HYDRODYNAMIC ORIENTATION OF SPERM HEADS FOR FLOW CYTOMETRY

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ABSTRACT Two specially shaped sample injection tubes orient flat-shaped mature sperm heads in a flow cytometer. Orientation allows control of a photometric difficulty experienced with some flow cytometers in the measurement of fluorescent and scattered light from sperm and other flat cells. Both relative DNA-stain content and a measure of cell flatness can now be determined simultaneously for each cell at the high rates possible with flow instruments.

INTRODUCTION

Changes in DNA content (1) and in head shape (2) of mature mammalian sperm have been proposed as methods for detecting genetic and reproductive damage in spermatogenic cells. Flow cytometers, operating at hundreds of measurements per second, should prove very useful in detecting such damage. Some instruments, notably those utilizing epi-illumination (3,4) are approaching the precision and stability required for measurement of DNA-stain content but are unable to provide morphologic information. Flow cytometers that detect the emitted fluorescence perpendicular to the axis of flow (5,6) are capable of similarly good precision in the measurement of DNA-stain content but only of spherical or cylindrical cells. In these instruments the measurement of flat mammalian sperm heads fluorescently stained for DNA content yields either grossly nonsymmetrical or bimodal frequency distributions (1,7). This occurs because the flat shape and high index of refraction of the sperm cause preferential emission of light from their narrow edge. The emission profile appears to be a weak function of angle everywhere except at the edge. From the shape of the histograms, it is inferred that the average intensity over the edge is approximately twice that of the flat face and the edge intensity varies sharply with angle (1). The observed fluorescence intensity thus depends upon the orientation of the cell with respect to the exciting light beam and the detector; the resulting fluorescence intensity distribution obtained for normal, randomly oriented sperm heads reflects both DNA-stain content and head shape. Hydrodynamic orientation of the sperm heads within the flow cytometer allows precise measurement of relative DNA-stain content and also provides a potentially useful measurement of morphological parameters such as cell thickness and degree of curvature of the flat face.

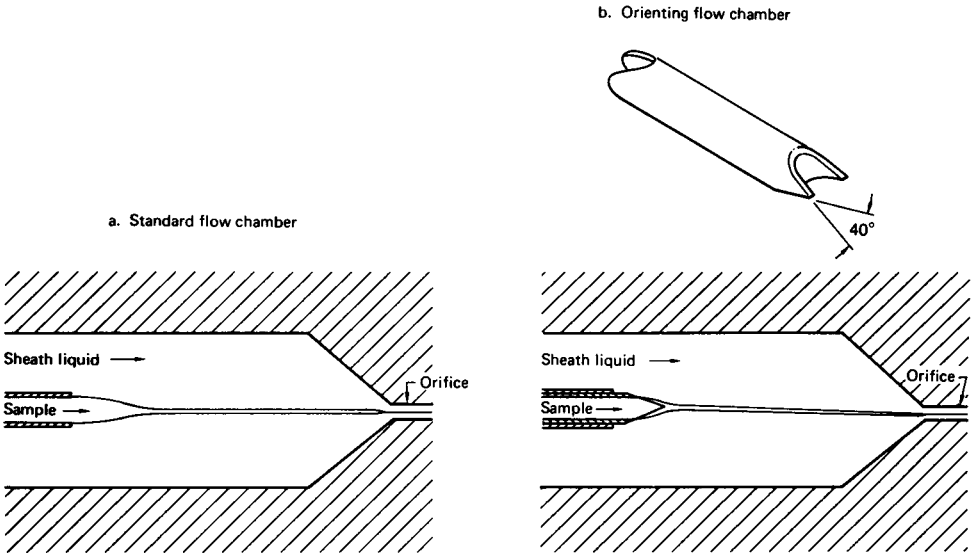

FIGURE 1 *a*. Sample tube arrangement in our standard flow chamber (6). The sample tube has an inside diameter of 0.5 mm, the sheath a diameter of 2.5 mm, and the orifice is 0.25 mm in diameter. The end of the sample tube is 12 mm from the entrance to the orifice and 13 mm from the laser beam. The resultant sample stream has a circular cross-section. *b*. A wedge-shaped tube is positioned inside the standard sample tube. The resulting change in flow conditions causes the formation of a thin, ribbon-shaped cell stream and a corresponding orientation of sperm heads in the plane defined by the flow axis and the two tips of the wedge-shaped tube.

METHODS AND RESULTS

Flow cytometers typically utilize a cylindrical fluid geometry such as shown in Fig. 1*a*. Cells in the sample stream flow out of the sample tube and are picked up by the sheath fluid, which carries them through the orifice where the stream diameter is reduced, confining the cells to the central axis of the flow chamber. The rapid change in velocity upon entering the sheath causes eolongated objects such as sperm heads to align with their long axis parallel to the direction of flow (8, 9). There is, however, no preferred orientation about this axis.

Orientation about the flow axis is accomplished by subjecting the cells to planar hydrodynamic forces achieved with two specially designed sample tubes. The first tube is illustrated in Fig. 1*b*; its shape was suggested in conversations with R. Stovel (10) and M. Fulwyler (11). The tube is made from 26-gauge hypodermic needle stock; outside diameter is 0.5 mm, inside diameter is about 0.25 mm. The end has been filed into a wedge shape, included angle about 40°, as shown in the figure. These diameters have not been optimized for orientation ability, but were chosen to allow the orientation tube to fit directly into the standard sample tube of our flow cytometer. Because of the wedge shape, the sample stream is drawn into a thin ribbon by the sheath liquid. As the sperm heads, moving at near-zero velocity in the sample tube, encounter the sheath, moving at about 100 mm/s, they are rotated so that their flat side is in the plane of the ribbon. This orientation has been observed at 4 mm from the end of the tube, probably occurs much closer, and is maintained through the orifice and at least to the intersection with the laser beam 10 mm beyond the sample tube. The sperm can be oriented at any desired angle with respect to the laser beam by rotation of the sample tube.

The ability of this design to orient sperm heads was demonstrated in several experiments. In

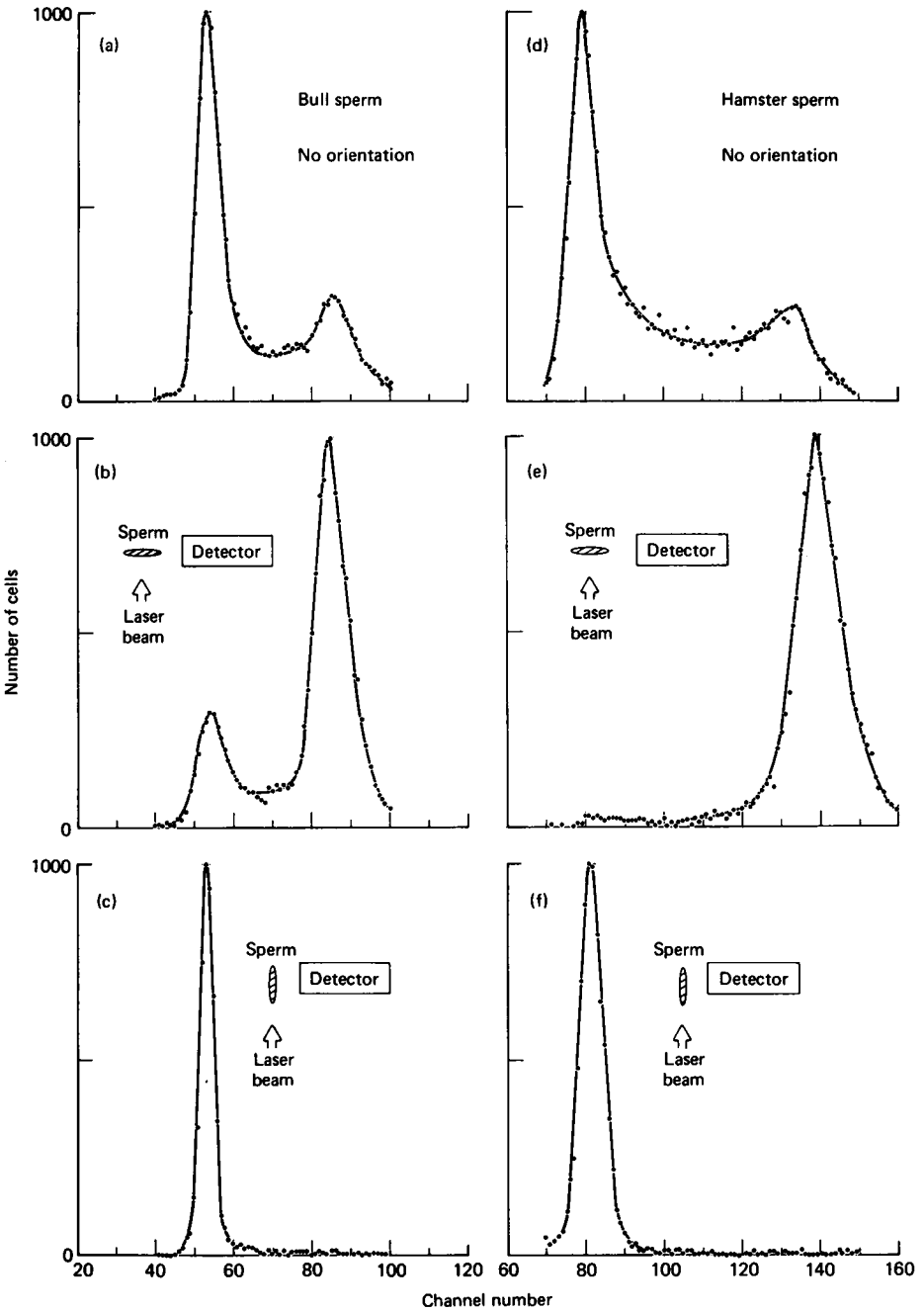

FIGURE 2 Fluorescence intensity distributions obtained for both oriented and nonoriented sperm heads. Bull and hamster sperm were stained by the acriflavine-Feulgen procedure. The inset diagrams indicate their orientation in the flow chamber with respect to the detector and laser illumination. The sperm are flowing perpendicular to the paper. The abscissa (channel number) is proportional to fluorescence intensity.

these tests the emitted fluorescent light was measured perpendicular to both the flow and laser beam axes, as illustrated in the inset diagrams of Fig. 2. In this figure the direction of flow is perpendicular to the paper. These measurements utilized $f/1$ light collection optics and a photomultiplier as detector. The laser light was focused to an elliptical cross-section of about 8 μm thickness along the flow axis and was polarized perpendicular to the flow axis.

The data of Fig. 2 are for bull and hamster sperm. Similar results were also obtained for mouse and ram sperm. For sperm heads randomly oriented about the flow axis, our flow cytometer yields the results shown in graphs 2*a* and *d*. If the sperm were spherical they would have produced a single peak (1). The small peak, around channel 85 for bull sperm and 133 for hamster sperm, is produced by those sperm with edges pointed within the field of view of the detector. In both cases about 25% of the sperm have such an orientation. The remainder of the sperm produces the low-intensity peaks in channels 53 and 81. The middle graphs, *b* and *e*, are the results with the orientation tube positioned to orient sperm heads with their edges pointed toward the detector. In graph *b*, 75% of the bull sperm signals now fall within the peak at high fluorescence intensity, reflecting the degree of orientation achieved. The remaining 25% of the sperm failed to orient because their tails were still attached. Sperm heads with attached tails cannot be oriented by this technique; they yield the lower intensity signal, e.g., channel 53 in Fig. 2*a*. In graph *e* virtually all of the hamster sperm are very well oriented; few of these sperm have attached tails. In the lower graphs, *c* and *f*, the flat side of the sperm head is toward the detector and virtually all of the sperm yield a small fluorescence value. In this case, the tailed-sperm failed to produce a high intensity peak because the degree of orientation was sufficient to keep their relatively narrow bright edge out of the field of view of the detector.

We have also achieved good orientation by a second method, placing a thin wire (200 μm diameter) across the end of the standard sample tube, as illustrated in the upper part of Fig. 3. The wire extends about 1 mm beyond the tube on both sides and is folded back and attached to

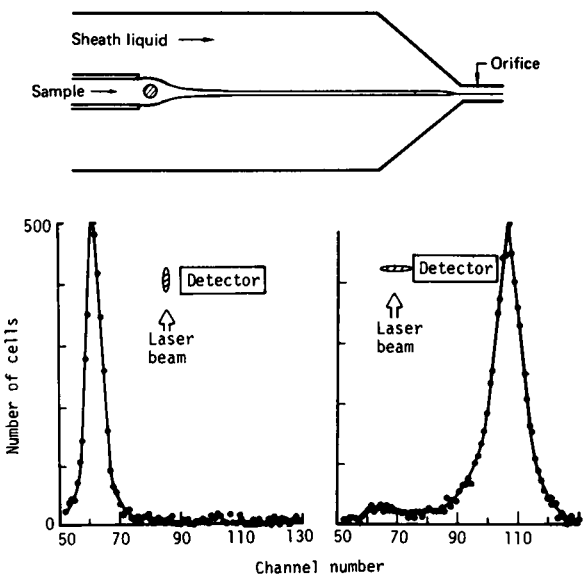

FIGURE 3 An alternate method of orienting sperm cells. The diagram shows the position of a 200-μm diameter wire placed across the standard sample tube, causing the formation of a thin, flat cell stream. The histograms were obtained using the same hamster sperm of Fig. 2, oriented as shown.

the tube. Although the orientation mechanism is not well understood, we believe it comes from the planar flow and velocity changes in the vicinity of the wire. The two histograms in Fig. 3 show that very good orientation was achieved with hamster sperm. Similar results were obtained with the wire placed at several positions between the sample tube and orifice.

In our experience sample tubes of various designs can be used to orient sperm cells, with differing degrees of success. Apparently the major requirement to produce orientation is that a very thin, flat cell stream be produced over a short interval through the application of hydrodynamic forces. Of the arrangements we have tried, the wedge-shape tube is the simplest to implement and is currently the preferred design. Orientation data referred to in the following discussions were taken with the wedge-shaped sample tube.

The presence of an angularly dependent fluorescence emission from sperm heads implies that

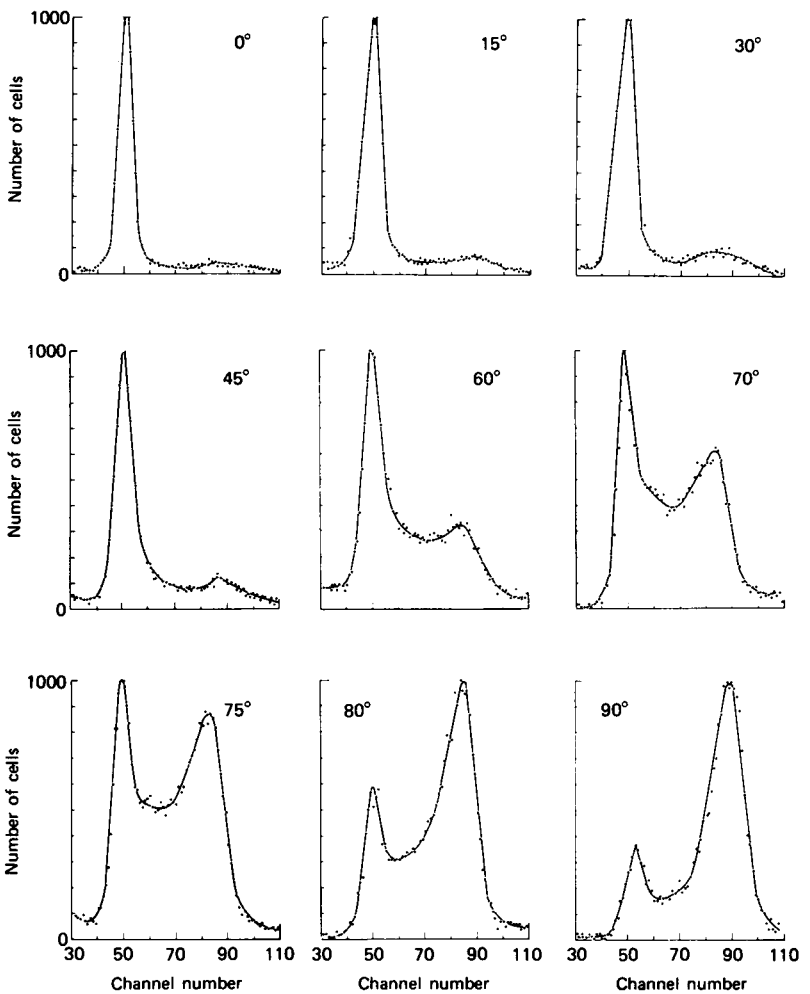

FIGURE 4 Fluorescence intensity distributions for oriented acriflavine-Feulgen-stained mouse sperm. The angle indicated is between the plane of the flat side of the sperm and the laser beam. The detector is at 90° to the beam.

there should be an illumination effect as well. Preliminary data indicate that this is indeed the case, but are not adequate to quantitate the magnitude of the effect.

An additional experiment was performed to measure directly the angular distribution of light emitted from acriflavin-Feulgen-stained mouse sperm. Although ideally the angular distribution should be measured by rotating the detector about the oriented sperm, physical limitations of our flow chamber required the rotation of the sperm instead. Consequently the observations include the emission and excitation effects folded together. Fig. 4 shows the results. The angle indicated is between the plane of the flat side of the sperm and the laser beam, with the detector positioned at 90° to the beam. We interpret these distributions as follows. Between 0° and 45°, 90–95% of the sperm display the low-intensity signal, confirming that their flat sides face the detector and indicating that in these orientations the signal is insensitive to angle. Beginning at 60°, edges of some sperm enter the field of view of the detector sufficiently to elicit high intensity signals. The greater the angle, the larger the fraction of sperm that give high signals, reaching about 80% at 90°. At approximately 70° the number of sperm in the low and high intensity regions is roughly equal. This angle is then the midpoint of the transition region between the two types of emission. This, plus the uniformity of the first four distributions, confirms that the flat face of the sperm has a more stable angular distribution than does the edge. The 60°–80° patterns suggest an intermediate population of cells giving intermediate intensity signals, but these never produce a distinct peak. This probably means that the distribution of orientations is not sufficiently narrow to resolve the transition region. Again the persistence of 20% of cells in the low intensity peak at 90° is probably due to sperm with tails still attached.

DISCUSSION AND CONCLUSION

Orientation of sperm heads controls the effect of cell shape on observed fluorescence and to a large degree it allows separation of the effects of DNA-stain content and cell morphology. One way to take advantage of this new capability is to orient the cells with their flat face toward the laser beam and to use a pair of detectors, one in the forward direction and the other at 90° to the laser beam. For any individual sperm cell the relative signals in the two detectors will depend upon the degree of flatness of the sperm head. A homogeneous population of well-oriented sperm will all yield a small signal in the forward (0°) detector and a large signal in the 90° detector. Abnormal (i.e. non-flat or less flat) sperm would yield slightly increased 0° signals and smaller 90° signals; round sperm would all produce the same intermediate signal in both detectors. The relative signal in the two detectors could then be used as a test for some forms of abnormal morphology. A frequency distribution of the ratio of the two signals would be a distribution of some yet to be defined function of flatness of the sperm heads. By gating on a specific value of flatness, relative DNA-stain content could then be measured for subpopulations of sperm heads. Eventually it may be possible to define the functional relationship between the ratio measurement and DNA-stain content and thus estimate DNA content for the entire population. No matter how well DNA-stain content is measured, the relationship to absolute DNA content will continue to be obscured by the effects of sperm condensation in the stoichiometry of the reaction. Intensity measured normal to the flat face of sperm heads is minimally affected by differences in their morphology. Recent measurements on oriented SWR/J mouse

sperm have yielded coefficients of variation[1] as low as 2%. With such resolution it should be possible to detect variability in DNA content as a consequence of biological damage, although in cases of severe morphologic changes it may be difficult to separate the effects of DNA content variability and shape of the sperm. Further experiments are required and are now underway to develop the full potential of this technique.

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[1]The coefficient of variation is the standard deviation divided by the mean, assuming a normal frequency distribution.