Extended Application of Flow Microfluorometry by Means of Dual Laser Excitation*

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Summary. A dual laser beam excitation device for flow analysis of biological particles has been developed. The aid of this arrangement is to increase the range of fluorescent agents employed so far in quantitative and qualitative cytochemistry. Combining an argon ion and a helium-cadmium laser two color fluorescence measurements were performed employing propidium iodide as a DNA stain and fluorescamine which stains total protein in fixed cells. Energy transfer processes between the antibiotic and DNA specific dye mithramycin and propidium iodide both being bound to nuclear chromatin were analyzed. Utilization of energy transfer processes is generally discussed as a mean to extract information about the structure and conformation of nuclear chromatin in situ. The application of a crypton ion laser with three lines near 400 nm and a single line at 350 nm having a light output in each range of nearly one Watt gives the opportunity of utilizing DNA fluorochromes which have an excitation maximum in the deep blue region. DNA spectra are shown employing mithramycin, the benzimidazol derivative 33258 (Hoechst) and the indol compound DAPI which has a high DNA specificity combined with a great stability under UV illumination. By separating two focussed laser beams at their intersecting points with the liquid sample stream the trajectory of each flowing cell crosses the beams sequentially, which causes a solitary dual excitation of each cell. The advantages of a solitary excitation device compared with a simultaneous one is discussed.

Introduction

Laser flow systems and cell sorters based on electronic sensing and separating of cells (van Dilla et al., 1968; Bonner et al., 1972; Holm and Cram, 1973; Arndt-Jovin, 1974; Crissman et al., 1975; Marx, 1975) can employ only a few

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numbers of fluorescent dyes which are excitable with 457 and 488 nm line of argon ion laser. Among these dyes are acriflavine (Sprenger et al., 1971), auramine O (Kramer et al., 1972), ethidium bromide (Ditttrich and Göhde, 1968), propidium iodide (Crisman and Steinkamp, 1973), and mithramycin (Crisman and Tobey, 1974; Tobey and Crissman, 1975), all being used for DNA analyses, fluoresceinisothiocyanate (Crisman and Steinkamp, 1973), used as a protein stain, and fluoresceindiacetate (Rotman, 1973), which enables the monitoring of cytoplasmic esterase activity within cells.

Beyond it some other fluorochromes have been utilized so far with respect to fluorescent labeling of cellular constituents. But these dyes only fluorescence after being excited with blue light near 400 nm. Consequently, we have developed a dual laser beam arrangement to expand the excitation spectrum of laser flow systems and to enlarge the number of employable fluorochromes.

**Materials and Methods**

HeLa cells were grown in monolayer culture. They were harvested after treatment with trypsin (0.06%) and then centrifuged at 100 x g for 15 min. Cells were then fixed in glutaraldehyde (1% in Hepes buffer, pH 7.4) for 18 h and stored at 4 °C in Tris buffer (0.18 M Tris-HCl and 0.18 M NaCl). DNA staining has been performed with propidium iodide (Crisman and Steinkamp, 1973), mithramycin (Crisman and Tobey, 1974; Tobey and Crissman, 1975) and DAPI (4'-6-Diamidino-2-Phenylindole) (Dann et al., 1971), at a final concentration of 10⁻³ μg ml⁻¹ which yields a strong fluorescence in the nuclei but not in the cytoplasm as it occurs at 0.1 μg ml⁻¹, a concentration used for detection of mycoplasma in tissue culture (Russel et al., 1975). Protein staining was carried out with FITC (Crisman and Steinkamp, 1973), and fluorescamine (Stöhr, 1975).

The flow systems used were the Cytofluorograph 4800A (Biophysics Systems) supplemented with an argon ion laser (10 mWatt at 488 nm) and a helium-cadmium laser (15 mWatt at 441 nm), and the FACS II cell sorter (Becton Dickinson) with an argon ion laser (1.4 Watt at 488 nm) and a crypton ion laser (1.2 Watt at lines 407, 413, 415 nm and 1 Watt at 350 nm).

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**Results**

Utilizing the Cytofluorograph as the flow system (Stöhr and Goerttlér, 1974) we combined the 488 nm line of an argon ion laser with the 441 nm line of a helium-cadmium laser. The latter beam has been brought to a level of the first one by a front surface aluminium mirror. Both beams have been focussed by the same arrangement of two cylindrical lenses. Different angles of entry into the first lens effected the separation of the two beams at their points of interaction with the liquid laminar stream in the flow cuvette. This arrangement performs a solitary excitation with two different wave lengths. Each cell traversing the cuvette crosses the two zones focussed illumination. Electronical circuits guaranty the correlation of the two fluorescence pulses, which occur during the traverse of a cell through the beams. With this double beam arrangement we investigated fluorescamine (FC) as a fluorochrome which stains total protein in fixed cells and the surface membrane of viable cells.