FLOW CYTOMETRY ESSENTIALS

INSIDE
THE BLACK BOX

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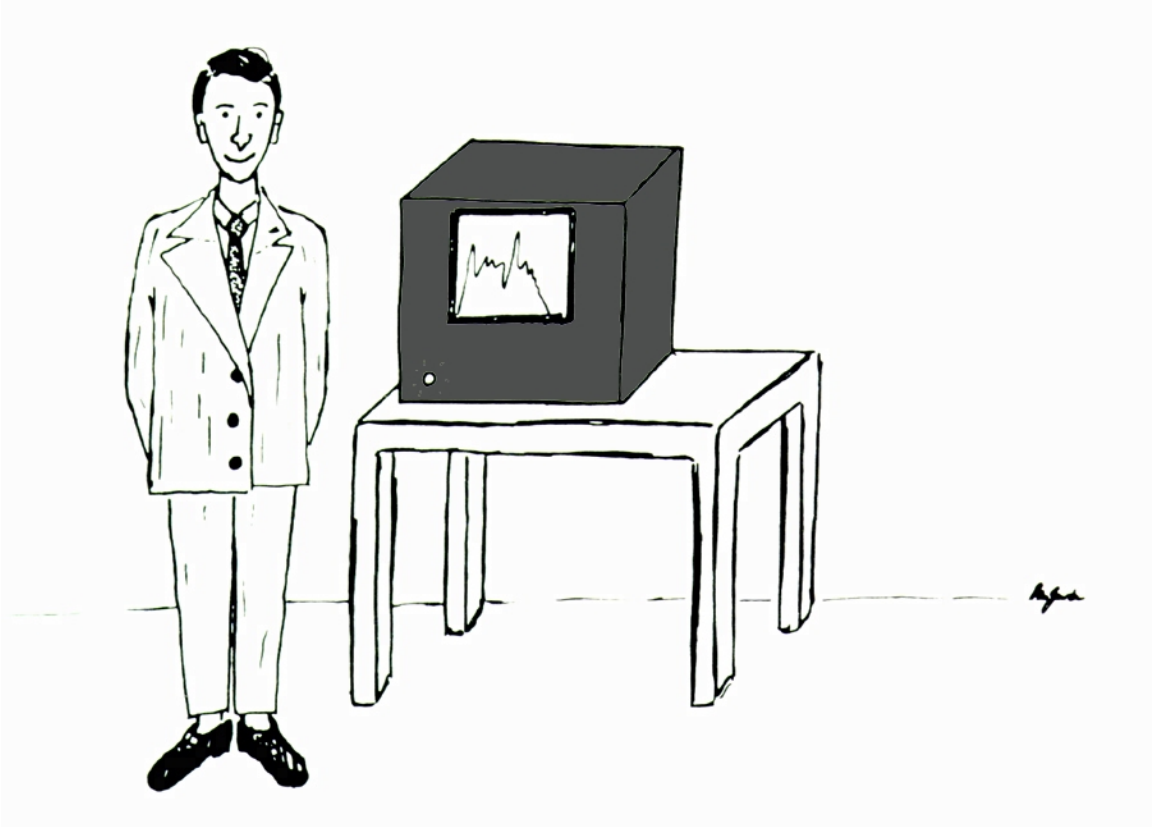
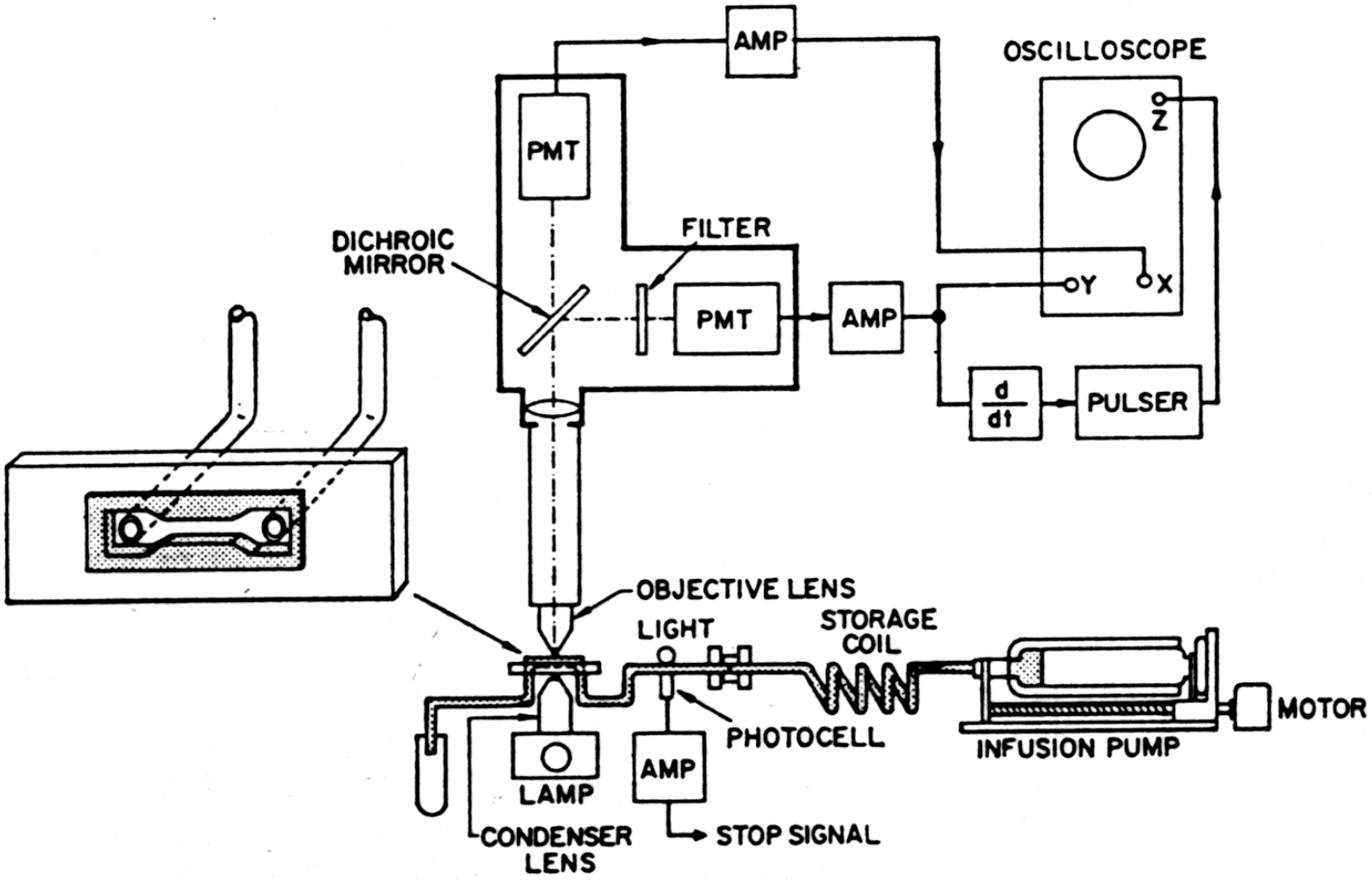
HOW NOT TO BE
A FLOW CYTOMETRIST



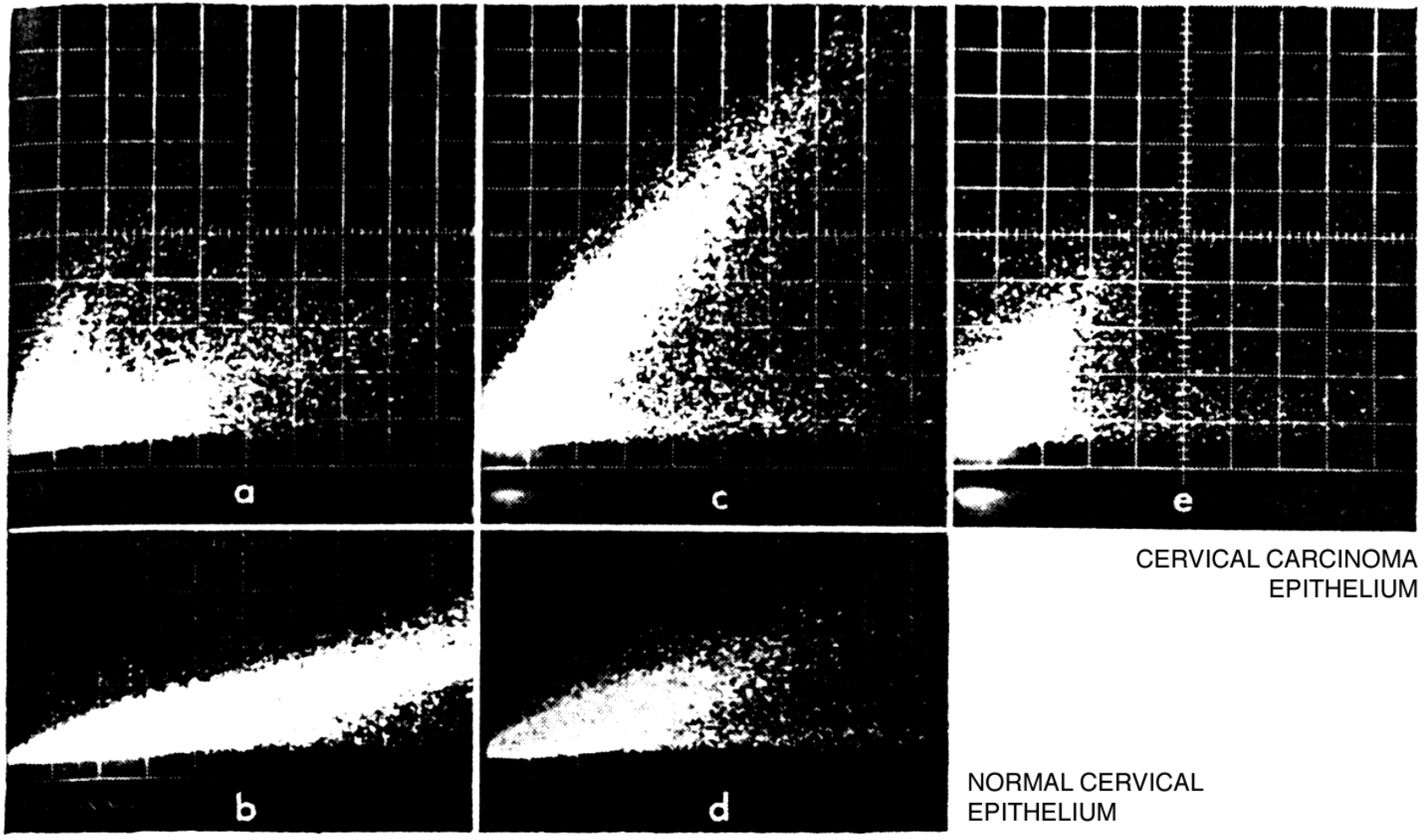
Drawing by Ben Givan

KAMENTSKY, MELAMED, AND DERMAN: "NEW INSTRUMENT FOR ULTRARAPID CELL ANALYSIS"



Kamentsky, Melamed, and Derman, Science 150: 630-631, 1965

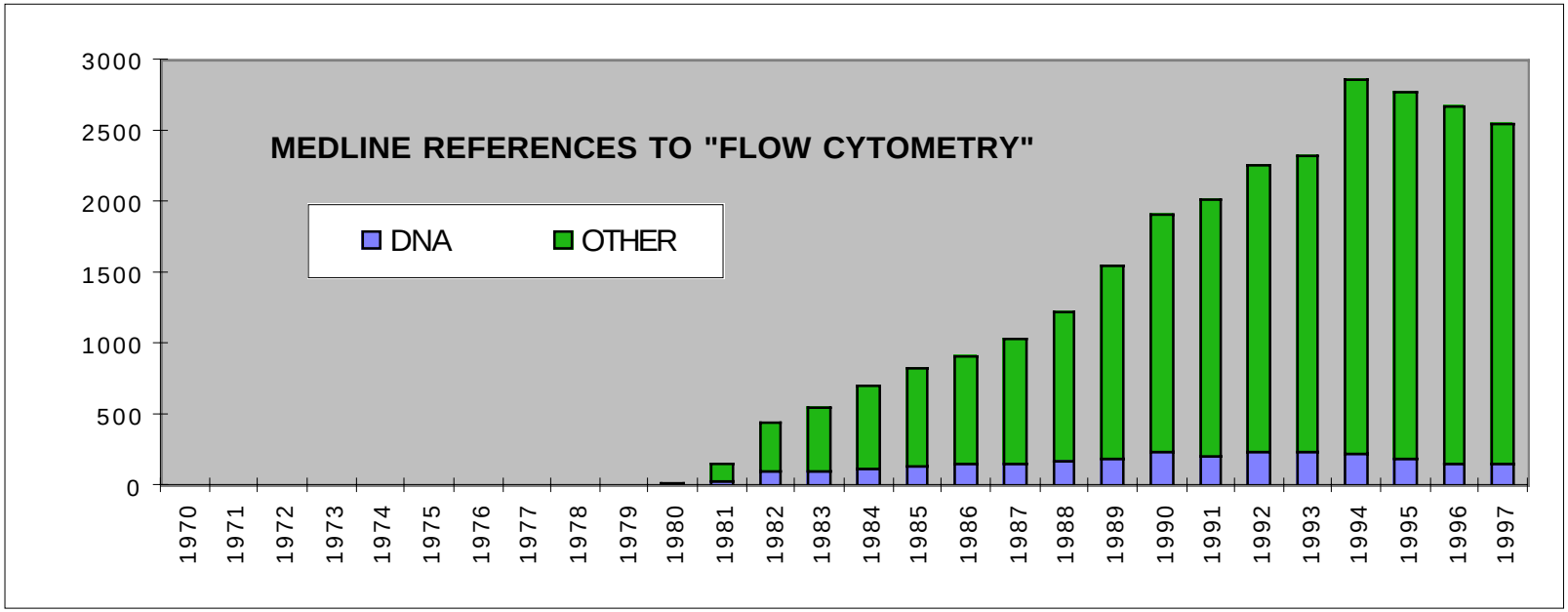
PAP SMEAR BY FLOW CYTOMETRY: 1965

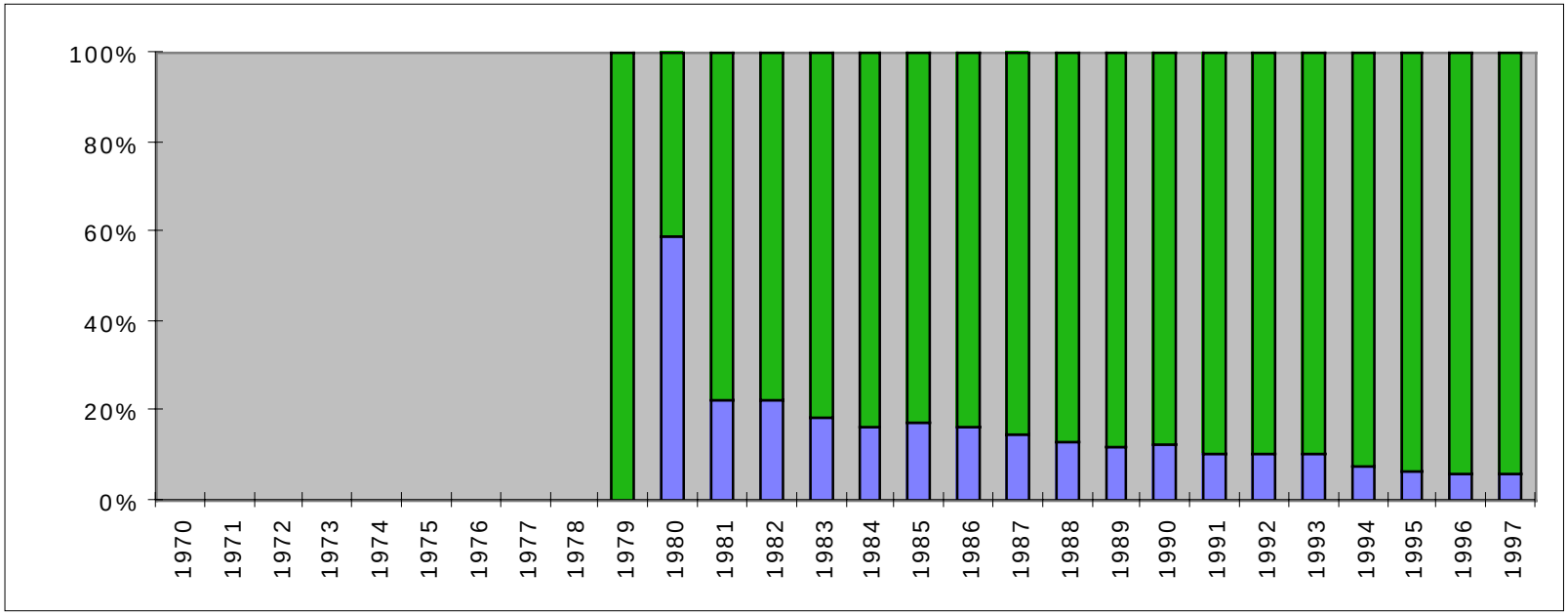


410nm BACK SCATTER

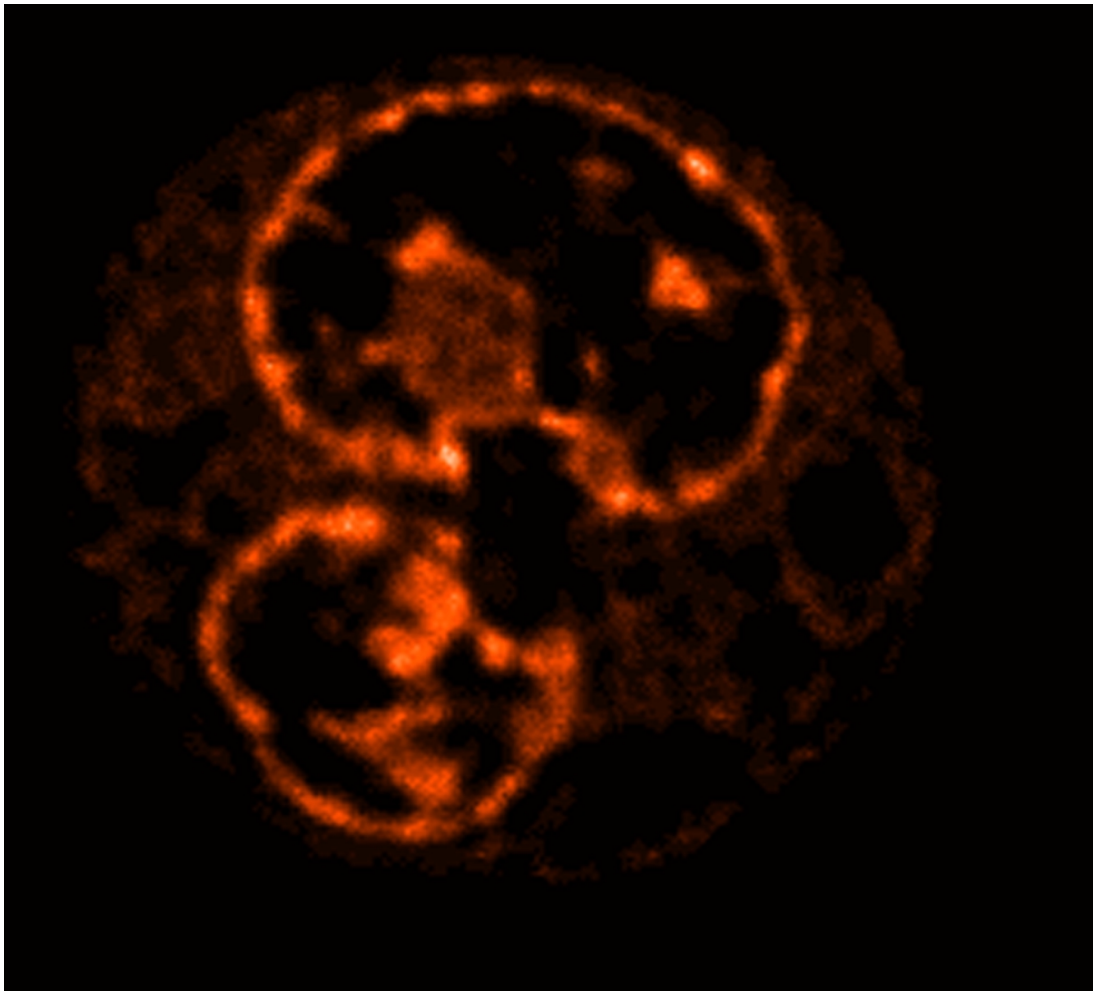
Kamentsky, Melamed, and Derman, Science 150: 630-631, 1965

WHEN?


MEDLINE REFERENCES TO "FLOW CYTOMETRY"
DNA
OTHER
3000
2500
2000
1500
1000
500
0
1970
1971
1972
1973
1974
1975
1976
1977
1978
1979
1980
1981
1982
1983
1984
1985
1986
1987
1988
1989
1990
1991
1992
1993
1994
1995
1996
1997

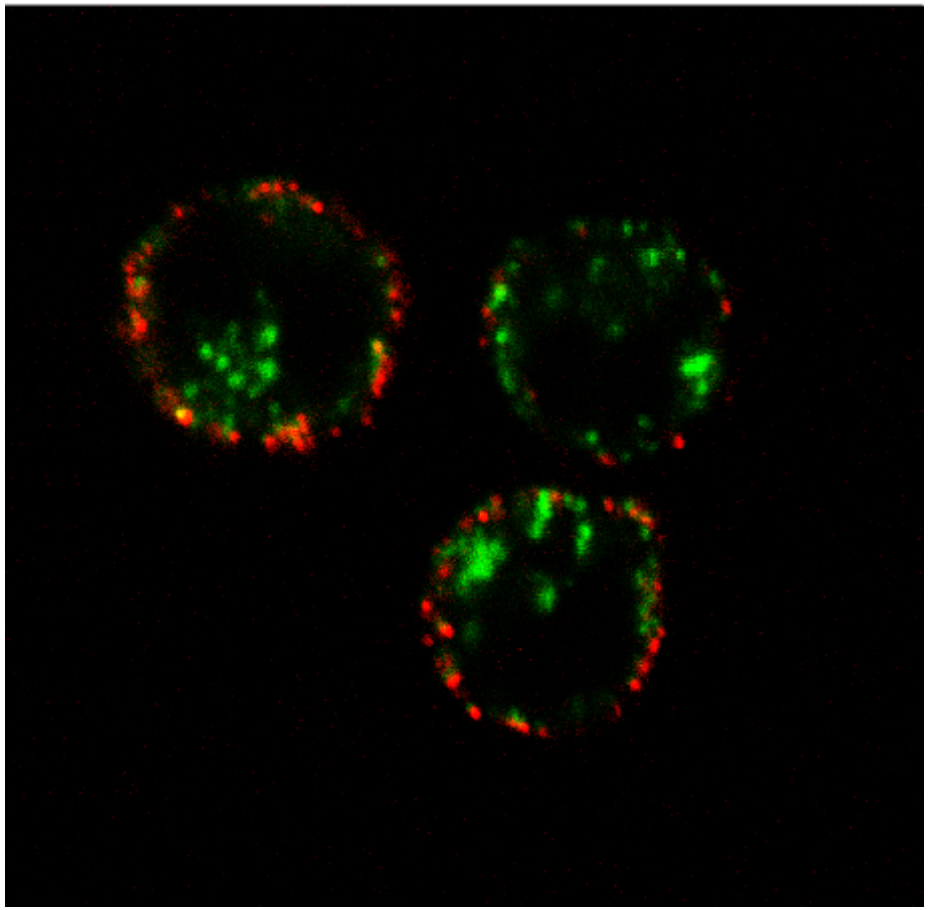

100%
80%
60%
40%
20%
0%
1970
1971
1972
1973
1974
1975
1976
1977
1978
1979
1980
1981
1982
1983
1984
1985
1986
1987
1988
1989
1990
1991
1992
1993
1994
1995
1996
1997

STARTING MATERIAL: STAINED CELLS:
WHAT A FLOW CYTOMETER CANNOT TELL YOU



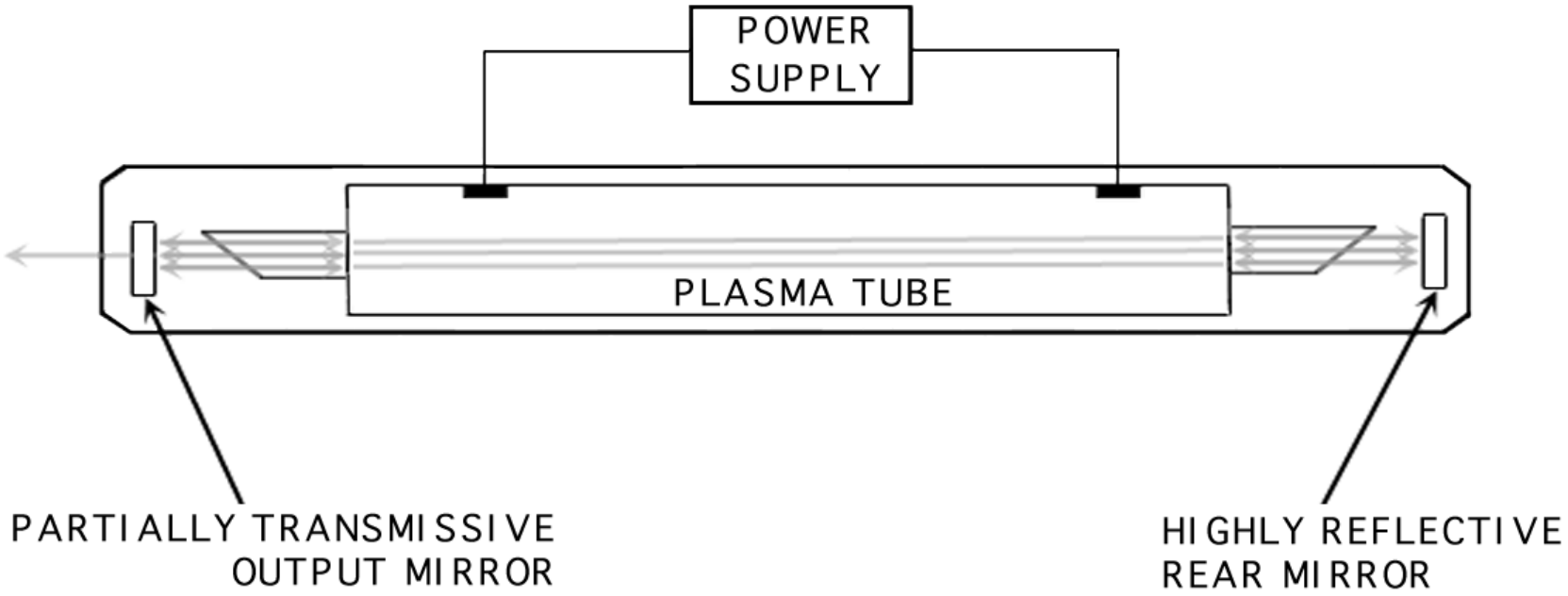
U937 CELL STAINED WITH PROPIDIUM IODIDE

EVEN MORE THAT A FLOW CYTOMETER CANNOT TELL YOU



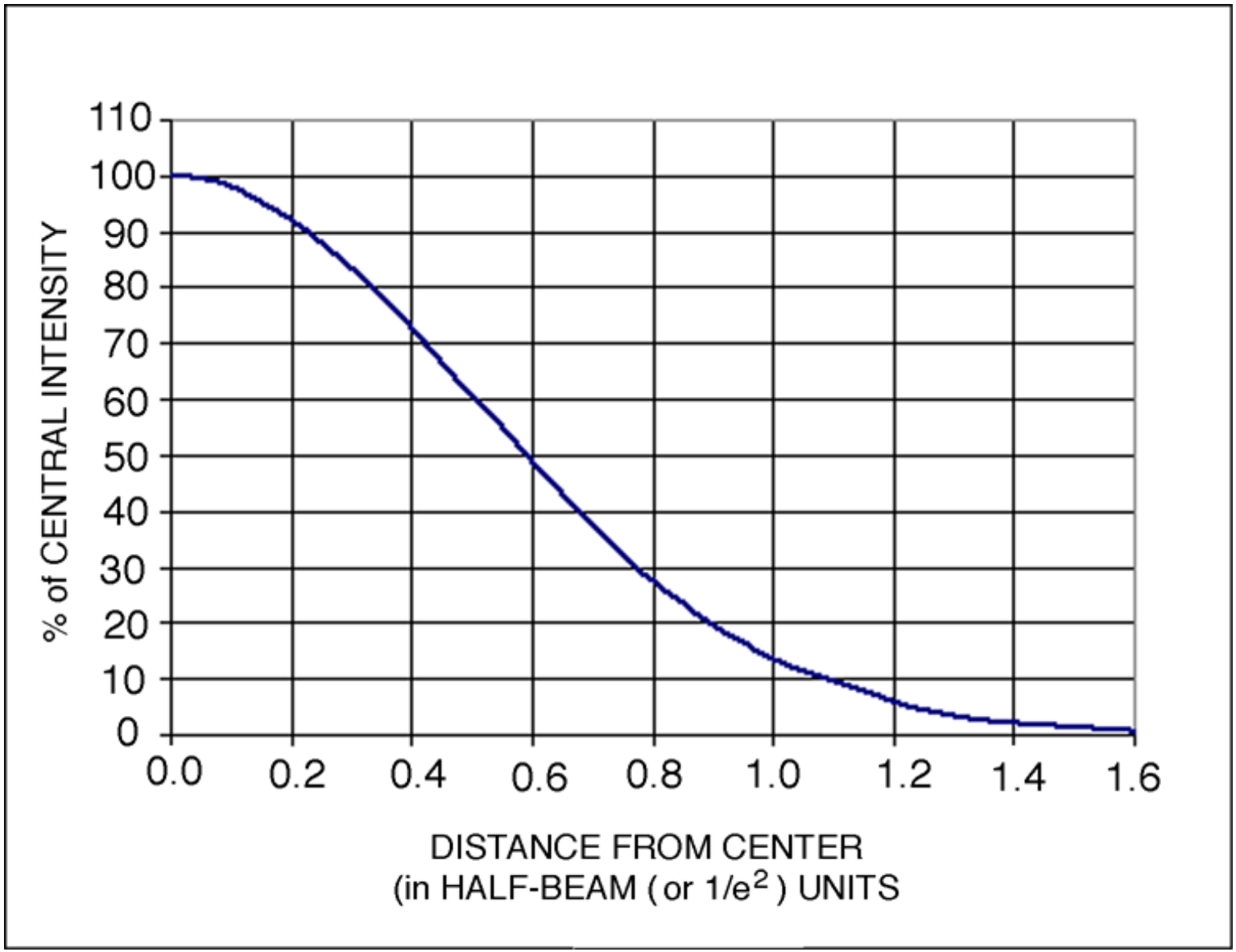
MONOCYTES: STAINED WITH FITC AND PE FOR FC RECEPTORS:
PAUL WALLACE, DEPT OF MICROBIOLOGY
KEN ORNDORFF, ENGLERT CELL ANALYSIS LABORATORY

A NOBLE GAS ION LASER



coherent color (eg argon ion laser: UV / 488nm / 514nm)
coherent direction

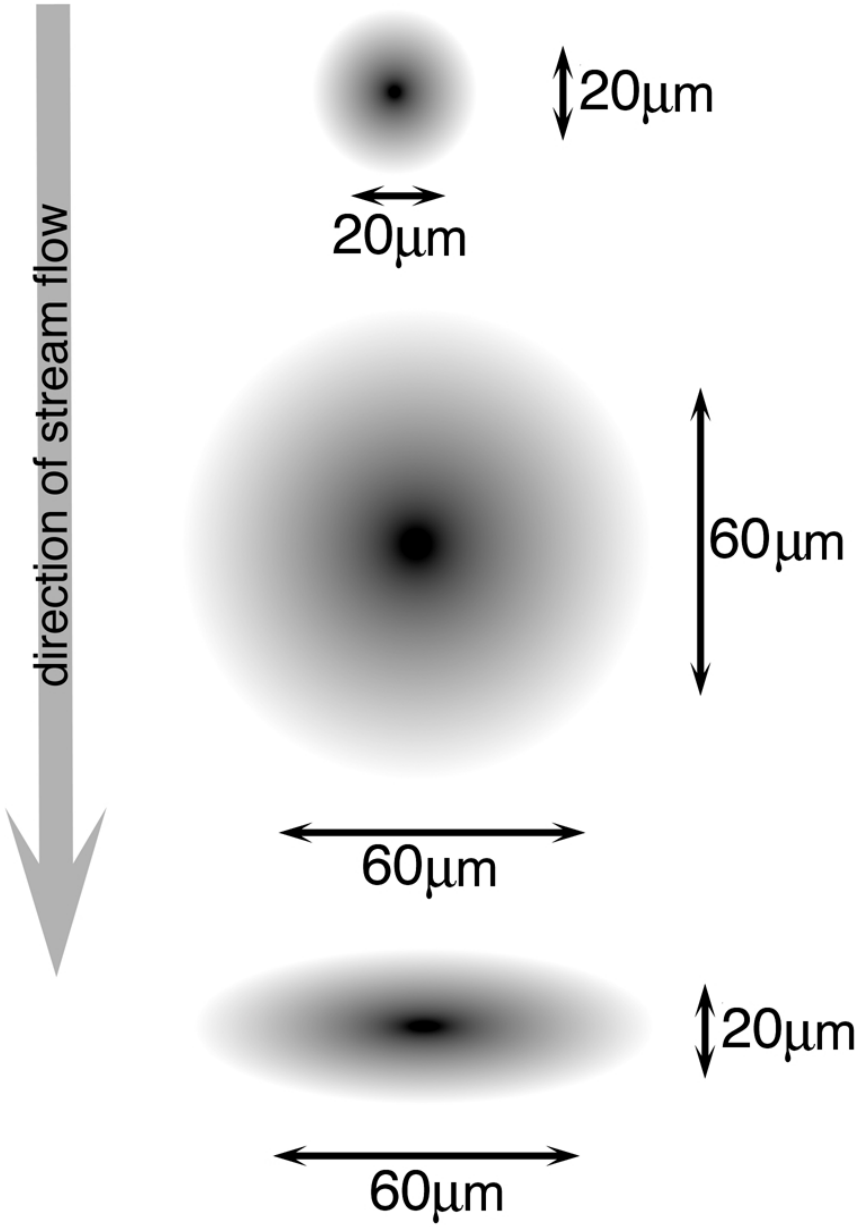
THE GAUSSIAN LASER BEAM PROFILE



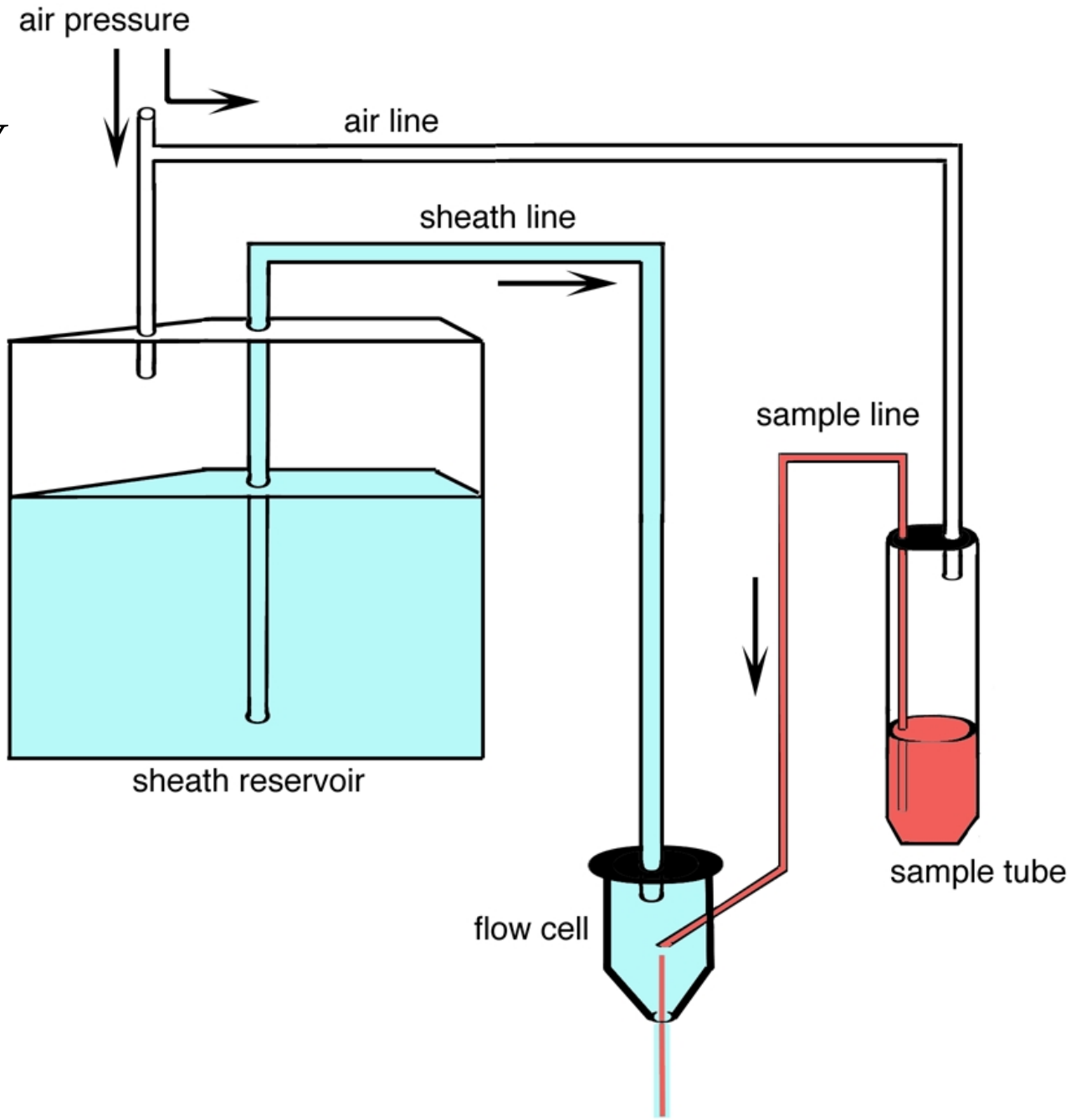
BEAM SHAPES:

MORE OR LESS ABILITY TO
ILLUMINATE CELLS EQUALLY
EVEN IF THEY STRAY FROM
THE CENTER OF THE BEAM

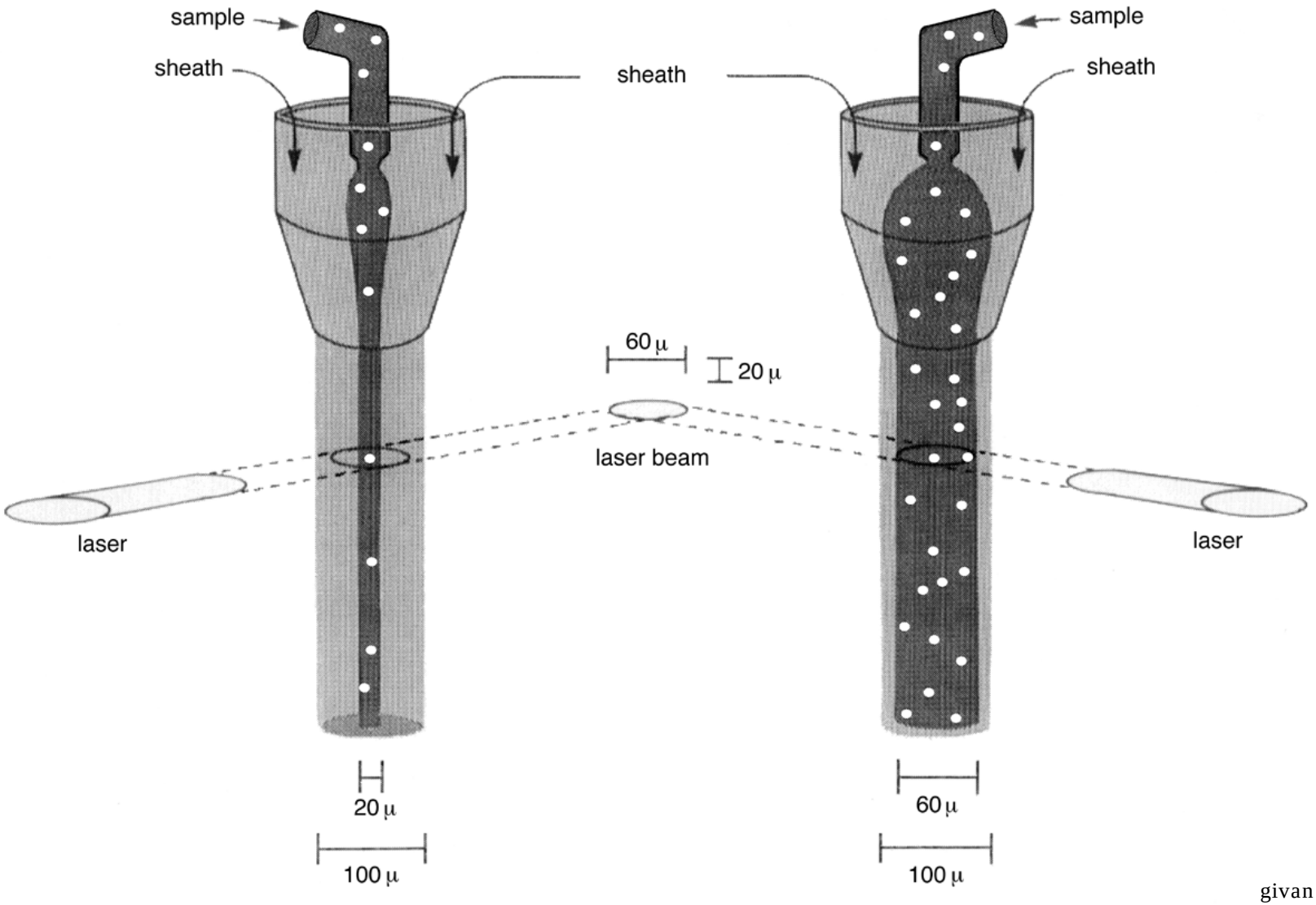
AND MORE OR LESS ABILITY
TO AVOID COINCIDENCE OF
TWO CELLS IN THE BEAM
EVEN IF CELLS FOLLOW
EACH OTHER CLOSELY
IN THE FLOW STREAM

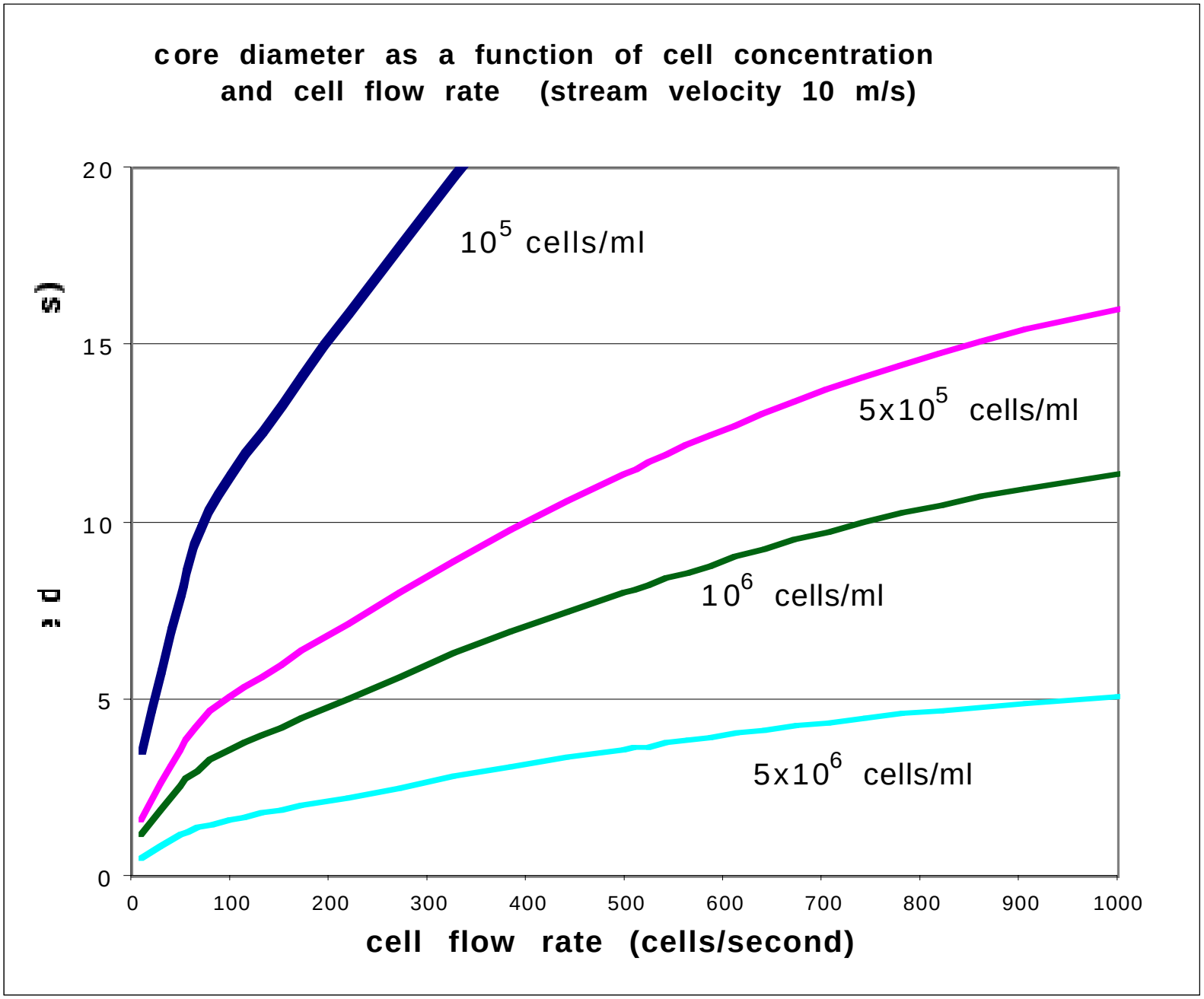


FLOW CYTOMETRY FLUIDICS

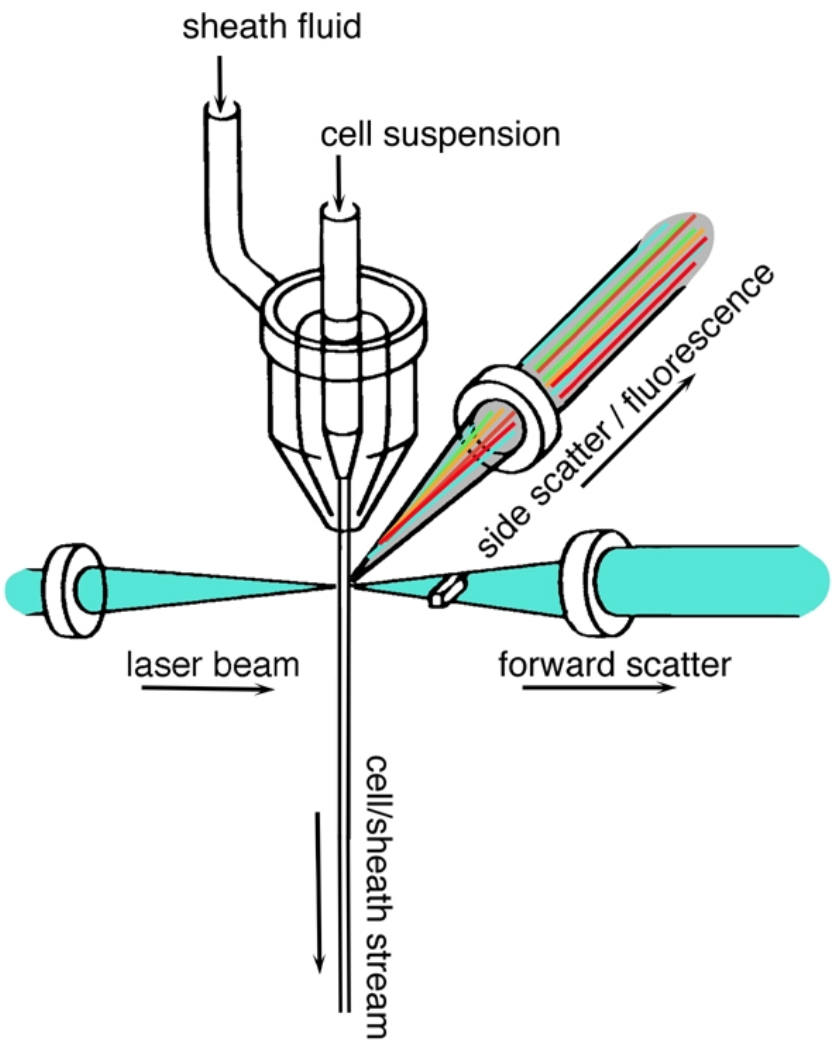


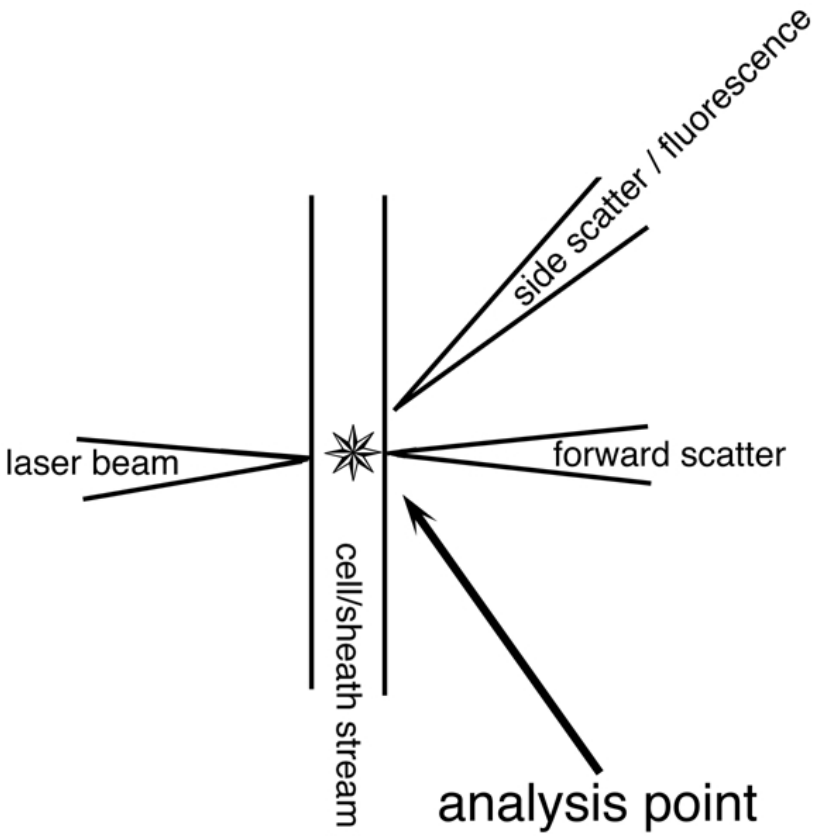
IF YOU ACHIEVE A FAST FLOW RATE BY MAKING THE SAMPLE CORE WIDE,YOU WILL HAVE WIDE CVs AND MULTIPLE CELLS MAY COINCIDE IN THE LASER BEAM



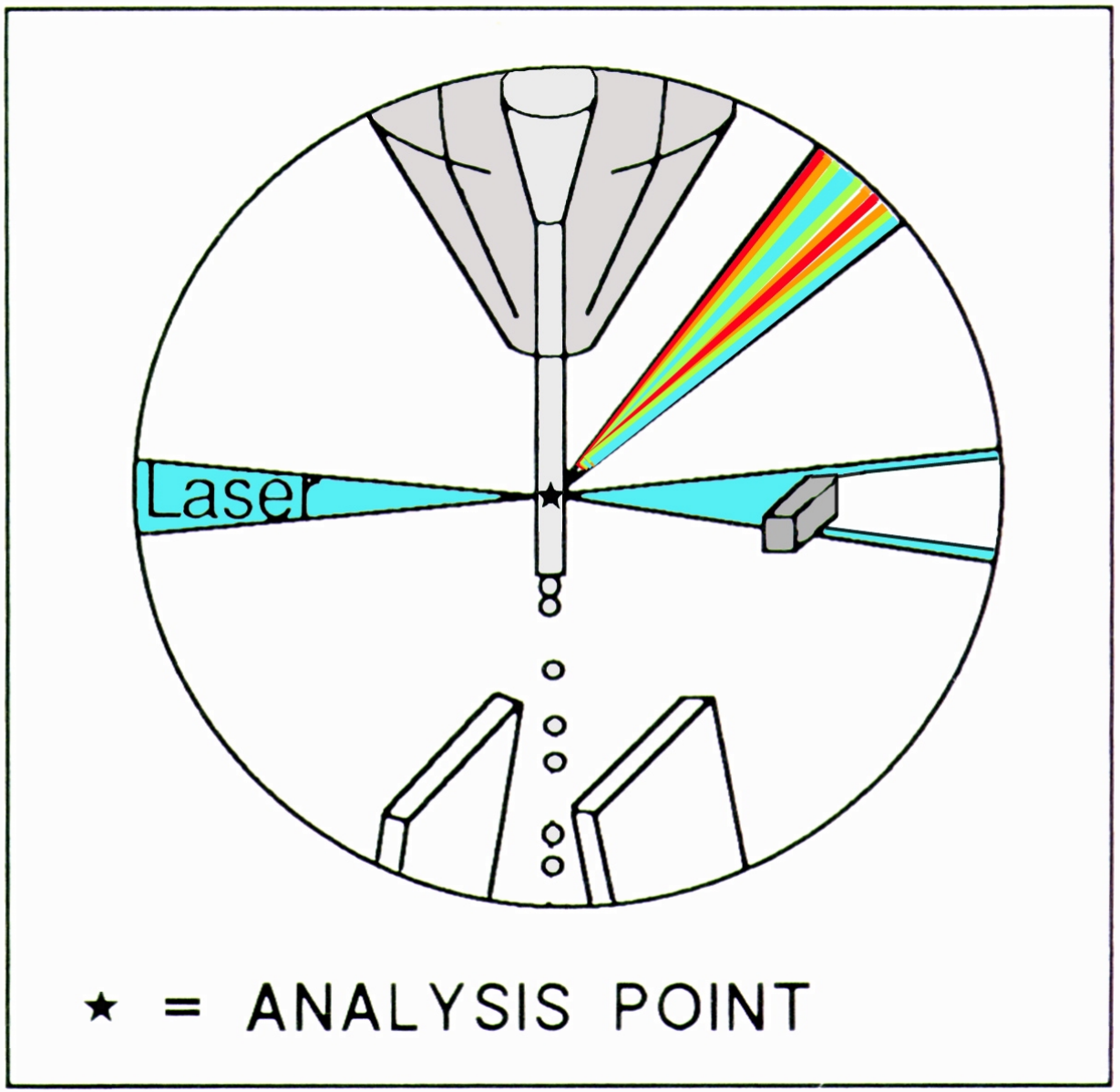

core diameter as a function of cell concentration
and cell flow rate (stream velocity 10 m/s)
20
15
10
5
0
0
100
200
300
400
500
600
700
800
900
1000
10^5 cells/ml
$5x10^5$ cells/ml
10^6 cells/ml
$5x10^6$ cells/ml
cell flow rate (cells/second)

ORTHOGONAL ALIGNMENT

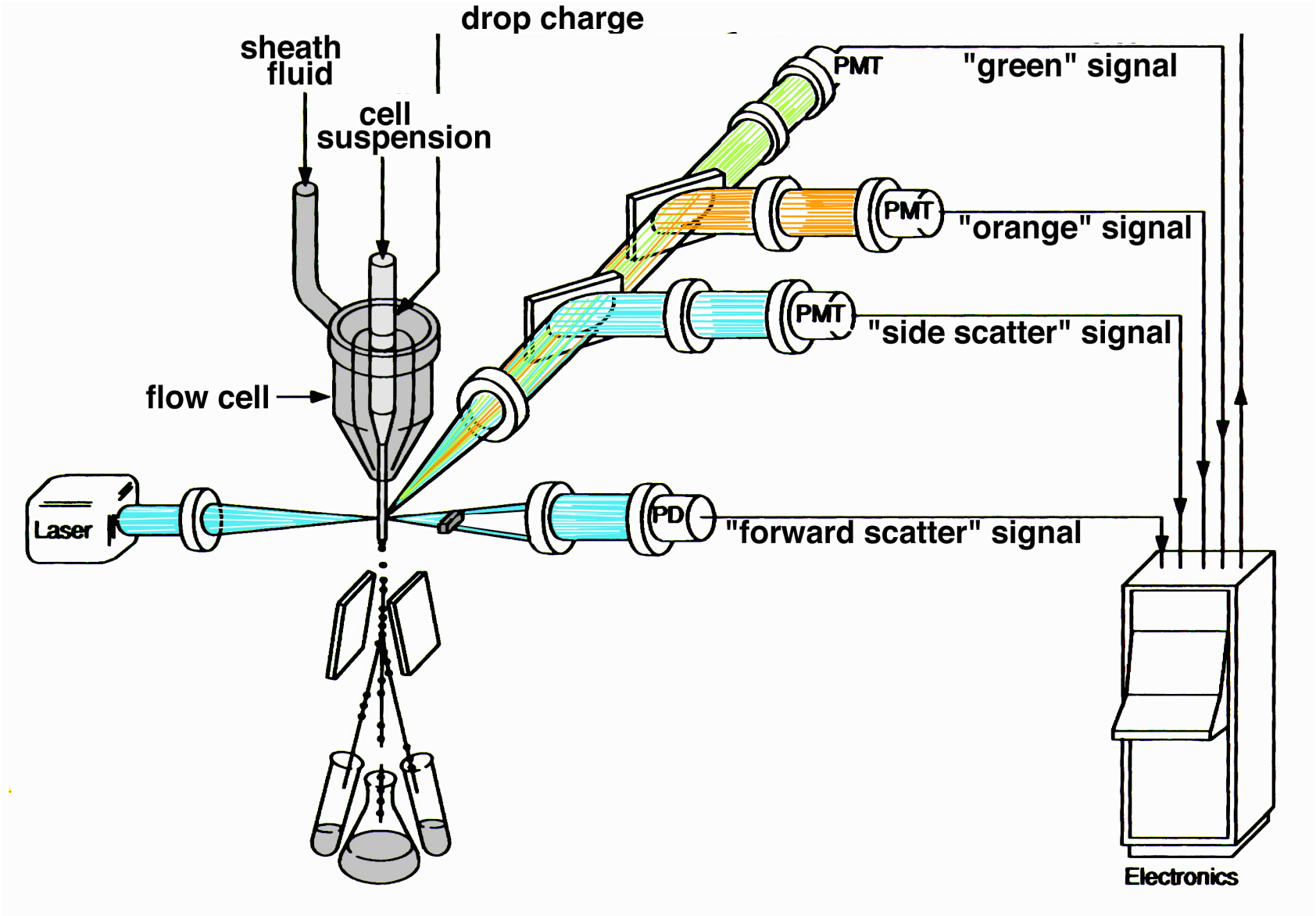




LIGHT FROM THE ANALYSIS POINT

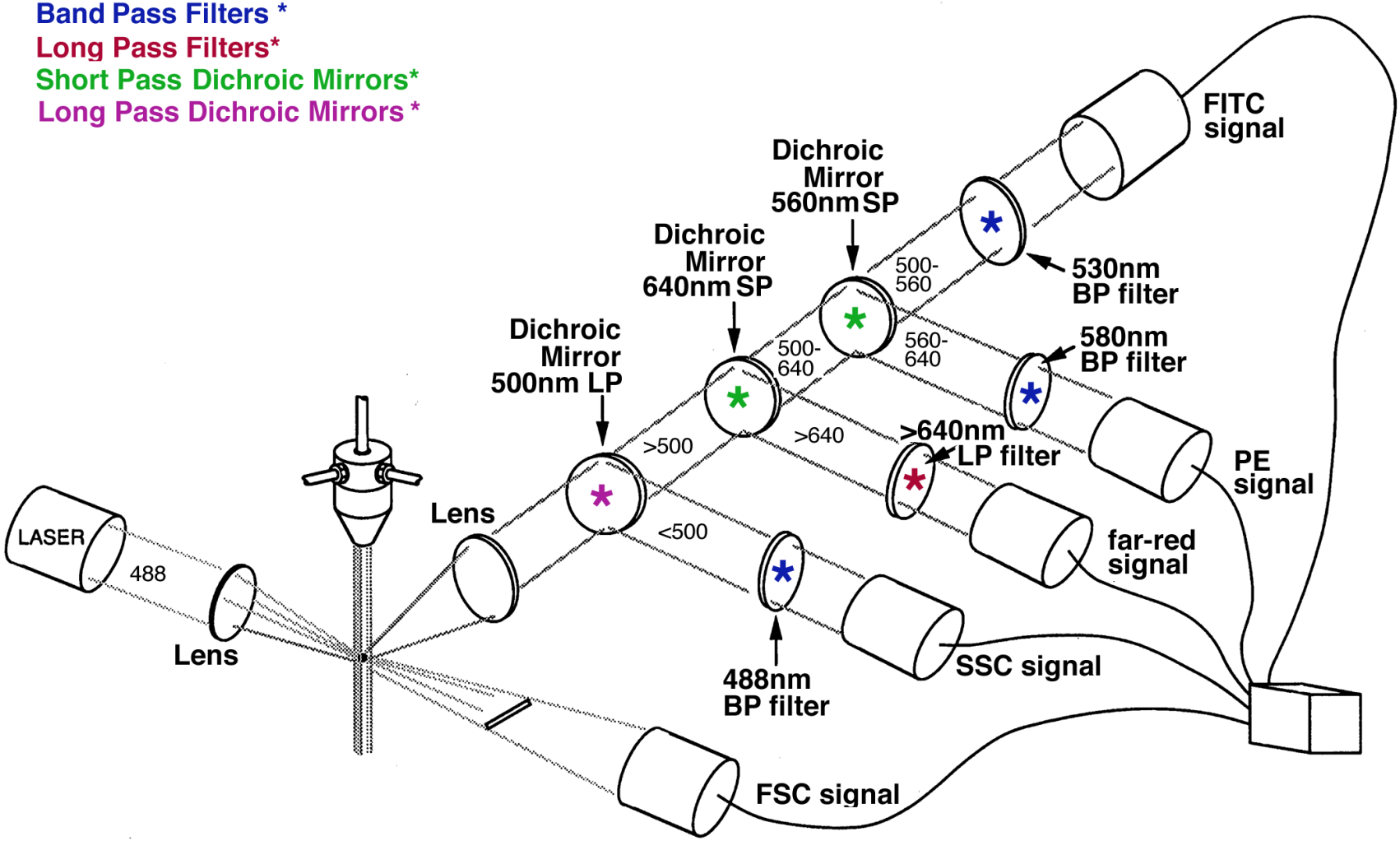


4-PARAMETER OPTICAL BENCH



PARTITIONING LIGHT WITH MIRRORS AND FILTERS

Band Pass Filters *
Long Pass Filters*
Short Pass Dichroic Mirrors*
Long Pass Dichroic Mirrors *



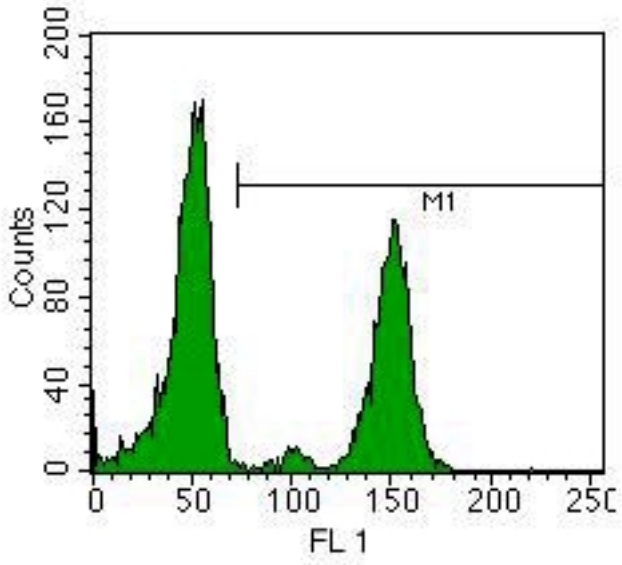
FCS DATA FILE

FCS2.0 256 978 1024 25023 0 0
\$FIL\IS20924003\$SYS\HP 200/300 SERIES PASCAL 3.X/BDIS CONSORT 30 VER E
12/86\$CYT\FACSTRAK\$DATE\24-Sep-93\$BTIM\13:13:51\$ETIM\13:14:
1\$MODE\L\$TOT\6000\$P1N\FSC-H\$P1B\8\$P1R\256\$P2N\SSC-H\$P2B\8\$P2R\256\$P3N\FL1-H\$P3B\8\$P3R\256\$P3S\CD3
Leu4 \$P4N\FL2-H\$P4B\8\$P4R\256\$P4S\CD19
Leu12\$G1N\FSC\$G1R\256\$G2N\SSC\$G2R\256\$G3N\FL1\$G3R\256\$G4N\FL2\$G4R\256\SAMPLE ID\481|A| \TUBE
NAME\CD3/CD19\END BATCH\FALSE\WBC
CONC\-999.9\ABSLYMPH\-999.9\$PAR\4\$P1E\0,0\$P2E\0,0\$P3E\4,0\$P4E\4,0\$NEXTDATA\0\$DATATYPE\I\$BYTEORD\4,3,2,1\$GAT
E\4\$R1I\1\$R2I\2\$R3I\3\$R4I\4\$R1W\0,255\$R2W\0,255\$R3W\0,255\$R4W\0,255\$GATING\R1 AND R2 AND R3 AND
R4\CREATOR\Simul SET\$SMNO\003\CONVERTED BY\FACSConvert v1.0a4\
x.....*<..84k...a...../$.*/j...V...Z.....:2..'0e...~$..~...`.....=;._7..I1+[.....;4..<4.]6#.-C4..02o.....:(.H6=.D3)n.....*3..>1..,'..i[c...o.&!..61..>1b...b...X....r1
0q...a..$..e\..65W....F/3..46a...n...r..!..8.Z.....:}.....6()'....:8..-7.B:0.92!...40..'./1%..7)..45y.....75o.&&..ZR..8+..<<..<9-.:.b.%%..=7e..."....;8.....
@1..ndP...N
..^.|.d...i.+6J
...89,. .-..>=n.....6"..,.631..70.CKDZ...3.. I..1..g___..I`..%m.."_.....44]..$.../..:<T...s...D ....6>@".!.T5%..45K.
.b.)..C2..|6...9.Y....b8'N...Z...~.....-%../)W..00..*..5:R...a...q.....A5..:1..02X..
]...G..)..05m...]....H2..y75..=3..0*..-:n....=-).t*3[..,..:3..(8>..(X...s.....81.'B=K..!.B'8..13.F7,R.....?2..?7.-3,Q
....=5..4-T..+..81.~0...84../'.*+%..*<`. .a...c...g.0+S.....-3X..,a.v
M.."..77b.....6.I
..X
.6..:*d...../*...%U.....15P...-....\86b..#I..9[.....+0..35m...h...>hT`..7(../...87[.....G7S.....<1N...[...K..
..46..32.},*V....1@..M5&.w52_..+..c\R..-p..&.w92..37p...e.. N...I
.*k...U..)..<8..=?Q
..K../_...p....1'...6...*&T
..S.....;%..57..03L....D45..7(k.(%Z.....4-..b]e.....:=d....#/"..`Vt ....4+b.. ..68..*".%:xc"..[...d..
..15T.. ..5/...5..;:.70#F
~...._V..6/1...j..#..62..0.Z....!,.t...n.....72.C)3j ..d.....=/o...q...y#.%b. "..>...5&p...[.. ..8A.G+"..4/o.....,0X.&.f...0...d.....14..1+u
..U....a82...:3[...../=..2-..50J..*.<8*..=*c7#$d.-/\
."..82.(4!b.....20z..#..;7k...Z..../.U.....]VI
..c.....=,.D:...98..4&..7'..85Z...C..0..63..82Y.3.1...W...r $(.I3...+?s..
..73.A0...99..=6p$....@0h...W...z..'q..0m.....,8..>=^...n.1<P...Y...~"....-*e...t.....45.A4/..$$..4/i...g..$W./.Q....{,5.222Y...r#....;*4
..u....J3.h.....&'L..#./..V...._....K-7h....+$3.$....5)N
}.1..9..';J....v37..,(T...S....s-6..72..0-.!.$..,3..E7n./.\....<4$N
..p....01;.M71L....}33..d[..41..`20..6..hY..<)S...Z
....=-f...h.....'4..1)p.....\SN.....6.a...U.....5+.d-%U....G72..9/X../..07Y
...\2$..-+..+(n....1--S...x.?5F.....1.p..%f.....7)..D-.F8&p()...@;..=3e .2..43V.....82..,0.)9U.. ...4..&4U...L

FCS DATA FILE (TRANSLATED)

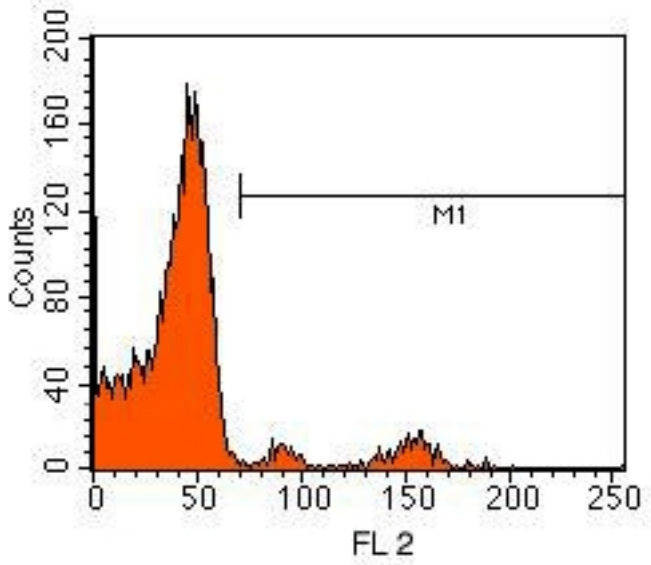
CELLS IN SEQUENCE	FSC-H	SSC-H	FL1-H	FL2-H
1	120	28	152	24
2	190	169	42	60
3	175	149	56	52
4	107	25	149	0
5	97	22	151	26
6	174	136	47	36
7	190	127	42	47
8	106	14	148	0
9	86	17	165	23
10	90	16	149	31
11	184	163	58	50
12	191	160	39	48
13	101	24	152	19
14	126	36	153	0
15	126	28	157	0
16	96	17	155	0
17	215	224	61	59
18	165	95	55	46
19	173	73	49	43
20	91	27	158	0
21	210	180	59	52
22	179	161	60	52
23	165	93	54	35
24	187	45	67	52
25	192	184	48	50
26	111	25	149	17
27	207	206	58	40

SINGLE COLOR HISTOGRAM WITH STATS



Total Events: 6000

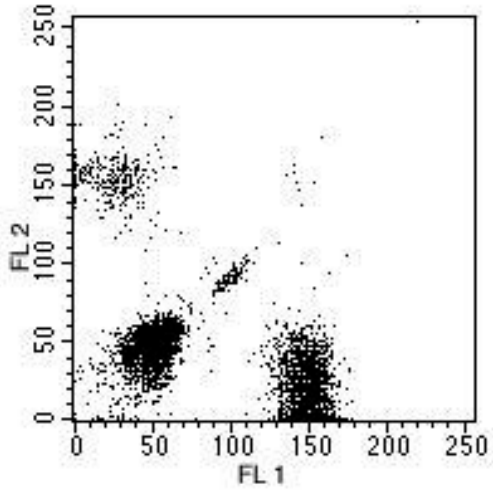
Marker	Mean	Median	Peak Ch	% pos
All	87.76	58.00	56	
M1	146.08	149.00	150	43

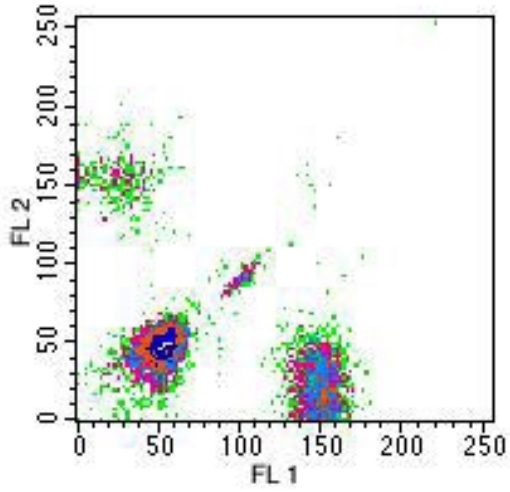


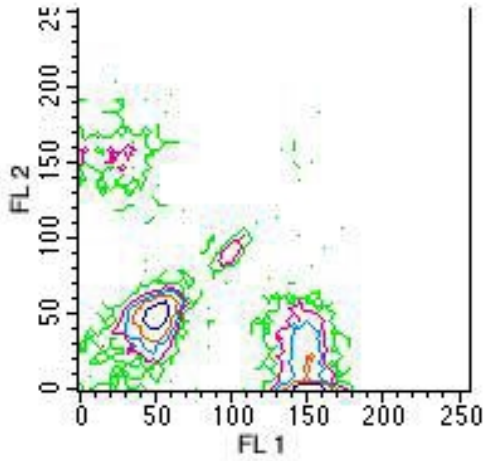
Total Events: 6000

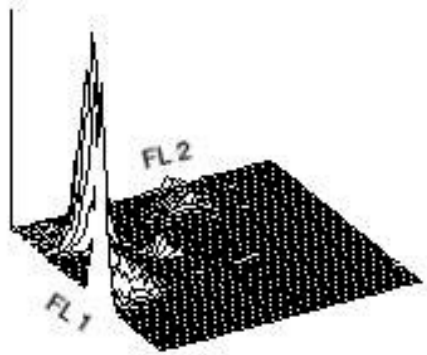
Marker	Mean	Median	Peak Ch	% pos
All	41.32	42.00	0	
M1	134.06	148.00	156	8

TWO-DIMENSIONAL PLOTS

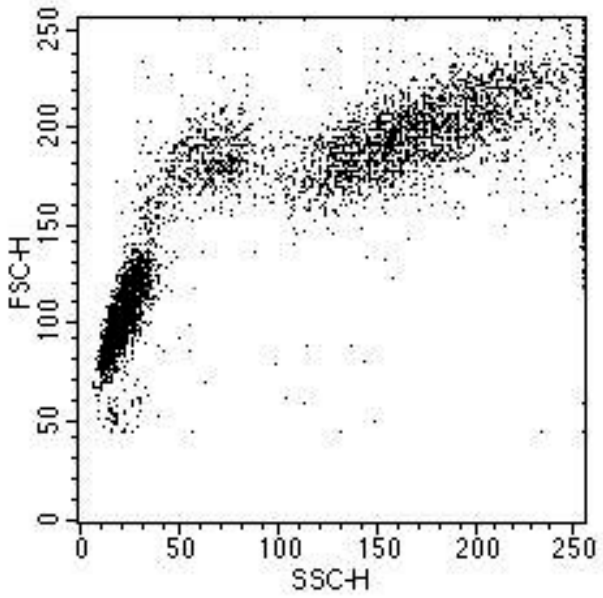


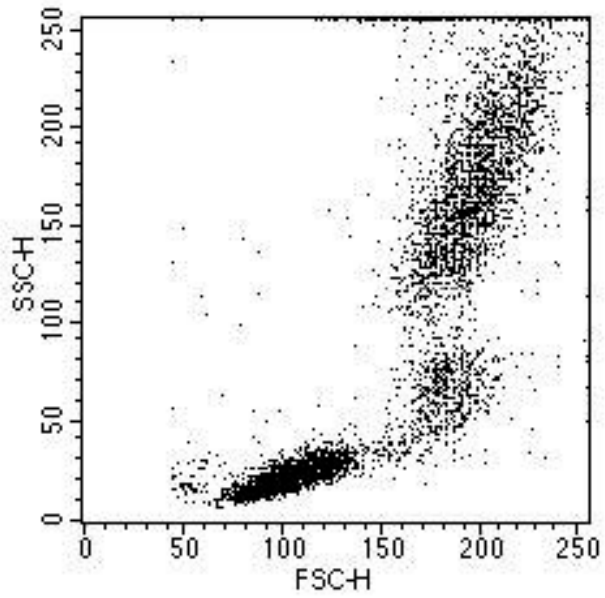






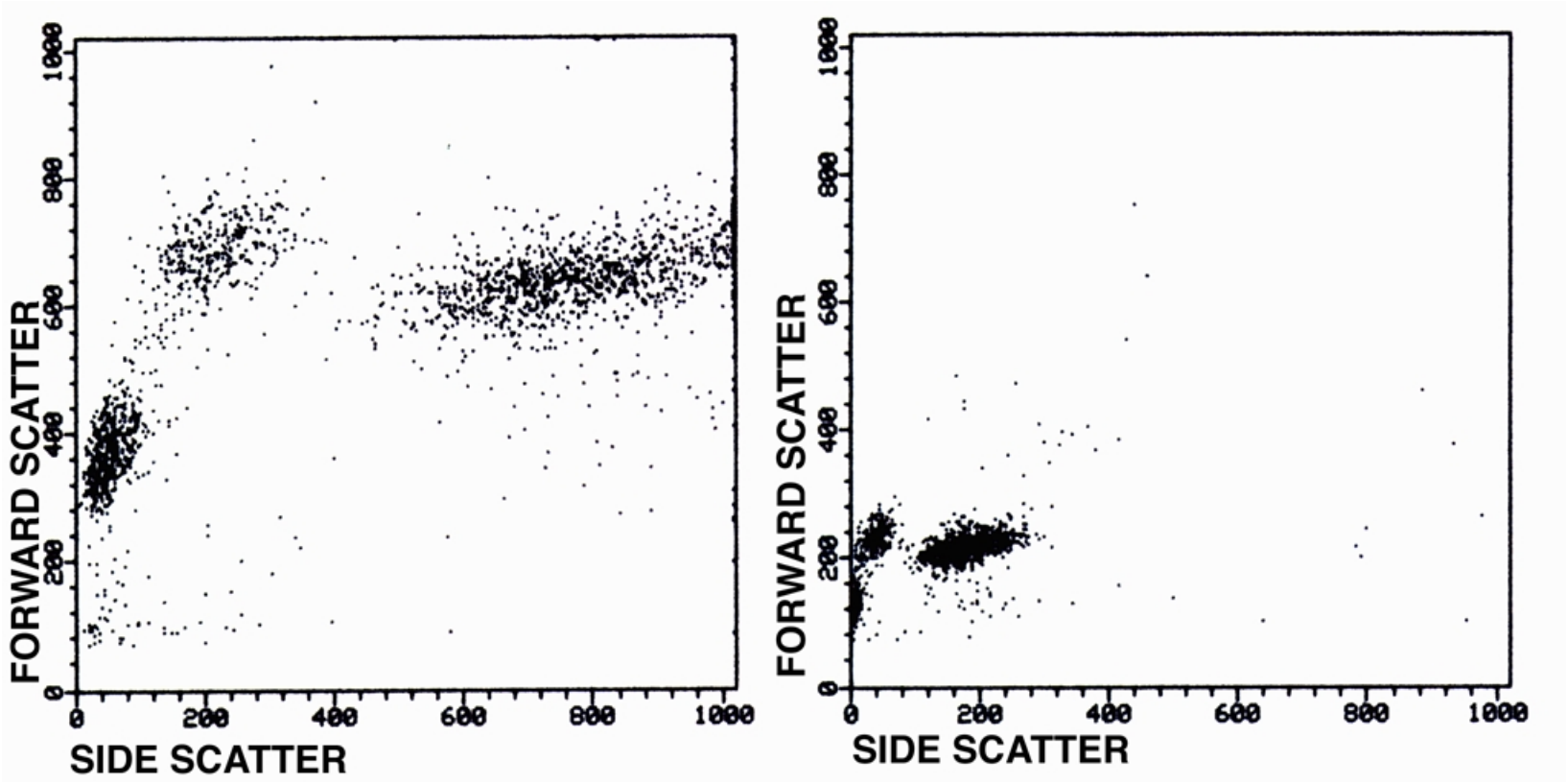
TWO-DIMENSIONAL SCATTER PLOTS FOR PHYSICAL CHARACTERISTICS OF CELLS



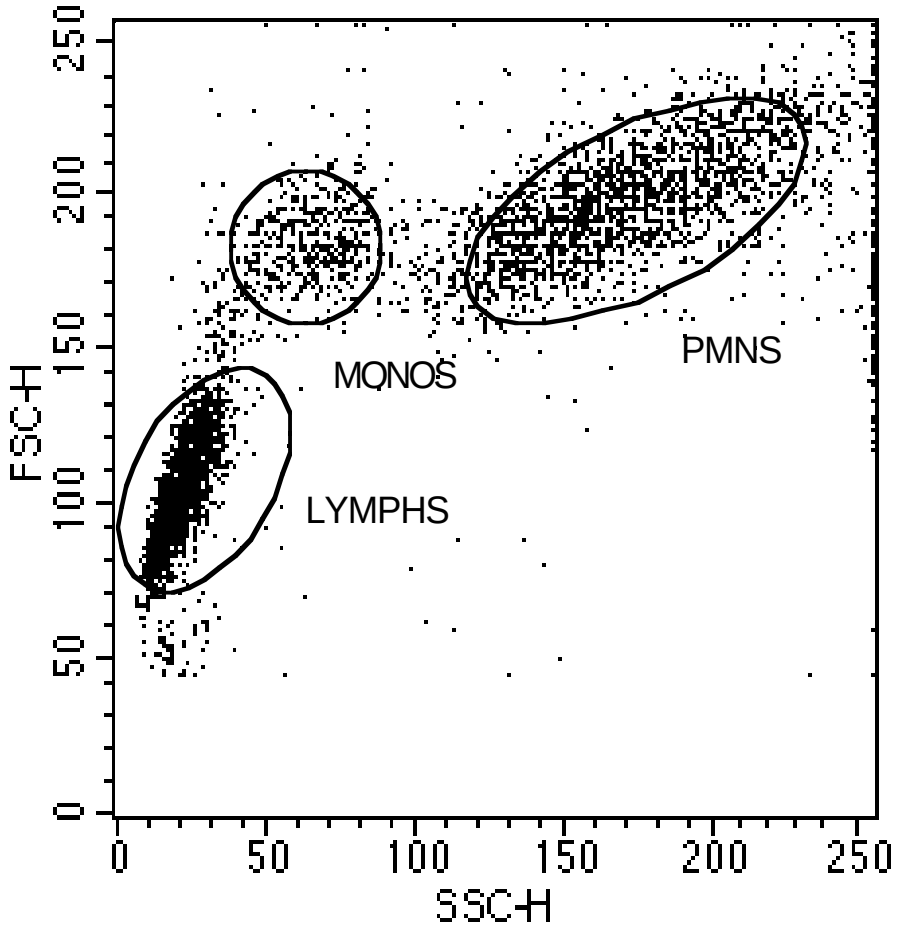


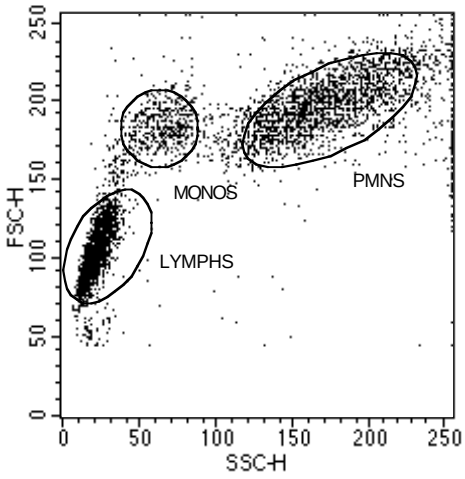
PLOT IT ANY WAY YOU LIKE

ELECTRONICS:
AMPLIFICATION OF THE SIGNALS

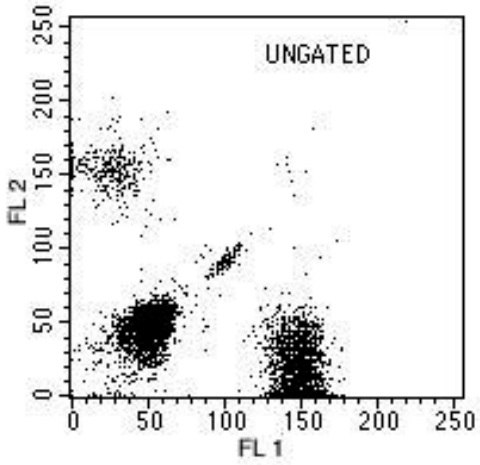


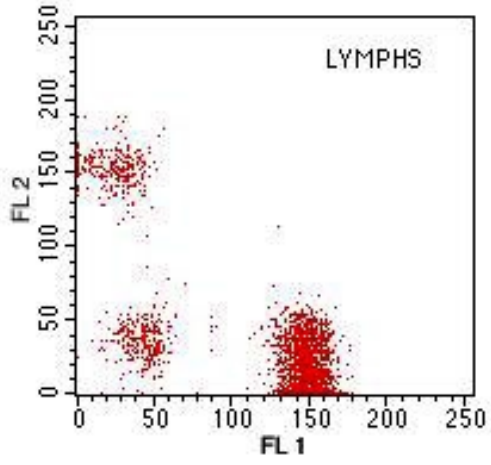
GATING ON FORWARD AND SIDE SCATTER

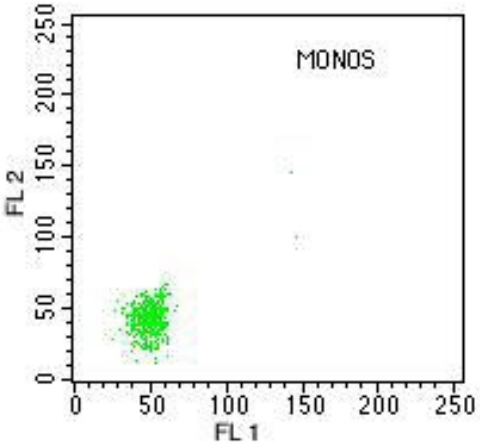


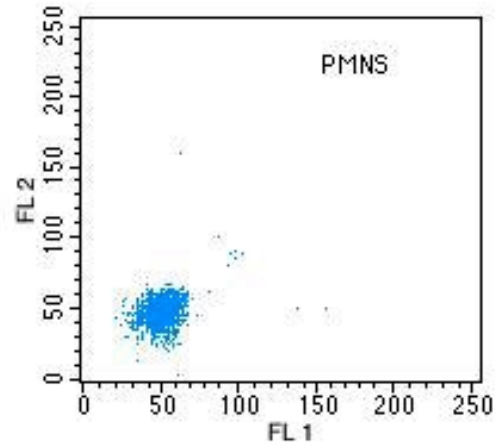


FLUORESCENCE OF GATED CELLS









INSTRUMENT VOCABULARY (1)

- LASER: illuminates cells
- SHEATH FLUID: surrounds the sample stream, aligning cells in the laser beam
- SAMPLE CORE: narrow stream carrying cells within the wider sheath stream
- NOZZLE/FLOW CELL: delivers cells into the sheath stream and then accelerates the flow so that cells arrive one by one at the analysis point
- ANALYSIS POINT: position at which the laser beam intersects the sheath stream and illuminates cells

INSTRUMENT VOCABULARY (2)

- FLUORESCENCE: light emitted from a fluorochrome after it absorbs light from the laser (always of a longer wavelength than the absorbed light)
- SCATTER: light that is reflected, refracted, or otherwise bounced off a cell (always the same color as the illuminating beam)
- FORWARD SCATTER (FSC or FALS): light scattered at small angles away from its original direction
- SIDE SCATTER (SSC or 90^{o}LS): light scattered to 90^{o} from its original direction

INSTRUMENT VOCABULARY (3)

- PHOTODETECTOR: detects light scattered or emitted by cells and converts it to an electronic signal
- AMPLIFIER: increases or decreases signals from cells so as to put them “on scale”
- COMPUTER: takes the light signal that has been detected by the photodetector, amplified by the amplifier, and digitized by the analog-to-digital converter, and stores it as a number in a file for analysis.

ANALYSIS VOCABULARY (1)

- PARAMETER: 4, 5,6, or more types of information that are provided by a flow cytometer about each cell -- for example, a cytometer with 5 photodetectors provides 5 kinds of information about each cell
- DATA FILE: list of numbers that form the total flow description of a cell suspension after it passes the analysis point
- HISTOGRAM/1-D HISTOGRAM: plots the data for cells in a data file with respect to one parameter
- DOT PLOT/2-D HISTOGRAM: plots the data for cells in a data file with respect to two correlated parameters

ANALYSIS VOCABULARY (2)

- REGION: a description, using flow cytometric intensity values, of a cluster of cells
- GATE: a restriction, using Boolean combinations of regions, on the cells that will be included in subsequent analysis
- MARKER: a more-or-less arbitrary dividing line between intensities that will dichotomize “negative” from “positive” cells
- QUADRANTS: markers in 2 dimensions that divide signals into “negative”, “single positive” (2), and “double positive” intensities

FCS DATA FILE (AGAIN)

CELLS IN SEQUENCE	FSC-H	SSC-H	FL1-H	FL2-H
1	120	28	152	24
2	190	169	42	60
3	175	149	56	52
4	107	25	149	0
5	97	22	151	26
6	174	136	47	36
7	190	127	42	47
8	106	14	148	0
9	86	17	165	23
10	90	16	149	31
11	184	163	58	50
12	191	160	39	48
13	101	24	152	19
14	126	36	153	0
15	126	28	157	0
16	96	17	155	0
17	215	224	61	59
18	165	95	55	46
19	173	73	49	43
20	91	27	158	0
21	210	180	59	52
22	179	161	60	52
23	165	93	54	35
24	187	45	67	52
25	192	184	48	50
26	111	25	149	17
27	207	206	58	40

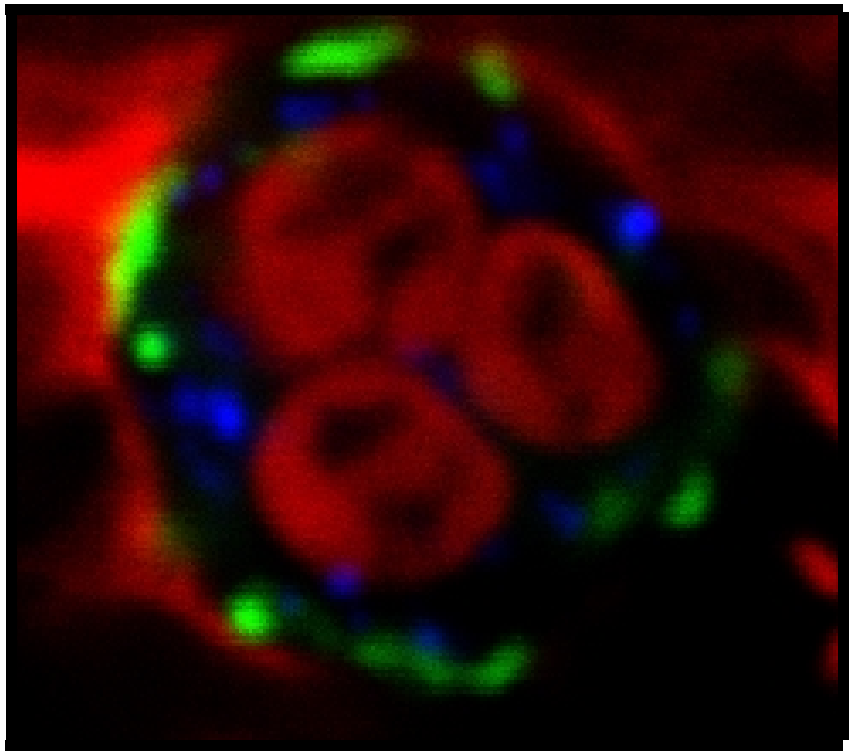
NEUTROPHIL STAINED for DNA, CD16, and IFNγ

WHAT DOES A CELL REALLY LOOK LIKE?

a cell (by flow cytometry)

- FSC = 224
- SSC = 603
- FL1 = 467
- FL2 = 894
- FL3 = 126

a cell (by confocal microscopy)



Webster’s New World Dictionary (1958) says that a "minimizer" is "one who tries to make religious or philosophical problems appear easily explained."

- With humility, we need to admit that flow cytometry is, essentially, a minimizing technique.
- It simplifies the uniqueness and elegance and complexity of a cell down to a set of 5 or so numbers.
- The power of flow cytometry comes from rapid, objective, and quantitative computing, allowing calculations, correlations, and statistical conclusions from those few numbers derived from each of many cells.
- Our intelligence and training should make us aware of all those important things that flow cytometry does not tell us about a cell.