A MEANS FOR ORIENTING FLAT CELLS IN FLOW SYSTEMS

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ABSTRACT Flattened cells, such as red blood cells, epithelial cells, and sperm of many species, cause problems for fluorescence-activated cell analysis and sorting machines because the flow systems of such devices are unable to control the orientation of these cells as they flow past the detectors. For this reason, the fluorescence or scattered light measurements for identical cells may vary greatly. A flow geometry is here described that orients flat cells in a coaxial flow system so that each cell presents the same aspect to the observation device. A wedge-shaped exit on the sample injection tube in a coaxial flow system is sufficient to produce the desired orientation effect when used with low sample flow rates. Data is presented showing the effect of orientation of fixed chicken erythrocytes on histograms of small forward-angle light-scattering measurements.

INTRODUCTION

In analysis of cells in flow systems, the cells are generally lined up in sequence by making a dilute suspension of the cells and injecting it axially in the center of a concentric flow tube containing a carrier fluid. The resulting coaxial flow is then tapered down to a small diameter. If laminar flow is maintained during the reduction of diameter, the cross-sectional geometry is preserved, and the cell-containing center stream tube is also reduced in diameter, thus confining the cells to a relatively narrow region near the flow axis. This process, sometimes called "hydrodynamic focusing," accurately positions the cells as they flow through a sensing station for observation by one or more of a variety of detection devices (1). The observation may occur internally, i.e., within some flow tube or chamber, or externally, i.e., after the fluid is ejected through a nozzle as a jet.

Describing the cells by three convenient, orthogonal axes, we may define "flat" cells as those in which the thickness along one axis is considerably smaller than along the other two. A problem arises in the analysis of such cells: the aspect of the cells as seen by the observation device varies with the orientation of the cell. Hydrodynamic forces arising during hydrodynamic focusing tend to align cells so that their long axis, if they have one, is parallel to the flow axis. Due to the axial symmetry of the flow, however, there are no forces that would give flat cells any preferred orientation with respect to rotation about their long axis; a disk-shaped cell will tend to travel edge-first after

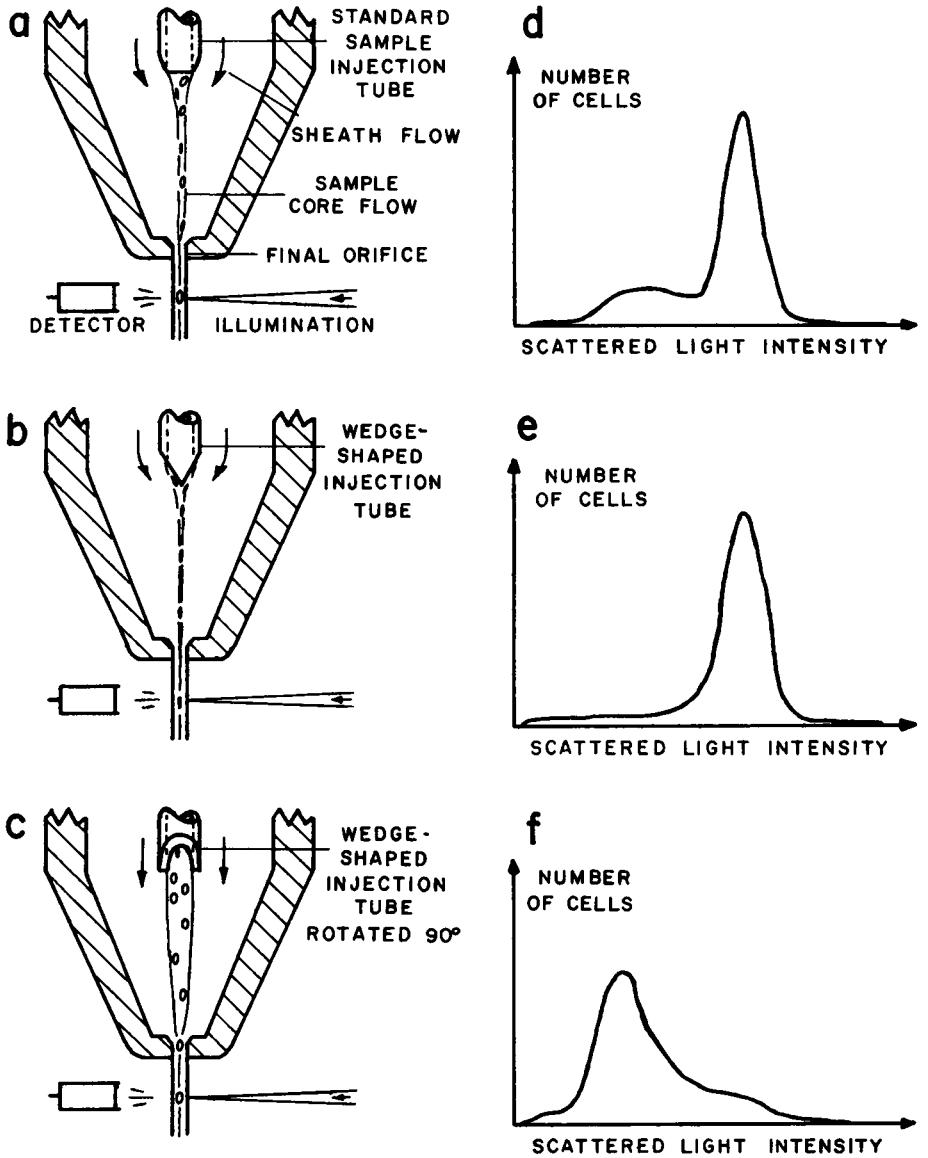

FIGURE 1 Normal and orienting flow configurations and resultant histograms. *a*. Schematic illustration, not to scale, showing cutaway view of standard, coaxial flow system with cell suspension being delivered through sample injection tube into sheath flow. The combined flow is then accelerated through a final orifice with illumination and cell detection occurring in the external jet. *b*. and *c*. The standard sample injection tube has been replaced by one with a wedge-shaped tip, shown edge on in *b* and rotated 90° in *c*. The sketches illustrate the orienting effect of this geometry on flat cells. In *b* the sheath flow converges around the tip so that at low sample flow-rates, the sample flow-core is flattened in this view. An observer in the plane of the paper will see the full face after the cell has passed through the nozzle. In *c*, with the injection tube rotated 90°, the sheath flow is seen to not converge around the injection tube. This allows the sample flow-core to remain broad in this dimension, resulting in a ribbon shape. The differential

focusing, but an observer watching it pass might see a profile that is round or flat or any elliptical shape in between.

Two commonly detected cell parameters, fluorescence and small forward-angle scattered light, both exhibit artifacts in flow system analysis due to this orientation effect. Two examples of cells that show such artifacts are fixed chicken red blood cells (CRBC), which are commonly used in flow system testing and calibration (2), and sperm of many species, used in DNA content studies (3). The problem also arises with the flat, epithelial cells of interest to workers involved in efforts to automate gynecological screening (4–6).

For some time, a means has been sought to circumvent the orientation artifacts that arise in these and other cell systems. One approach, described by Van Dilla et al. (3), places the illumination and observation along the flow axis so that cells with a long axis are always seen end on, and further orientation is not required. Kay and Wheeless (6) and Kachel et al. (7) demonstrated another approach wherein the geometry of the focusing portion of a flow system is not axially symmetric, but rather converages at different rates in orthogonal directions resulting in hydrodynamic forces that tend to orient flat objects in the flow. Fulwyler (8) showed that when an injection tube with a wedge-shaped exterior at the exit is mounted in a microtube of rectangular cross-section such that two flow regions converge around the injection tube exit, orientation of flat objects results. The purpose of this paper is to report that the wedge-shaped injection tube concept has been combined with the existing concentric flow geometry of a cell sorting and analysis system, resulting in a simple means for orienting flat cells in flow systems.

METHODS

We wished to apply the ideas of Kay, Kachel, and Fulwyler to the type of flow systems used in the fluorescence-activated cell sorter, developed in our laboratories at Stanford and marketed commercially as the FACS-II or III by Becton Dickinson FACS Systems (Mountain View, Calif.) (9,10). In this system, cell suspensions are injected axially through standard 22-gauge (0.028-inch outer diameter) hypodermic tubing into a sheath flow regime that then is tapered down and ejected through a 50-μm diameter orifice. Cell detection takes place in the external jet.

A fairly simple modification of the sample injection tube exit proved sufficient to orient fixed CRBC. Whereas the standard injection tube has a conical taper near the exit (Fig. 1 *a*), to facilitate smooth merging of the sample and sheath flow regimes, it was found that a wedge-shaped exit like that used by Fulwyler in his microtubes produces the desired orientation effect

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convergence of the flow in the orthogonal direction at low flow-rates causes flat cells to tend to lie flat within the ribbon core. *d*, *e*, and *f* show histograms of small forward-angle scattered light vs. relative number of cells for glutaraldehyde-fixed CRBC obtained with the configurations of *a*, *b*, and *c*, respectively. The standard configuration of *a* results in the bimodal distribution of *e*. The cells in the two populations are identical but appear as separate populations because of the orientation artifact. When the cells are oriented by means of the wedge-shaped injection tube of *b* and *c*, the histograms *e* and *f* result, where the cells appear predominantly in one of the two peaks of the standard distribution.

when the sample flow rate in the injection tube is sufficiently slow. The wedge-shaped exit is made by grinding two opposite flat faces on the end of the 22-gauge hypodermic tubing used for the sample injection tube. The faces form an angle of approximately 20° with respect to the tube axis, making a total wedge angle of 40°.

RESULTS

Observations of flow using this type of injection tube exit, made by putting ink in the sample flow for visibility, showed an interesting effect. If the average velocities of the sample and sheath flows were made nearly equal, the core of the coaxial flow region would retain the cylindrical cross-section of the injection tube. As the sample flow rate was reduced, however, the cross-section of the core would tend to flatten, ultimately assuming a flat ribbon shape at very low sample flow rates (Fig. 1 *b,c*). The width of the ribbon remained equal to the inner diameter of the injection tube, while the thickness depended on the flow rate. Of even more interest was the fact that CRBC's in the sample flow tended to orient themselves with their flat faces parallel to the ribbon.

Analysis of small forward-angle light scatter of glutraraldehyde-fixed CRBC demonstrated that the orientation induced at the sample injection point is maintained as the flow is reduced in diameter and accelerated through the jet orifice.

Scattered light analysis in standard nonorienting flow systems normally shows a bimodal distribution when a population of CRBC is displayed as a histogram (Fig. 1 *d*). To the extent that scattered light is a measure of size, these histograms indicate a mixed population of "small" and "large" cells, but it has been demonstrated (2) that the population is uniform and that the presence of two peaks is an artifact due to the flatness of the cells; cells seen edge on to the detector scatter less light over the detected angular range than cells that are not seen edge on.

The histograms of Figs. 1 *e* and *f* were acquired with the wedge-shaped sample injection tube in a modified FACS-II nozzle assembly on the Stanford prototype cell sorter. At very low flow rates, the orientation effect caused the majority of the CRBC to appear in either the "small" (edge-on) peak or the "large" peak, depending on which way the injection tube was oriented.

DISCUSSION

It is evident that the wedge-shaped injection tube can be useful in resolving orientation artifacts in flow systems. However, two factors limiting its potential usefulness should be pointed out. First, effective orientation requires very low sample flow rates, thus limiting analysis rates to a few hundred cells per second. Secondly, as can be seen from the histograms of Figs. 1 *e* and *f*, the orientation of CRBC, even at low flow rates, is not complete; some small percentage remains unoriented. At the present time, it is unclear whether the lack of complete orientation is due to some inherent limitation of the orienting effect, such as the aspect ratio of the ribbon, or is related to the fact that CRBC are not completely flat.

Although detailed parametric studies have not yet been performed to determine the

optimum geometry for a wedge-shaped injection system, preliminary results indicate that the orientation effect is not very sensitive to geometric changes such as wedge angle, diameter, ratio of inner to outer diameter, or axial distance from the final orifice. Some evidence suggests that the orientation effect becomes stronger as the wedge angle is made sharper. It is quite possible that other flow geometries may have an even stronger orienting effect, and perhaps allow higher flow rates.

This research was supported by a grant from the National Institute of General Medical Sciences (GM-17367).

Received for publication 1 December 1977.

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