and spread on microscope slides.

The cytological and the cytochemical principles, which have been presented in preliminary form elsewhere (*1–3*), and which will soon be described in detail (*4*), involve staining the cells with a basic fluorescent dyestuff or *fluorochrome* under conditions that favor selective and stoichiometric combination of the dye with chemical constituents that are located principally in the nucleus of the cell. Each stained cell when illuminated with long-wave ultraviolet radiation acts as a self-luminous body, whose light emission can be measured with a microphotometer. The *primary* or the intrinsic fluorescence that is due to the natural cellular constituents is negligible in comparison with the *secondary* fluorescence induced by the dye-binding.

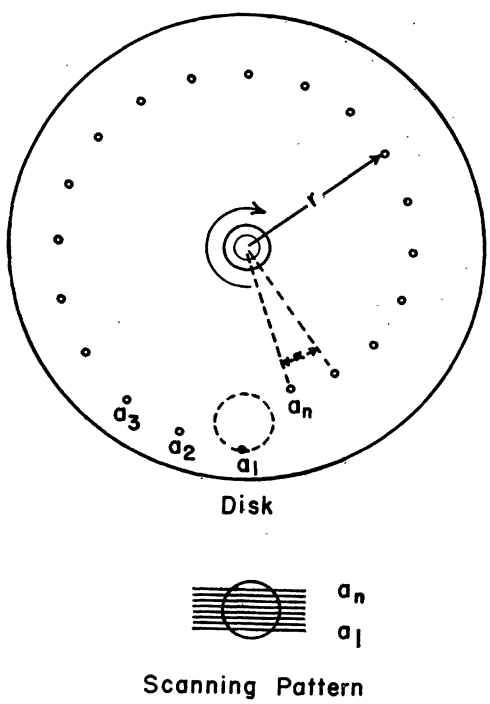

FIG. 1. Nipkow scanning disk. A spiral of apertures, a_n, spaced at an equal angular interval, $a = 360°/n$, to produce n lines in the scanning pattern.

Cancer cells, in comparison with normal cells derived from the secretions of certain anatomical sites, such as the cervix uteri, combine with more fluorochrome and emit on the average per unit area two to three times the fluorescent light of normal cells. This conclusion is derived from a microfluorometric analysis and a statistical treatment of samples of the order of 100–300 cells in the vaginal smear and in the pleural fluid. Data for the cells of other anatomical sites, such as the urinary, the respiratory, and the gastrointestinal tracts, have not yet been obtained.

There exists, therefore, a quantitative means of differentiating normal and cancer cells which exfoliate

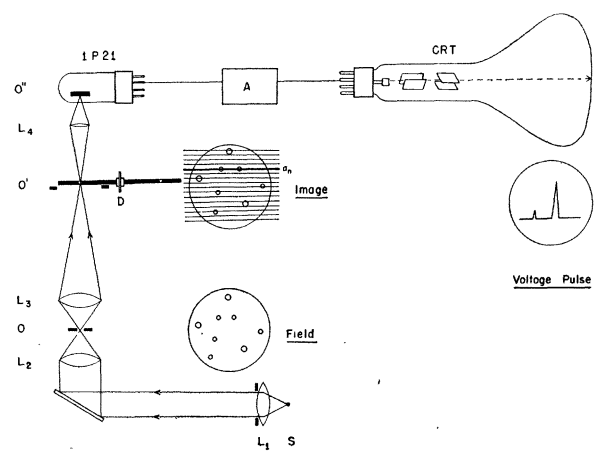

FIG. 2. Microfluorometric scanning of cells with oscilloscope presentation. *S*, source of Köhler illumination; L_1, field lens; L_2, condenser; *O*, object; L_3, objective; *O'*, image; *D*, scanning disk; L_4, auxilliary lens; 1P21, photomultiplier; *A*, amplifier; and *CRT*, cathode-ray tube, with voltage pulse from cells scanned along line, a_n.

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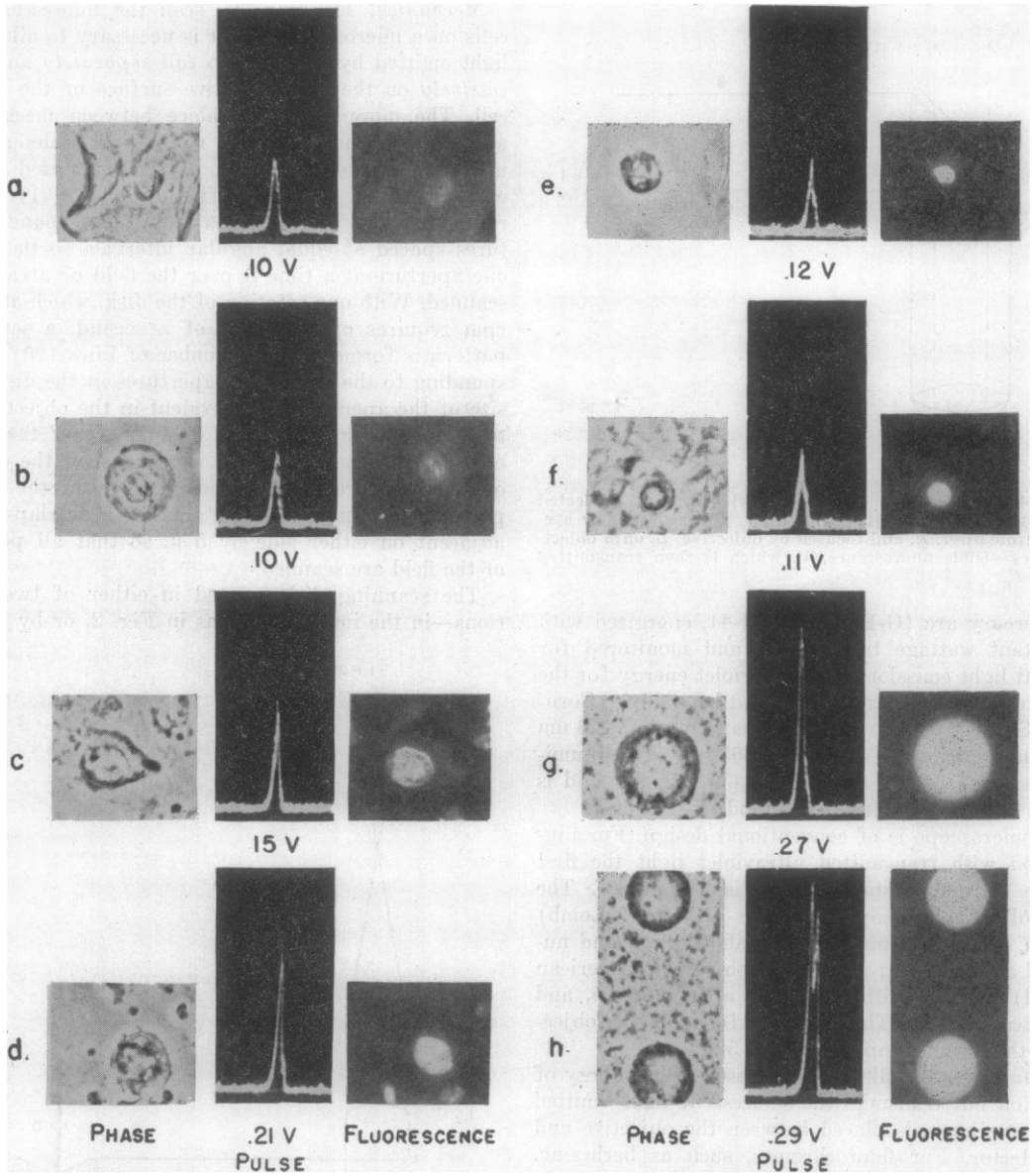

FIG. 3. Microfluorometric scanning. Fluorescence photomicrographs, oscilloscope tracings of voltage pulse and phase photomicrographs of cells. Vaginal smear: (*a*) squamous, (*b*) parabasal, (*c*) abnormal (Class III), and, (*d*) cancer (Class IV) cells. Pleural fluid: (*e*) histiocyte, (*f*) mesothelial, (*g*) cancer (Class V), and (*h*) two cancer (Class V) cells.

from the cervix uteri, even in the early preinvasive stage of cancer. The microfluorometric scanner described here is under development as an adjunct to the Papanicolaou technique (*5*) for the cytological diagnosis of cancer, in the hope that this instrument can be used to facilitate the large-scale screening of the population for neoplastic disease, particularly for cancer of the cervix in the early stages, when appropriate treatment will afford a high rate of cure.

INSTRUMENTATION

The microfluorometric scanner consists of the following components: (1) a constant source of the long-wave ultraviolet light, (2) an ordinary light microscope with an automatic mechanical stage, (3) a scanning disk (Fig. 1), (4) a photoelectric detector, and (5) a voltage pulse discriminator and counter. By means of this device, the fluorescent light from each cell *separately* and *successively* is permitted to fall on a photocell. A pulse of light energy derived from each cell is converted into a voltage pulse, which is amplified and analyzed in terms of its ability to activate an electronic counting circuit set to respond to a certain voltage input and to register thereby the presence of certain types of cells. For display the voltage pulse is also presented on the screen of a cathode-ray oscilloscope (Figs. 2, 3).

Fluorescence microscope. The source of illumination

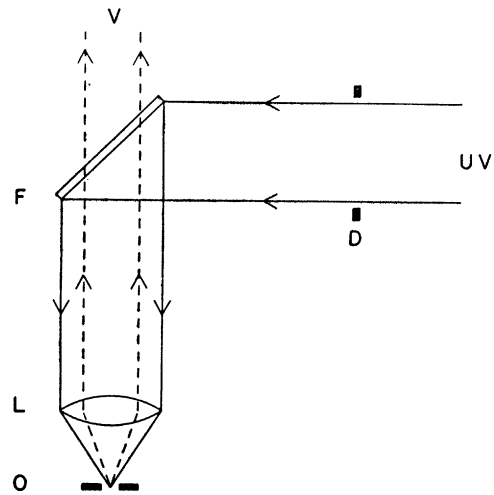

FIG. 4. Fluorescence microscope with vertical reflected illumination. Incident ultraviolet light, *UV*, reflected by special dichroic filter, *F*, and focused by objective, *L*, onto object, *O*, excites visible fluorescence, *V*, which is then transmitted through *F*.

is a mercury arc (G-E, Type AH-4), energized with a constant wattage transformer and monitored for constant light emission. The ultraviolet energy for the excitation of fluorescence is isolated by a filter (Corning C. S. 7-83) and consists of a band of about 40 mμ which has maximum energy at 365 mμ. The illuminator is fitted with a quartz lens (Fig. 2, L_1) and is used in accordance with the method of Köhler.

The microscope is of conventional design. For illumination with transmitted ultraviolet light the first surface of the substage mirror is aluminized. The optics of the microscope condenser (Bausch & Lomb) is glass, with minimum spherical abberration and numerical aperture 1.0; that of the objective (American Optical) is glass with numerical aperture 0.85, and magnification ×50. The depth of focus of the objective in the present application is ±5μ.

A nonfluorescent filter, which absorbs the energy of excitation but transmits the fluorescent light emitted by the specimen, is placed between the objective and the detector. For fluorochromes, such as berberine, that emit in the yellow region, Corning filter C. S. 3-61 or Polaroid UV 420 are satisfactory, but for preferred dyestuffs that emit in the green or the blue regions, a satisfactory filter combination is not readily obtained because of the transmission by the source-filter of the strong emission lines of the mercury arc in the visible blue-violet region. For this and other reasons an investigation has been made of the potentialities of vertical reflected illumination by the use of a special dichroic filter[3] with high reflectance for the ultraviolet and low reflectance for the visible, and with transmission properties that are reciprocal to the reflectance (Fig. 4). The performance of this filter system is such as to make likely its adoption in the microfluorometric scanner.

[3] Obtained through the courtesy of A. F. Turner, Bausch & Lomb Optical Company, Rochester, N. Y.

Mechanical scanning. To scan the fluorescence of cells on a microscope slide it is necessary to allow the light emitted by each cell to fall *separately* and *successively* on the photosensitive surface of the photocell. The more practical choice between mechanical and electronic methods is a mechanical scanner[4] that utilizes a Nipkow disk (*6*) of the type used in the early work in television. The disk (Fig. 1) has a radius of 4 cm and contains a spiral of round apertures spaced at equal angular intervals so that only one aperture at a time is over the field or area being scanned. With one rotation of the disk, which at 1,800 rpm requires one thirtieth of a second, a scanning pattern is formed with a number of lines (20) corresponding to the number of apertures in the disk. The size of the apertures is equivalent in the object plane to a circle with a diameter of 10 μ, or of the order of a cell nucleus. The radial separation of the centers of the apertures corresponds to 5 μ in the object plane. Each scanning path therefore overlaps those adjacent on either side by 5 μ, so that all portions of the field are scanned.

The scanning disk is used in either of two positions—in the image plane, as in Fig. 2, or by optical

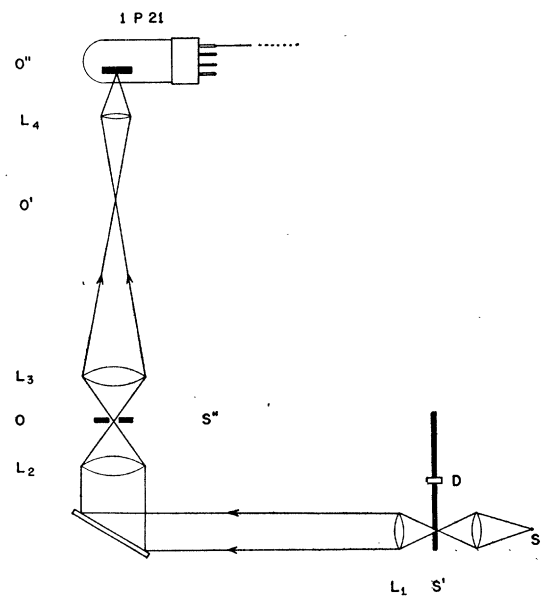

FIG. 5. Microfluorometric flying-spot scanner. *S*, source of critical illumination; *D*, scanning disk at image of source; L_4, ocular lens with high eye-point. Other symbols as in Fig. 2.

projection in the object plane, as in Fig. 5. When the disk is placed in the image plane, a conventional microscope field of about 0.2 mm diameter is illuminated. With one rotation of the disk the light from each self-luminous body in the field passes separately and successively through one of the scanning apertures and is focused on the photosensitive surface of the photocell. For a disk as described the linear

[4] We are indebted to M. G. Brown and M. L. Polanyi, of the American Optical Company, Stamford, Conn., for the principle of using the Nipkow disk in microscanning.

velocity of the apertures is 754 cm/sec, and the light impulse from a cell nucleus of average diameter, 10 μ, has a duration of 66 μsec.

When the flying-spot scanner is used (Fig. 5), the scanning disk is optically projected in the object plane. A flying spot illuminates the microscope field at any instant with a circular area of light 10 μ in diameter and scans the entire field in one rotation of the disk. Each object fluoresces separately and successively, and the light pulse therefrom is focused on the photocell for about 66 μsec. This system is preferred in terms of both mechanical and electronic performance and potentiality.

Aside from the scanning of a field, the microscope slide as a whole is scanned in a few minutes by means of an automatic mechanical stage,[5] which moves the slide back and forth in the x-direction and advances it intermittently in the y-direction.

Photoelectric detector. A lens (Fig. 2), which has as conjugate foci the image-plane of the microscope and the cathode of the photocell, produces a reduced image of the scanned, self-luminous body in the plane of the photosensitive surface. By this means the light energy passing through any scanning aperture during its entire transit falls on a small central spot of the photocathode, and variations in the sensitivity of the photocathode with position are minimized.

The detector is a type 1P21 photomultiplier tube (RCA), operating with a voltage of 675 v between the cathode and the last dynode and with 75 v per dynode stage. This affords a satisfactory signal-to-noise ratio.

The load resistance used with the photomultiplier tube is 5 megohms, which, together with the input capacity of the preamplifier that follows, produces a voltage pulse of somewhat longer duration than that of the light pulse of 66 μsec obtained in the scan of one nucleus with diameter 10 μ.

Preamplifier. A Type 9002 electron tube, which is connected as a cathode follower and used after the photomultiplier, serves as a preamplifier. This circuit reduces the capacitive load on the 1P21 tube and provides an output impedance that is low enough to permit the convenient use of several feet of cable between the output of the preamplifier and the input of the pulse-width discriminator. The amplitude of the voltage pulse at the cathode of the 9002 tube is of the order of 0.1–0.3 v, depending upon the type of cell scanned.

Pulse height-width discrimination. The discriminator circuit that follows the preamplifier performs two functions. First, a pulse-height discriminator rejects pulses that arise from cells with a lesser magnitude of fluorescence and at the same time passes pulses from cells with fluorescence energy above the level of interest and presumably in the range of diagnostic significance.

A second function of the circuit is to discriminate between pulses of different widths. This requirement must be met because the heterogeneous distribution of

[5] Designed by C. R. Stryker, of the Sloan-Kettering Institute.

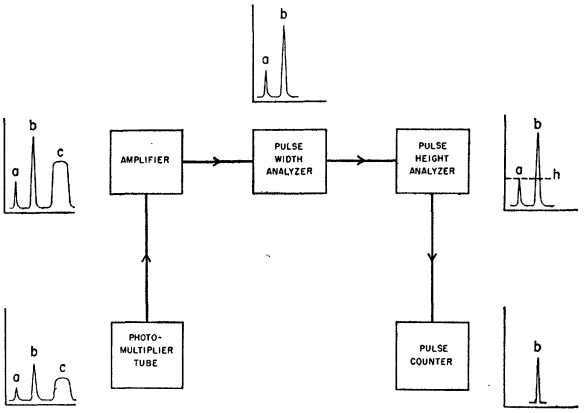

FIG. 6. Block diagram of electronic components of microfluorometric scanner. Voltage pulses of a normal cell, *a*, cancer cell, *b*, and a cell mass, *c*, are analyzed in terms of width (wide pulse *c* is rejected) and height (only pulse *b* is counted).

cells on a microscope slide is such that not only isolated cells but groups or clusters of cells occur. Such clusters, if compact and not optico-electronically resolved, give rise to pulses that are greater in height than those from individual cells. However, since the clusters are several cell dimensions in width, the associated voltage pulse is several times wider than that derived from a single cell, and such a pulse can be rejected electronically on the basis of width. For this a pulse-width discriminator passes a band of pulses with widths corresponding to those derived from nuclei of various sizes and with a range of diametric dimensions. In the present work this range is set at 10–18 μ, which is equivalent to a band pass of 66–119 μsec in the pulse-width discriminator. A pulse derived from a luminous object greater than 18 μ in diameter—i.e., of the order of twice that of two average normal nuclei—is rejected.

The final requirement which the circuit must satisfy is that the pulse-height and -width discrimination should proceed so that one operation does not electronically exclude the other. Further work on the design of circuits is required in order to fulfill this condition. The use in parallel of circuits for pulse-height and -width (7) discrimination, together with a coincidence circuit, may afford the simplest solution to the problem.

Pulse-counting. The output of the discriminator circuit is led into an amplifier (Tracerlab SC-15), which has a resolution time of less than 5 μsec and an input sensitivity of 1 mv. The output of this amplifier, derived from a multivibrator, is a negative pulse with a duration of 1 μsec and an amplitude of approximately 2 v. The output of the pulse amplifier is fed into a scaler (Tracerlab SC-100), where the pulses are counted at a maximum rate of 60,000 counts per minute.

The operation of the microfluorometric scanner composed of the foregoing components can now be summarized. Let one assume that the flying spot of illumination sweeps across a field which contains (Fig. 6)

a normal cell (*a*), a cancer cell (*b*), and a cluster of cells (*c*). The output of the photomultiplier tube consists of three pulses of differing amplitude and width which correspond to the intensity and the distribution of the light in the fluorescing cells. After amplification the pulse-width discriminator rejects pulse *c*, derived from the cluster of cells. The remaining pulses, *a* and *b*, are led into the pulse-height discriminator, whose level, *h*, is set above the height, *a*, of the normal pulse. A portion of pulse *b*, derived from the cancer cell, then energizes the counting circuit, and the presence of a cancer cell is recorded.

Oscilloscope presentation. The voltage pulse can be led from the preamplifier to a cathode-ray oscilloscope (Sylvania Model 400) for presentation on an observation screen (Fig. 2). The fluorescence and phase photomicrographs and the voltage pulses for various cells that exfoliate from the cervix uteri and the pleura are given in Fig. 3. The cytological criteria and the classification of Papanicolaou (*8*) are used. The voltage pulses are photographically recorded from the oscilloscope screen as each cell is scanned. Representative normal cells of the vaginal smear (squamous and parabasal epithelial cells) and of the pleural fluid (mesothelial cells and histiocytes) have voltage pulses of the order of 0.1 v. Cancer cells of the vaginal smear (Class IV) and the pleural fluid (Class V) have voltage pulses of 0.2 and 0.3 v, respectively. Abnormal cells (Class III) in the vaginal smear with morphological features suggestive of, but not conclusive for, cancer have intermediate values, of the order of 0.15 v. The fluorescence intensities of the cells in Fig. 3 are approximately representative of the average for samples of the order of 100 cells. The statistical evaluation of this material, which will appear in a forthcoming publication (*4*), indicates that 20 per cent of cells of Class III, 60 per cent of Class IV, and 83 per cent of class V have fluorescent light intensities above the maximum for the normal cells in a smear.

In view of the foregoing consideration, the development of a microfluorometric scanner for the automatic searching and detection of cancer cells in preparations of exfoliated cells appears to be feasible. This instrument, it is hoped, will serve as an adjunct to the Papanicolaou technique in the screening of the population for certain types of neoplastic diseases. Moreover, with modifications in technical and staining protocol to be described elsewhere, such an instrument has potential application in other fields: (*a*) clinical hematology, for the differential counting of cells of the peripheral blood and the bone marrow; (*b*) biology, in the counting of the relative numbers of resting, dividing, and polyploid cells in a fixed tissue culture, a spread of whole cells, or a preparation of isolated nuclei; and (*c*) radiology, for the study of the degenerative change in the nuclear chromatin of radiated cells that exfoliate from the serous and mucous membranes.

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Technical Papers

Series-Aiding Phototube Bridge[1]

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The series-aiding phototube bridge circuit, in conjunction with a light source, comprises a photometer that is ideally suited for measurements of light absorption with very high precision and sensitivity. The circuit schematically consists of a Wheatstone bridge arrangement of two resistors and two phototubes. The tubes are connected anode-to-cathode with a vacuum-tube voltmeter connected from a point between the resistors to the common connection between the phototubes. A small battery or dry rectifier can supply the bridge potential. In this circuit, the dynamic resistance of one phototube acts as a load resistance for the other phototube so that very large output voltages are obtained for small changes in relative light intensity on the tubes. Such a circuit measures the ratio of the light intensity on the two tubes and hence is independent of the intensity of the light source. When Type 935 phototubes are used, a sensitivity of 1 v for 1% change of light intensity is achieved when a total of 30 v potential is applied across the bridge. At 90 v the sensitivity is 7 v/%. Thus a high voltage is provided for a small change in light intensity. The vacuum tube voltmeter that operates in connection with this bridge must operate on circuit resistances ranging from 10^7 to 10^{13} ohms.

Best performance of the photometer employing this

[1] Abstract of a paper presented at the Gordon Research Conferences, New London, N. H., on Aug. 1, 1951.