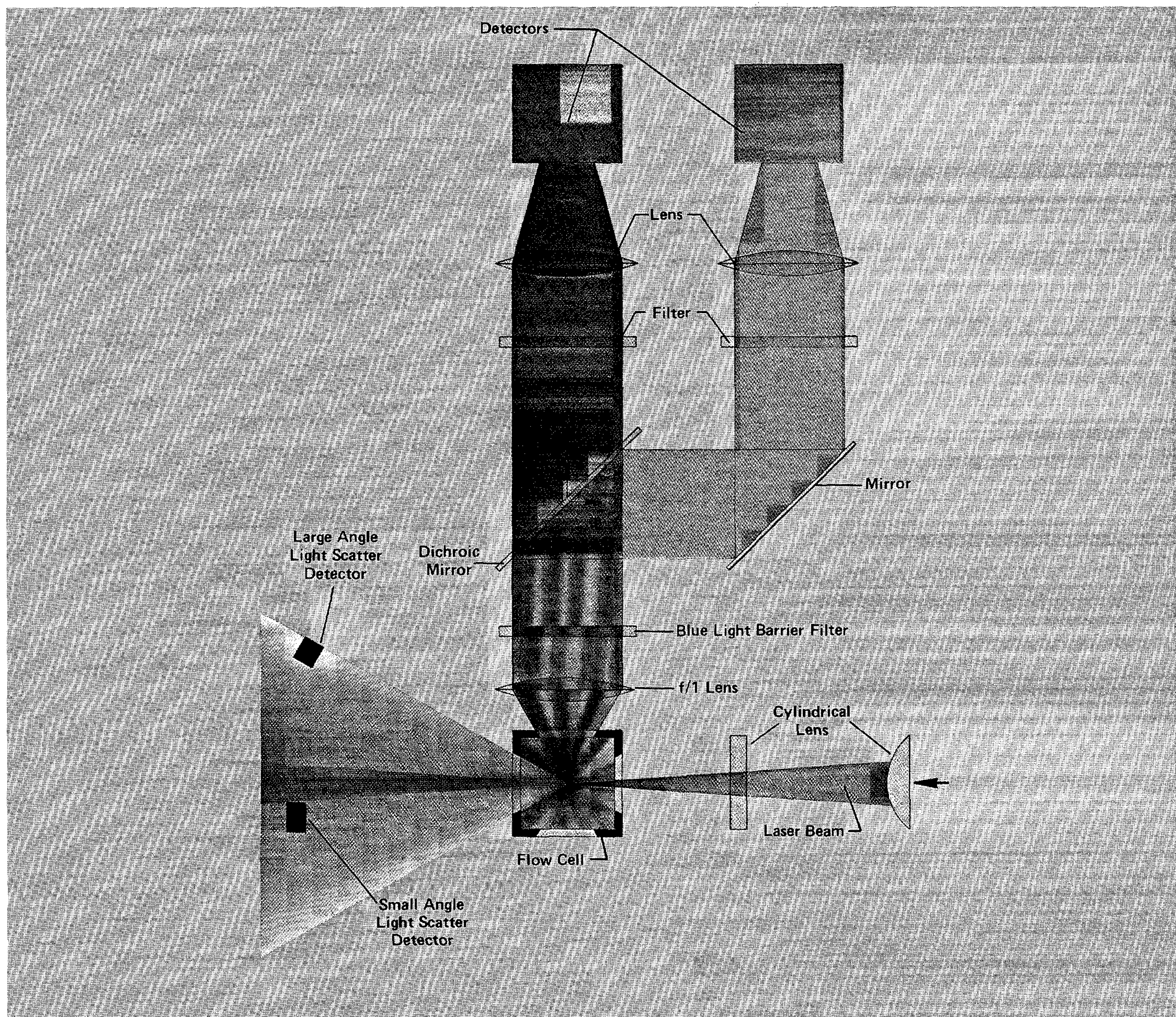

Fig. 5. Flow cytometer optics. Cylindrical lenses of different focal lengths focus the laser beam into an ellipse at the center of the flow cell. As a stained cell passes through the laser beam, a pulse of fluorescent light is generated and a portion of it is collected by f/1 light-collection optics. An interference "barrier filter" prevents scattered laser light from entering the highly efficient photomultiplier detectors. In the model shown, there are two photodetectors to measure green and red fluorescence originating from different parts of the cell. A dichroic mirror reflects the longer wavelength red fluorescence and transmits the shorter wavelength green fluorescence.

Concurrent with the emission of cell fluorescence, the blue laser light is scattered. The scattered light provides a means of obtaining information concerning the structure (morphology) of the scattering object. Since the size, shape, and mass (hence the refractive index) of a cell are the morphological features usually desired, an analysis of the light-scatter pattern produced by a cell may permit cell identification by providing a "signature" related to these physical properties. If light scatter signals from two detectors at different angles are compared, a signal can be generated that will discriminate fluorescent signals from debris and thereby enhance the purity of the signals from cells.

Two general types of laser illuminating light beams have been used. Earlier flow cytometers used spherical lenses to focus the laser beam into a spot of about 75-micron diameter at the cell stream intersection point to give light pulses about 40 microseconds long. The system shown here uses cylindrical lenses with different focal lengths to form an elliptical beam about 5-7 microns across its minor axis and about 100 microns across its major axis. Because the intensity distribution of the laser beam is Gaussian, these dimensions correspond to the points where the intensity of the laser beam is about 1/10th the intensity in the center of the laser beam. Cells traverse this laser beam in about 3-4 microseconds, so standard electronic circuitry for gamma ray spectroscopy can be used.

Fig. 6. This diagram represents the various signals that are handled by the electronics and the PDP-11 computer. The time between signals is calibrated and the signals are held so that all appropriate signals from the same cell can be compared and decisions can be made based on their values. This is all done before the cell arrives at the droplet break-off point. An induced charge can be placed on the few droplets most likely to contain the cell of interest, and these can be sorted. With this system purities exceeding 90% have been achieved. The operator interacts with the PDP-11 through a keyboard terminal and a cathode ray display.

Basic Fluorescence Measurements

Most applications of flow cytometry involve measurement of the fluorescence induced in a cell stained with a fluorescent dye, as the cell passes through a laser beam. Analysis of the light pulse determines the concentration and location of the stained biological molecules within the cell.

To illustrate, we consider a cell stained with two fluorescent dyes, a yellow stain specific for the DNA in the cell nucleus and a green stain specific for the protein in the cytoplasm (Fig. 7). Exciting the cells with the 448-nm line of an argon laser generates two fluorescent signals, a red/yellow signal from the nucleus and a green signal from the cytoplasm.

The flow chamber ensures that each cell passes through the center of the laser beam and is illuminated uniformly by the laser light. In most LASL flow

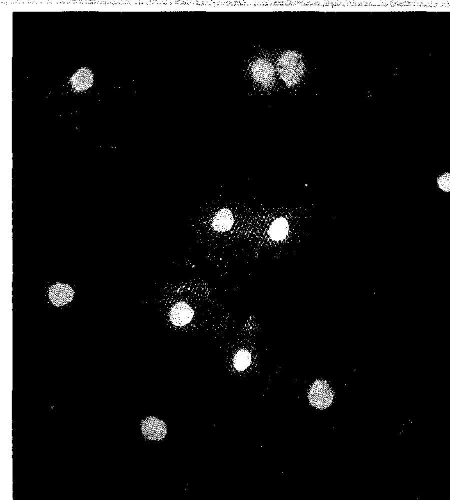

Fig. 7. Normal squamous cells stained with fluorescein isothiocyanate (green) and mithramycin (yellow). The cellular cytoplasm is green and the nucleus is yellow/red. The picture was taken through a fluorescent microscope with dark-field illumination.

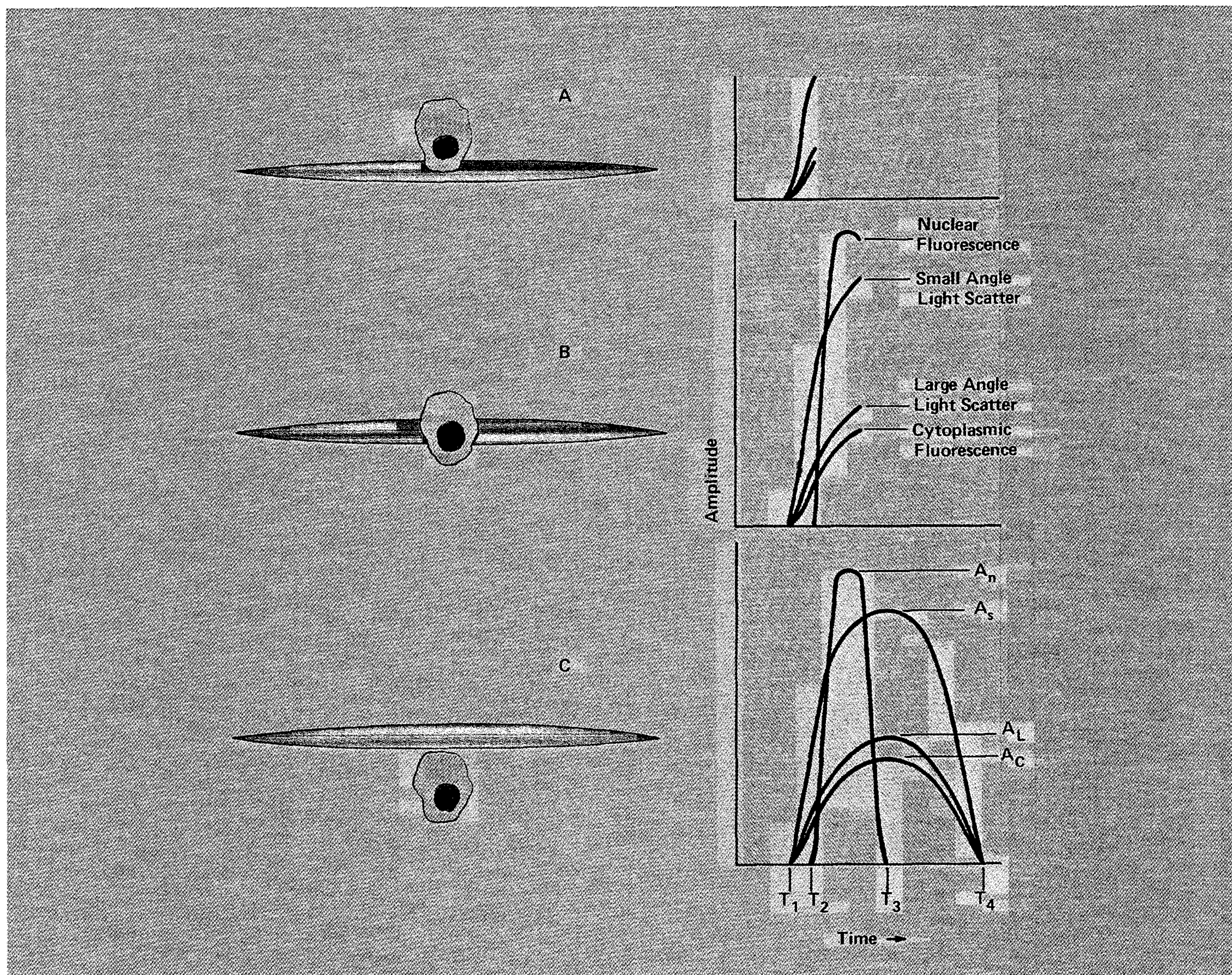

Fig. 8. The origin of electrical pulses that are generated as a two-color stained cell passes through the laser beam is illustrated here. In A, as the cell enters the laser beam, three signals are initiated: small- and large-angle light scatter and the excitation of green cytoplasm fluorescence. In B, the yellow nucleus fluorescence has been excited. In C, the complete pulse shapes from the four detectors can be seen. Several useful signals can be obtained from these voltages pulses. The amplitude of each signal (A_L, A_S, A_C, A_N) gives a measure of the density of the stain within the cell and the amount of light scattered. The length of the pulses above the threshold setting ($T_4 - T_1$ and $T_3 - T_2$) gives a measure of the cell and nuclear diameters, and the area under each curve gives a quantitative measure of the total amount of fluorochrome and the total amount of light scattered. The experimenter can decide which signals are best for analysis. In reality, the light-scatter signals are considerably larger than the fluorescence signals. Adjustment of amplifier gains allows one to display the signals overlayed on the same scale. Nuclear and cytoplasmic diameters can be obtained from the length of the pulses

cytometers the laser beam is shaped to an elliptical cross section at the intersection with the sample stream. The slit of laser excitation light provides a low-resolution scan of each cell as it passes through the laser beam. The fluorescent signals (Fig. 8) are measured at 90° from the laser beam optic axis to minimize the background light in the fluorescent light detectors (that is, to give dark-field illumination). The fluorescence signal intensities are proportional to the amount of yellow and green fluorescent stain and thus to the amount of DNA and protein. The use of interference filters to separate the colors of light into the light detectors produces separate signals. The signals are analyzed individually by a two-dimensional pulse-height analyzer. The integrated intensity of a fluorescent pulse yields the DNA content (or protein content) of the cell, and the duration of the pulse yields the nuclear diameter (or cytoplasmic diameter).

A precise determination of DNA content was not possible with the first flow cytometers because of their poor resolution. For example, all normal, nonreplicating cells should have the same DNA content. However, the DNA distribution for a population of such cells measured with early flow cytometers