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EMOSS: An Epiillumination Microscope Objective Slit-Scan Flow System[1]

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An epiillumination microscope objective slit-scan flow system has been fabricated utilizing two dimensional slit scanning with hydrodynamic sample stream focussing. Low resolution (4 μm) analysis of cellular fluorescence is facilitated by the definition of a stabilized flow plane through hydrodynamic focussing. Coincidence of the region of stabilized flow with the focal plane of the microscope objective will allow for the collection and subsequent imaging of fluorescence from cells oriented along this plane. Two orthogonal slit-scan contours are generated as a cell traverses the excitation region. It is hoped that the need for a three dimensional system will be precluded by preferential orientation of the cells in the region of stabilized flow. Cellular fluorescence is collected by a high numerical aperture epiillumination optical system and imaged onto two orthogonal slits. Two photomultiplier tubes are used to detect fluorescence.

It is anticipated that the epiilumination microscope objective slit-scan flow system will be used with a variety of fluorescent stains and markers, as well as extended to the research of light scattered by cells. (Steen, H. B., Cytometry 1:26-31, 1980

Key words: Acridine orange, epiilumination, flow cytometry, fluorescence, imaging, microscopy, slit scan

Investigations have shown multidimensional slit-scanning to be an extremely useful technique in analytical cytology. Three dimensional fluorescence slit-scanning has been documented as effective for classifying cells from the female genital tract into pattern classes of normal and abnormal (Wheeless *et al.*, manuscript submitted for publication, 10). Additionally, because of the large feature set made available through use of this technique (8, 9) it should have broad application to other biological areas. The application to other areas, however, is limited by both the complexity and expense of slit-scan systems developed to date. To facilitate the widespread use of this technique, the design of less complex multidimensional slit-scan systems is needed. This need has led to the development of the epiillumination microscope objective slit-scan flow system (EMOSS) which incorporates two dimensional slit-scanning (1, 8) with hydrodynamic sample stream focussing (7).

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Materials and Methods

Instrumentation: The EMOSS flow system generates two orthogonal slit-scan contours from each cell as a cell traverses a fluorescence excitation region. The system is composed of four basic units: 1) specimen delivery; 2) fluorescence excitation; 3) collection and imaging of fluorescence; and 4) slit-scan detection. The sample stream jets from a glass capillary, through air, to flow over the surface of a glass coverslip. An epiillumination system is used to excite cellular fluorescence as the cells flow over the coverslip. Fluorescence is collected by an optical system and imaged to two image planes. A slit aperture is located in each image plane, the two slit apertures being mutually orthogonal. Photomultiplier detectors located after each slit provide slit-scan contours as the cells flow through the excitation region.

Specimen delivery: Specimen delivery is accomplished following the methods employed by Steen and Lindmo (4, 7). Cells flow in suspension from a syringe pump to a glass flow nozzle where a coaxial water sheath is introduced. The flow is laminar, consisting of a 50 μm specimen stream centered within the 250 μm diameter sheath. Exiting the flow nozzle at an oblique angle to a glass coverslip, the jet streams through air and onto the glass surface. The flow remains laminar with the sample stream occupying a central stabilized position. After passing through the fluorescence excitation region, the sample and sheath are removed by a gentle suction applied through a hypodermic needle oriented at an oblique angle to the coverslip in line with the flow nozzle.

Fluorescence excitation, collection and imaging: The optical layout of the EMOSS flow system is presented in Figure 1. Excitation

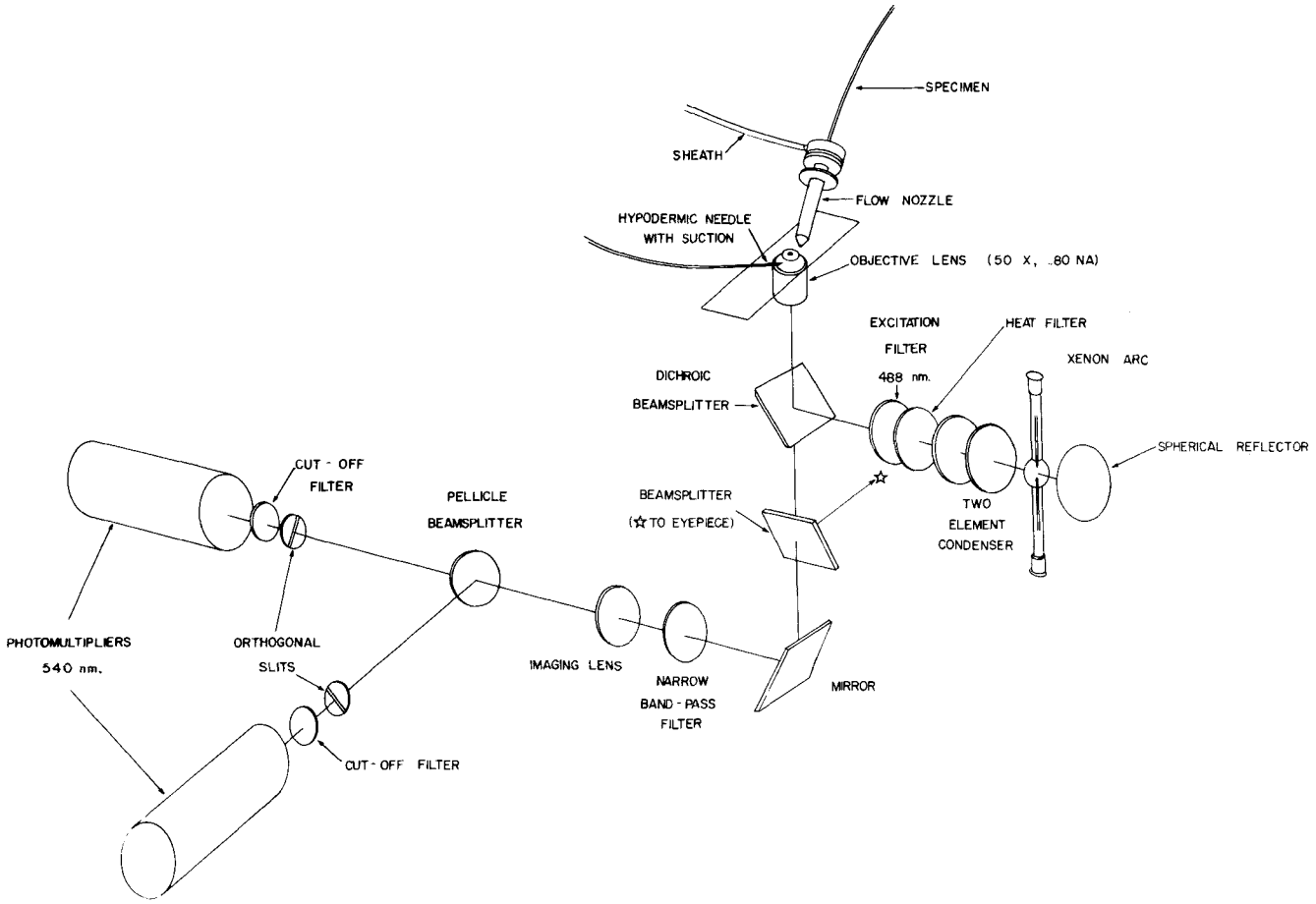

FIG. 1. EMOSS flow system.

radiation is generated by a 150 watt xenon arc lamp using critical illumination. The "hot spot" of the arc is imaged to the flow plane providing an irradiated region through which the sample stream is hydrodynamically focused. The excitation beam is filtered using a multilayer dielectric filter (designed with a sharp cut-off at 490 nm) followed by a dichroic mirror which directs the excitation beam to an infinity-corrected microscope objective (50×, 0.80 NA, oil immersion).

The excitation spot is imaged to a region of approximately 150 μm in diameter within the specimen stream. Such a region is of suitable dimensions to allow irradiation of all portions of a cell during measurement. Cellular fluorescence is collected by the objective, transmitted by the dichroic mirror, and subsequently directed to an eyepiece and detection optics. Fifteen percent of the fluorescent signal is directed to an infinity-corrected wide field eyepiece (10×) used with a barrier filter. The remaining 85% of the signal is directed to the detection portion of the system.

Slit-scan detection: The fluorescent signal delivered to the detection portion of the system is bandpass filtered (540 nm ± 20 nm bandwidth) and imaged to two detector channels by a lens and pellicle beam splitter. Each detector channel contains a slit aperture in the image plane, a photomultiplier tube and amplifier circuitry. Currently the slit apertures being used have an effective width of 4 μm. The two slits are mutually orthogonal, each at 45° to the apparent direction of cell flow (Fig. 2). Cells in the plane of the coverslip are, therefore, slit-scanned in two orthogonal directions as they flow through the excitation region.

Methods: The EMOSS flow system has been fabricated and tested. Fluorescent microspheres of varying sizes and degrees of brightness were utilized to check system alignment and provide for system calibration. After alignment and calibration, clinical gynecologic cytology specimens were analyzed to evaluate system performance. Techniques for collection, preparation, and staining of cytologic material follow those reported previously (2). Cellular material was collected by scraping the uterine cervix with a plastic spatula with subsequent suspension in a preservative solution. Cell dispersal was accomplished by syringing (5). Cells were then stained in suspension using a 0.01% Acridine Orange solution, washed and resuspended in Millonig's glutaraldehyde buffer and refrigerated until use.

Results and Discussion

Preliminary unfiltered contours of normal cells are presented in Figure 3. Each of the contour sets presents two orthogonal contours generated as a cell image moves over the pair of orthogonal slits. In sets A-C, cytoplasmic and nuclear boundaries are in evidence and processing of the contours for nuclear fluorescence, nuclear size and cell size is straightforward. The lack of multiple nuclear peaks in either contour of sets A-C indicates the cells to be single and uninucleate.

In contrast, sets D-F depict contours illustrating cell overlap or binucleation. In D, the lower contour appears to be from a single uninucleate cell. However, the upper contour shows two nuclear peaks, indicating binucleation and/or cell overlap. In contour sets E and F, the upper contours provide insufficient information to classify correctly the cells. The presence of a second orthogonal contour in both cases allows for resolution of a second nuclear peak permitting correct classification of cell overlap or binucleation.

The design of the EMOSS flow system allows for the generation of two orthogonal slit-scan contours. For cells aligned in the plane of the coverslip, the generation of the two

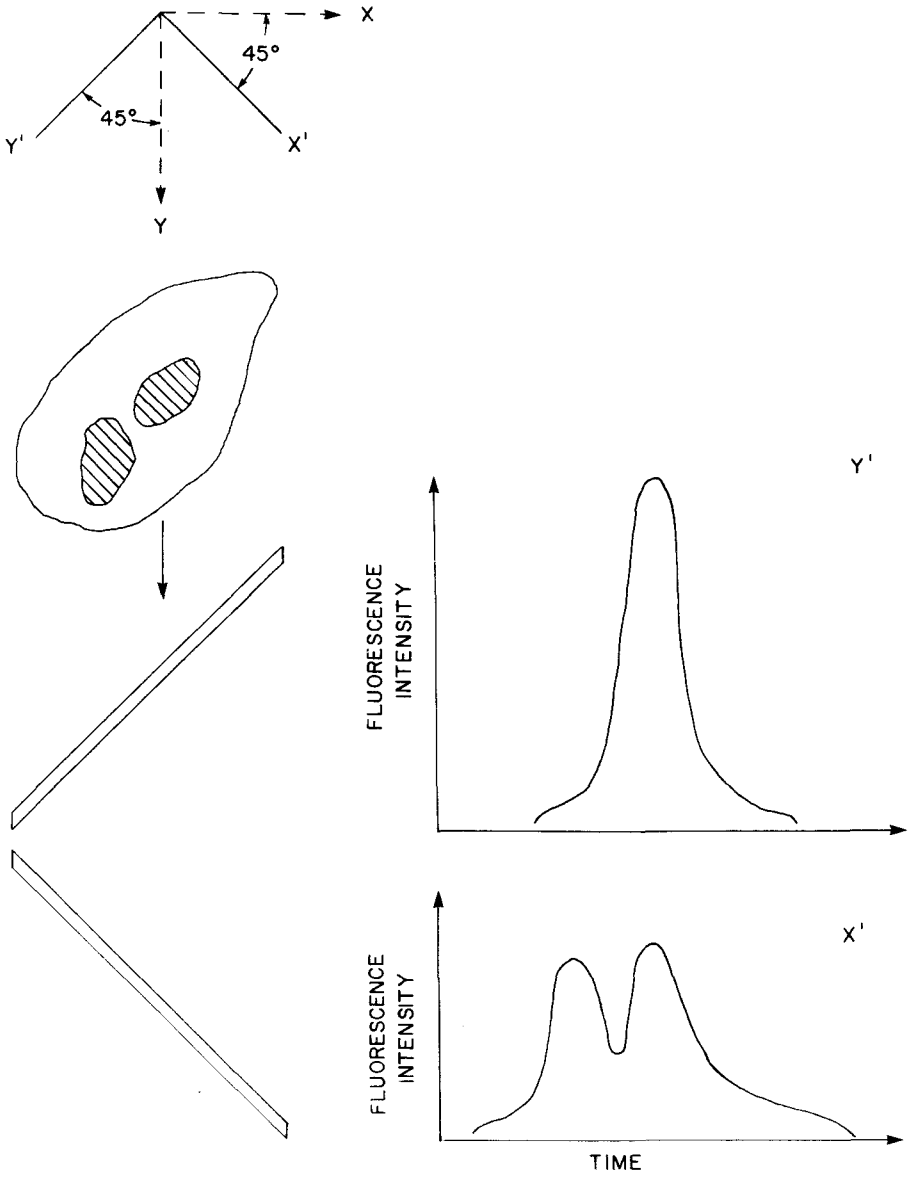

FIG. 2. Binucleate cell recognition through generation of two orthogonal slit-scan contours. This illustration depicts a binucleate cell with slit-scan contours for +45° and −45° slit orientations. Nuclei oriented along the Y′ -axis are resolved in the slit-scan contour taken along the X′ -axis but not in that taken along the Y′ -axis. A single slit-scan contour of a binucleated cell may result in misclassification of the cell due to the inability of the system to resolve the two nuclei. Multidimensional slit-scan contours permit recognition of most binucleated cells as well as cell clumps and overlapping cells.

orthogonal contours allows for the recognition of most multinucleated cells, overlapping cells, and cell clumps. In previous systems (1, 8) it has been necessary to generate three orthogonal slit-scan contours to allow recognition of multinucleation, overlaps, and clumps. It is hoped that a preferential orientation of the cells in the region of stabilized flow will preclude the need for a three dimensional system (4, 6). Data is currently being collected to test this assumption.

The contours shown in Figure 3 were obtained using a high NA (NA = 0.80) microscope objective. A high NA objective, while allowing for greater resolution capabilities and increased image irradiance will produce a decreased depth of field. Through the use of a high NA objective, only a thin section through the cell will be focused. This phenomenon is referred to as optical sectioning (3). The implications of this phenomenon upon the performance capabilities of the EMOSS flow system are currently under study (Weller *et al.*, in preparation).

Additional applications of the EMOSS flow system have been considered. The xenon arc lamp currently used in this system is characterized by a relatively flat spectral output between 400 and 800 nm. This lamp can readily be replaced by a number of arc lamps (such as a mercury arc), extending the spectral range of illumination into the ultraviolet. Through the use of appropriate excitation and collection filters, the EMOSS flow system can be used with a variety of dyes and markers.

The two channel configuration of the EMOSS flow system is well-suited to slit-scanning of fluorescence over multiple spectral bands. Additionally, through the use of two slits of different widths, slit-scan contours of the same cell may be generated at two resolutions. Most importantly, the two channel design incorporates many features of previous multidimensional slit-scan instrumentation into a compact optical system of simpler configuration than that found in earlier systems.

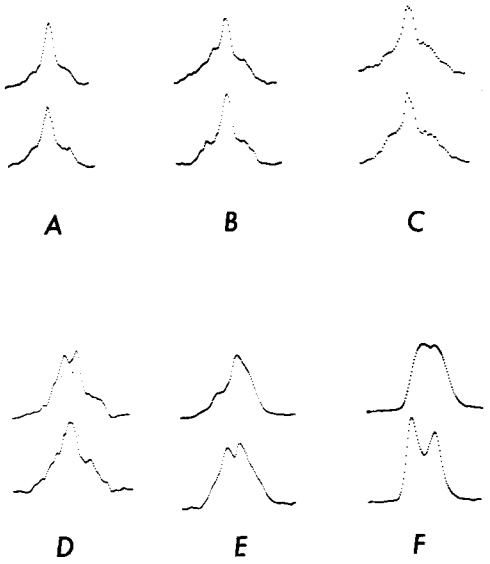

FIG. 3. Orthogonal slit-scan contour sets. *A-C*, contours generated by normal, single uninucleate cells; *D-F*, contour sets in which multiple nuclear peaks resolved along one dimension suggest binucleation or cell overlap.

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