The use of a Spherical Multiparameter Transducer for Flow Cytometry[1]

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Abstract: Fused silicon dioxide, multiparameter flow transducers with 50 µm internal square cross section and circa 60 µm length can simultaneously measure DC and RF impedance, as well as fluorescence and multiple angle light scattering. One of these spherical 'Automated Multiparameter Analyzer for Cells' transducers was mounted in an EPICS[TM] CVA flow cell housing and installed on a research prototype equipped with an argon ion laser. The signal produced by the spherical transducer with EPICS[TM] DNA-Check beads was 1.73 times greater than that produced with the standard cylindrical flow cell. Similarly, with EPICS[TM] Immuno-Brite beads the average ratio was 1.96.

The Coulter impedance and light scattering measurements were similar to those produced with the conventional cylindrical outside flow cell, although the internal cross section of the sphere was square and that of the cylinder was circular. The theoretical arguments of Leif and Wells have been demonstrated to be correct. At present, monolithic, spherical fused silica transducers are the optimum design for combined electro-optical, multiparameter flow cytometry analyzers.

Key Terms: Automated Analysis, Fluorescence, Coulter, Light Scatter, Automated Multiparameter Analyzer for Cells, Complete Cell Analysis, Cell Volume, Focused Flow, Immunohematology, Electro-optical Transducers

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Introduction:

The possibility of an "Automated Multiparameter Analyzer for Cells, AMAC," based on the simultaneous acquisition of multiparameter, electro-optical data was first suggested by Leif (5). In order to enumerate the five classes of blood leukocytes: lymphocytes, monocytes, neutrophils, eosinophils and basophils, the present commercially available Coulter blood cell analyzers employ electro-optical transducers to simultaneously measure the combination of two electronic impedance and one light scattering parameter (11). The addition of fluorescence measurements to these parameters permits the subclassification of these cells. The simultaneous measurement of all parameters both simplifies the electronics and ensures that all of the measurements obtained are from the same event. Since the size of the laser beam along the direction of flow is customarily considerably smaller than the physical length of the Coulter orifice, which in turn is smaller than the effective electronic length (4), the effect of coincidences can be minimized.

The present electro-optical transducer for the "Automated Multiparameter Analyzer for Cells" is a sphere with an inner square flow channel (6). Leif and Wells (9) have performed theoretical calculations comparing a spherical outside transducer with a square, flat walled outside transducer. They estimated that the signal acquired through the spherical surface would be 2.14 times that through a flat wall. They also described the increase in spherical aberration due to the flat wall, which significantly limits the optical resolution. The minimum spot size for a spherical flow cell was calculated to be 1.8 micron. Other flow transducers including electro-optical units have been described by Steinkamp (14). Watson described (17, 19) the addition of two spherical lenses to a square-cross-section flowcell. One of these was mirrored and the other was constructed of a higher refractive index glass and served as a focusing lens. He claimed a theoretical sixfold increase in light gathering efficiency versus the square walled flow cell. However, the addition of the two lenses has two disadvantages. Firstly, fabrication from three elements rather than 1 results in larger transducer, which in turn increases the working distance and size of the collection optics. And thus can preclude the use of some microscope objectives. Secondly as has been reported by Condrau et al. (1), "The main source of this luminescence (background emission) is the optical glue applied for the construction of the flow cell." The design of ellipsoidal transducers (13) has been optimized by Watson (18, 19). His design employs a highly efficient ellipsoidal-spherical flow cell with an internal square 250 x 250 μm square internal capillary and external mirroring. However, the optical detection advantage of his geometry would be degraded by the addition of the inlet and outlet required for electronic impedance measurements, and spherical surfaces are much less expensive to produce than ellipsoidal surfaces. The FACS Analyzer (2, 10, 12) was the first commercially available flow cytometer capable of combined DC impedance (Coulter volume), fluorescence and wide angle light scattering measurements. The use of a spherical transducer has two significant advantages compared to the FACS Analyzer: 1) the spherical surface eliminates the use of the glycerine optical coupling fluid and 2) low and median angle light scattering measurements can be made. Previous studies have demonstrated that the spherical transducer can also be employed with a mercury arc in a configuration similar to the FACS Analyzer (7). Thomas has described (16) and marketed another combined electro-optical instrument.

This instrument is unique in that it employs a triangular cross-section flow cell and it is constructed out of quartz pyramids in a similar manner to the AMACIII (15). A description of a research unit which could extend the capability of a commercially available, totally optical four part differential leucocyte analyzer to include an immunofluorescence measurement has been recently published (3).

Figure 1 is an idealized drawing of a spherical flow transducer. The transducer is shown with an inlet on the top and a radiation source to the right. The radiation enters through a small flat on the right side of the sphere. Fluorescence and 90° light scatter are collected orthogonally to the excitation by the lens shown in back of the transducer. Electrodes for impedance measurements are located upstream and downstream of the orifice. Both DC (Coulter volume) and radio-frequency, RF, impedance (Coulter conductivity) are sensed. Light scattering in the low to median angle (11) regions can be detected in the forward scatter direction.

Light emanating from the center of a spherical transducer is minimally deviated and the emitted angle from the surface of the spheres is smaller than the internal angle (9). Light emanating from the center of a flat transducer (15) is refracted away from the normal to the surface and the emitted angle from the flat surface is substantially greater than the internal angle (9). A conventional long working distance objective with a numerical aperture of 0.65 collects, from one quadrant of the sphere, all of the light originating from the center of the square internal flow channel. Mirroring the side of the sphere opposite the objective adds the reflected light to that already collected by objective. A lens of 0.95 numerical aperture is required to collect the light from one side of the square flow channel with a flat outer surface. Long working distance objectives with a 0.95 numerical aperture are <u>not</u> commercially available. However, an expensive lens with a 0.90 numerical aperture and a working distance of 1.3mm in air, which in fused silica is less than 1mm., is available from Mitutoyo. A 1mm wall thickness would make construction of the inlet and outlet of a Coulter orifice difficult. Chromatic aberration with a spherical surface is very low; whereas, with a flat surface, it is significant.

Materials and Methods:

A fused silica, spherical flow cell with a radius of 1.7 mm., a 50 micron square aperture, and without mirroring was compared to the standard Coulter cylindrical flow cell, which has a comparable aperture diameter and outside dimensions. Both had cup shaped inlets and outlets. The standard flow cell is used in the COULTER[R] VCS, STKS and MAXM clinical hematology instruments. Measurements were made on a prototype equipped with a 20 milliwatt argon ion laser. The beam was focused with a Profile Beam Shaping Assembly (Coulter PN 1580) and the fluorescent and orthogonally scattered light was collected and separated with a standard EPICS[R] Elite assembly. The spherical and cylindrical transducers were mounted in EPICS[R] CVA flow cell housings (Figure 2).

EPICS [TM] Immuno-Brite (PN 6603473, 10 microns diameter), COULTER[R] Latron Control (PN 7546914, 5.1 microns diameter), and EPICS [TM] DNA-Check beads (PN 6603488, 10 microns diameter) were employed to compare the spherical with the conventional flow cell.

Thirty three microliters of whole blood were reacted for 2 minutes with 10 microliters of T8(CD8)-FITC/T4(CD4)-RD1(phycoerythrin) (COULTER[R] CYTO-STAT, PN 6603802) and then lysed and quenched with STKS reagents (PN 7546917). Ten thousand events were taken in list mode and then analyzed on a 486 PC clone with proprietary software.

Results:

A comparison of the fluorescence distributions produced with Immuno-Brite beads is shown in both linear and log form in Fig. 3. The ratios of the modes of the peaks of linear fluorescence from the spherical and cylindrical flow cells are shown in Table 1. The difference in fluorescence is detectable even in the logarithmic presentation. With Latron beads (Table 2), the spherical flow DC CV was (Fig. 4) slightly lower (2.8 versus 3.0) than that of the cylindrical flow cell and the RF was considerably lower. The RF value was probably an artifact of the particular cylindrical flow cell studied. The median angle light scatter CV for the cylindrical flow cell was lower (3.3 versus 4.6). The median angle light scatter detectors were standard for the STKS and MAXM instruments and therefore, not optimized for the spherical flow cell.

Five parameters were compared with DNA-Check beads (Fig. 5). The DC CVs (Table 3) were 0.93 for the spherical flow cell and 0.56 for the cylindrical flow cell. The RF was better on the spherical than the cylindrical flow cell, presumably an artifact. Median angle light scatter was comparable with the sphere CV, 2.88, only slightly higher than the cylinder CV, 2.51. Ninety degree light scatter also gave a slightly higher CV with the sphere. Fluorescence with the spherical flow cell gave a CV of 3.14 and a mean channel of 2,320; whereas, with the cylindrical flow cell, the CV was 3.72 and the mean channel was 1,342.

The two-dimensional leukocyte distributions obtained by plotting Coulter volume against median angle light scatter (Fig. 6) were essentially the same for the two flow cells. The spherical flow cell produced (Fig. 7) a well defined dual label fluorescence distribution of lymphocytes. Thus, we have demonstrated that a spherical flow cell makes the same measurements as the conventional cylindrical flow cell to produce a five-part differential count, but with greater sensitivity for immunofluorescence.

Discussion:

The CVs of the physical parameters (DC, RF, and median angle light scatter, MALS,) obtained with both transducers were smaller for the DNA-Check beads compared to the Latron beads. This is as expected since the average diameter of the DNA Check-beads is almost twice that of the Latron beads. The increased light collection efficiency of the sphere is due to both the wider angle of acceptance and capacity to mirror the opposite side. The comparison of the electronic impedance studies demonstrates that the larger area of a square compared to a circle orifice does not result in a decrease of the signal- to-noise ratio. A simple explanation is that the current flow is reduced in the corners of the square flow channel. The development of a monolithic, multiparameter flow cell, capable of simultaneous fluorescence and physical measurements described in Leif et al. (8) has finally been completed. The use of this technology will permit

rapid, inexpensive complete cell analysis of leukocyte classes and subclasses.

Acknowledgments:

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Legends:

Figure 1. The light enters the sphere through the flat on the right. The objective is shown in back of the sphere.

Figure 2. Electronic photograph of a CVA with spherical flow cell. Both the sample and the sheath flow upward.

Figure 3. Single parameter fluorescence distributions of EPICS^{TM} Immuno-Brite beads. Top panel: results with the spherical transducer. Bottom panel: results with the conventional cylindrical flow cell.

Figure 4. Coulter^{R}Latron bead study. Left: DC impedance (Coulter Volume). Middle: Radio frequency impedance. Right: median angle light scatter. Top panel: spherical flow cell. Bottom panel: cylindrical flow cell.

Figure 5a. Electronic impedance studies with DNA-Check beads. Left: DC impedance (Coulter volume). Right radiofrequency impedance (RF). Top panel: spherical transducer. Bottom panel: conventional cylindrical transducer.

Figure 6b. Median angle light scatter (MALS).

Figure 7c. Left: single parameter ninety degree light scatter. Right: single parameter fluorescence distributions of EPICS^{TM} DNA-Check beads. Top panel: results with the spherical transducer. Bottom panel: results with the conventional cylindrical transducer.

Figure 8. Whole blood preparation. Coulter electronic cell volume (DC) plotted against median angle light scattering (MALS).

Figure 9. Two parameter fluorescence distribution gated on the lymphocyte distribution shown in Figure 6. The cells are stained with a COULTER CYTO-STAT preparation. Log CD8 (T8 FITC) is the abscissa; log CD4 (T4 RD1) is the ordinate.
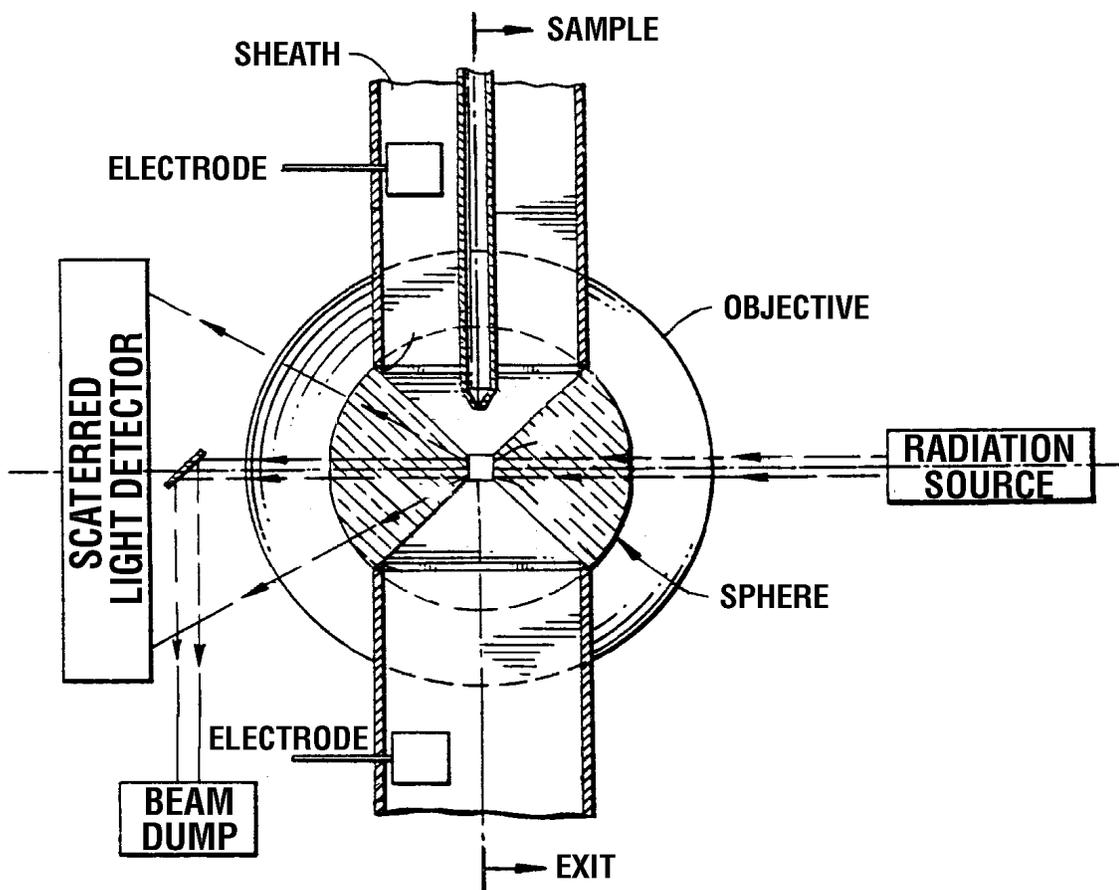
Figure 1. The light enters the sphere through the flat on the right. The objective is shown in back of the sphere.
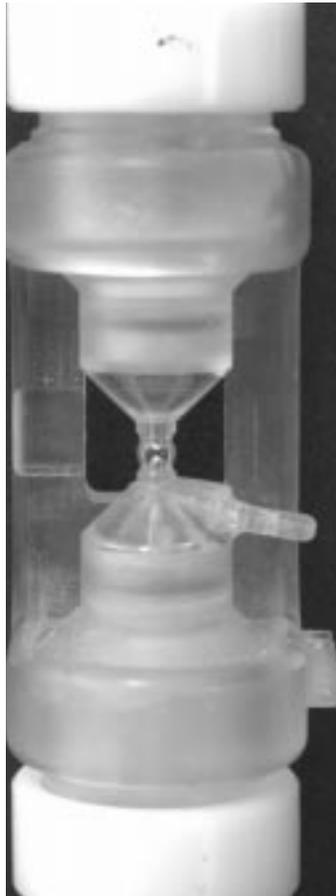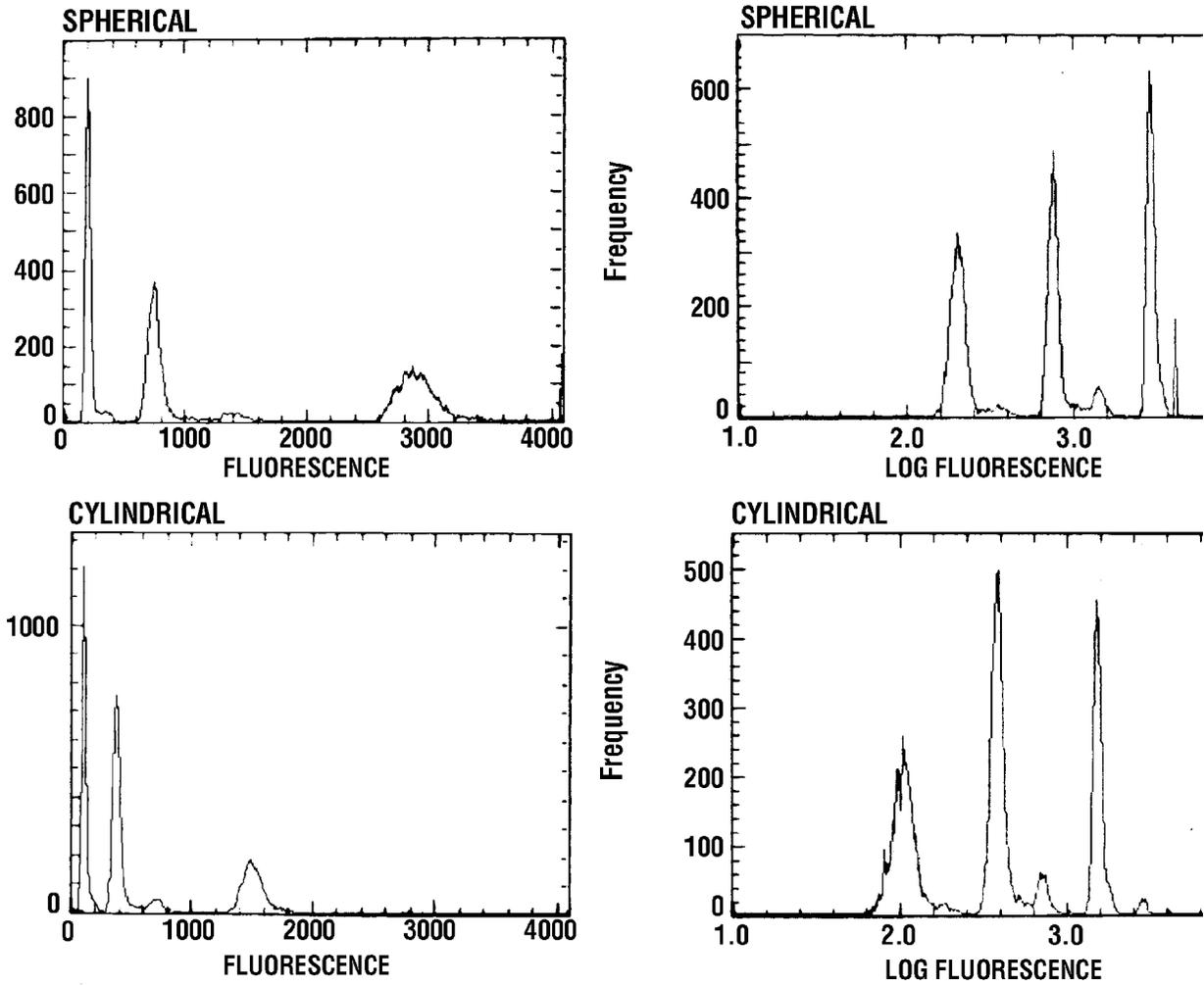
Figure 2. Electronic photograph of a CVA with spherical flow cell. Both the sample and the sheath flow upward.

IMMUNO−BRITE BEADS

Figure 3 Single parameter fluorescence distributions of EPICS[TM] Immuno-Brite beads. Top panel: results with the spherical transducer. Bottom panel: results with the conventional cylindrical flow cell.
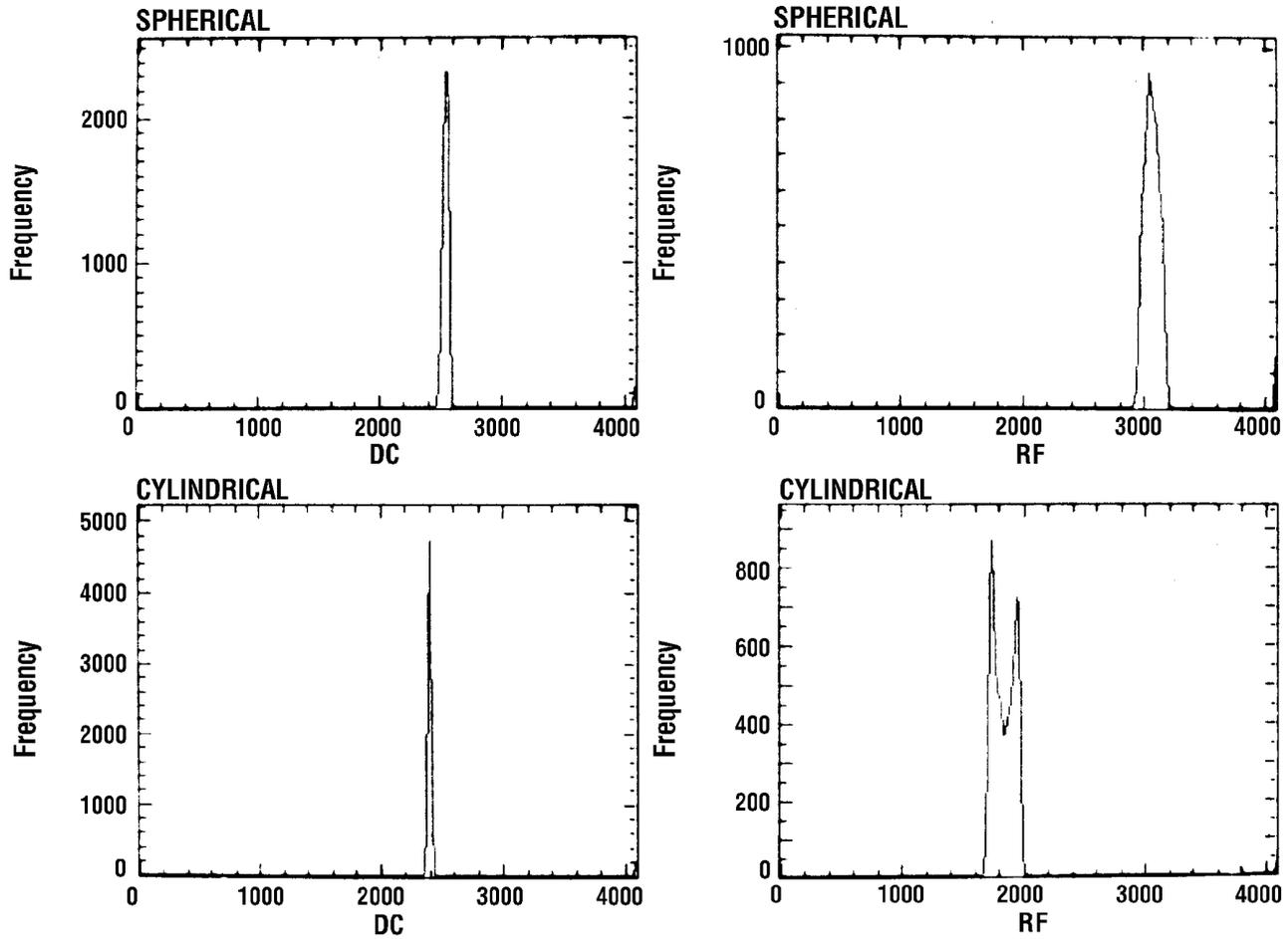
SPHERICAL **SPHERICAL** **SPHERICAL**

CYLINDRICAL **CYLINDRICAL** **CYLINDRICAL**
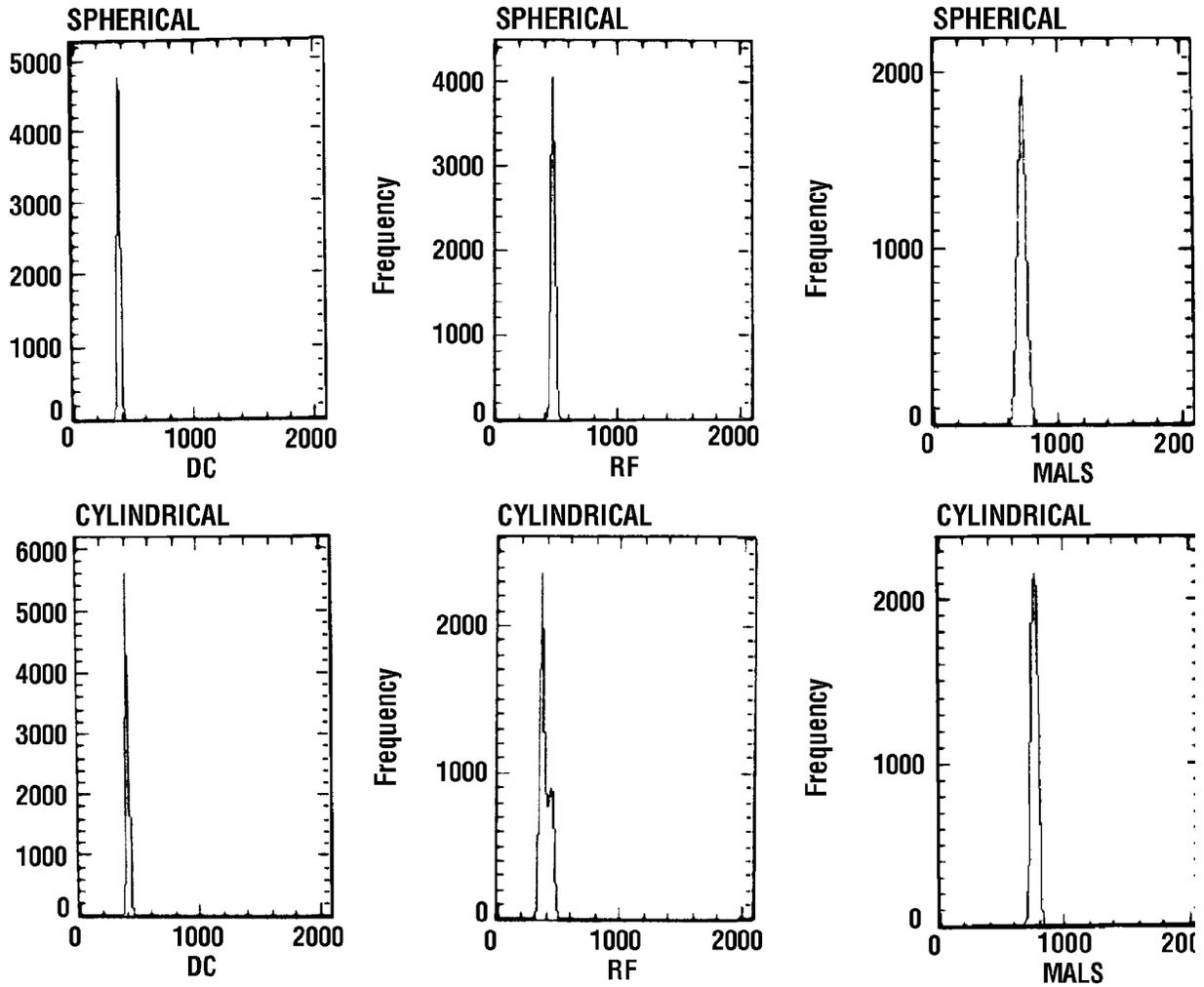
LATRON BEADS

Figure 4. COULTER[R] Latron bead study. Left: D.C. impedance (Coulter volume). Middle: Radio frequency impedance. Right: median angle light scatter. Top panel: spherical flow cell. Bottom panel: cylindrical flow cell.

DNA—CHECK BEADS

Figure 5a. Electronic impedance studies with EPICS[TM] DNA-Check Beads. Left: DC (Coulter volume). Right: radiofrequency impedance (RF). Top panel: spherical flow cell. Bc conventional cylindrical flow cell.

SPHERICAL

CYLINDRICAL

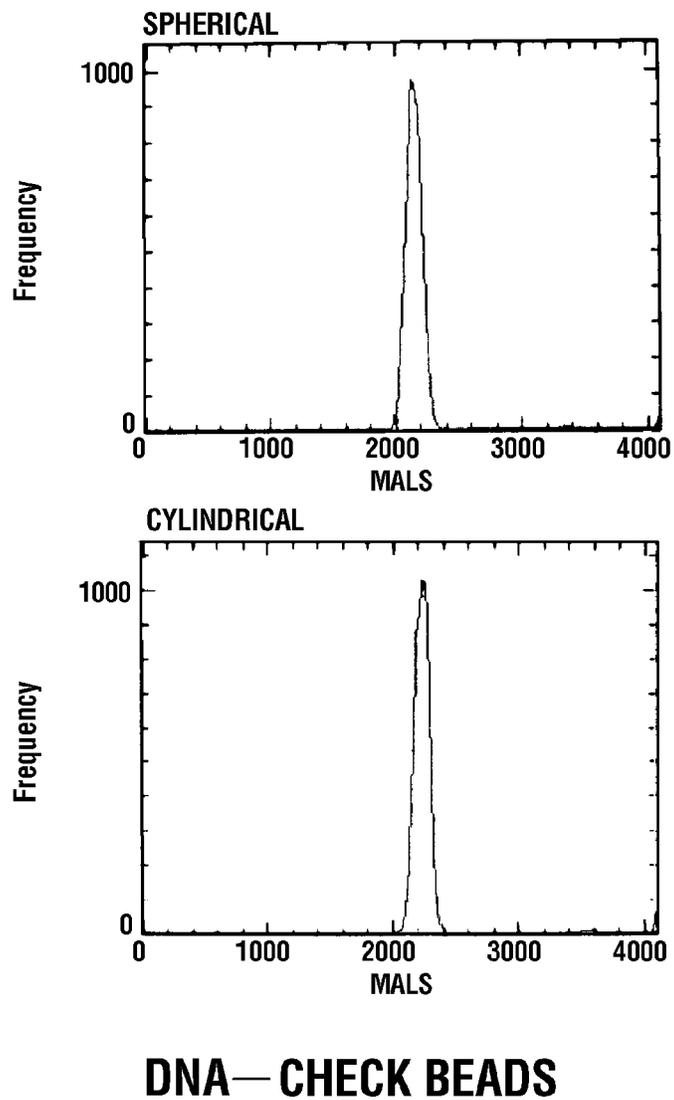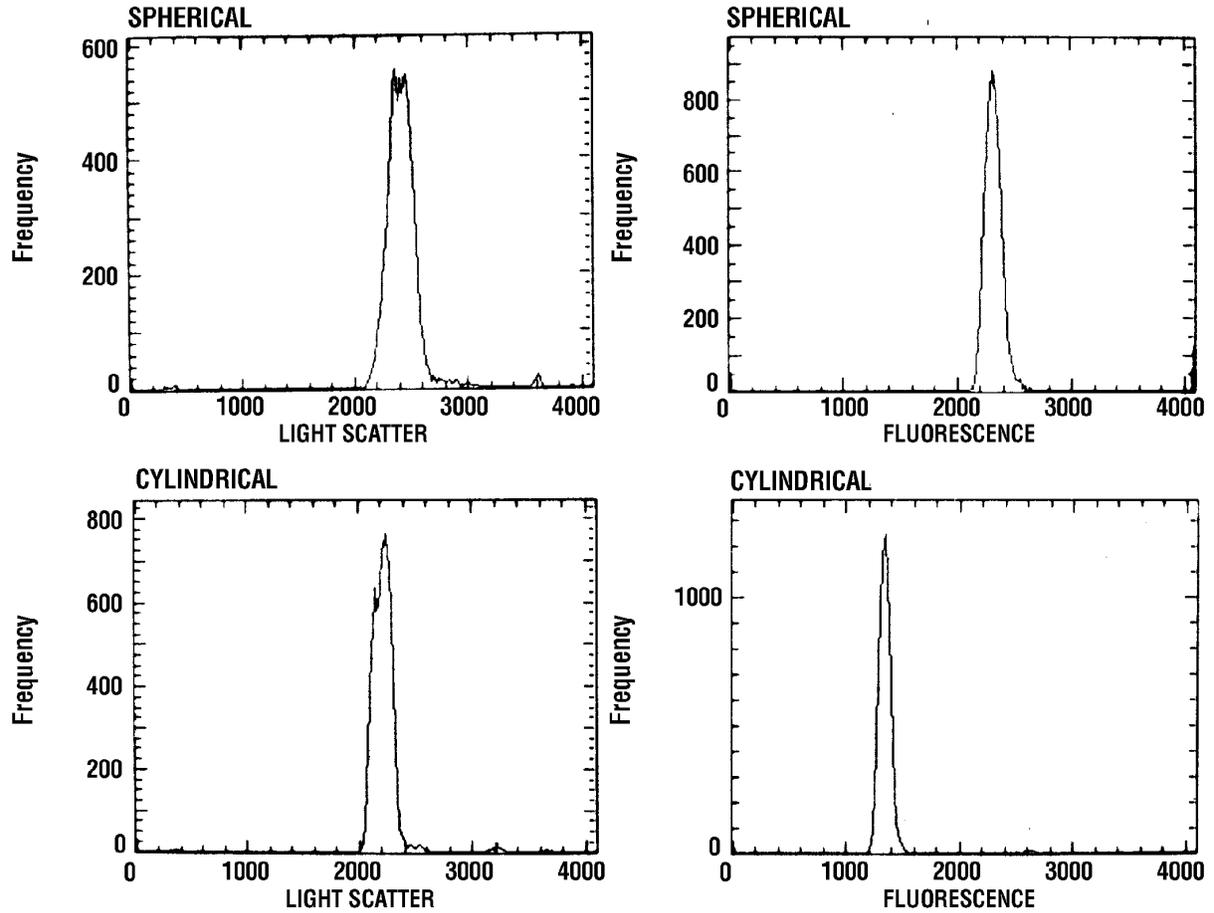# DNA—CHECK BEADS

Figure 5b. Median angle light scatter (MALS).

DNA−CHECK BEADS

Figure 5c. Left: single parameter ninety degree light scatter Right: single parameter fluorescence distributions of EPICS^{TM} DNA-Check beads. Top panel: results with the spherical transducer. Bottom panel: results with the conventional cylindrical transducer.

SPHERICAL

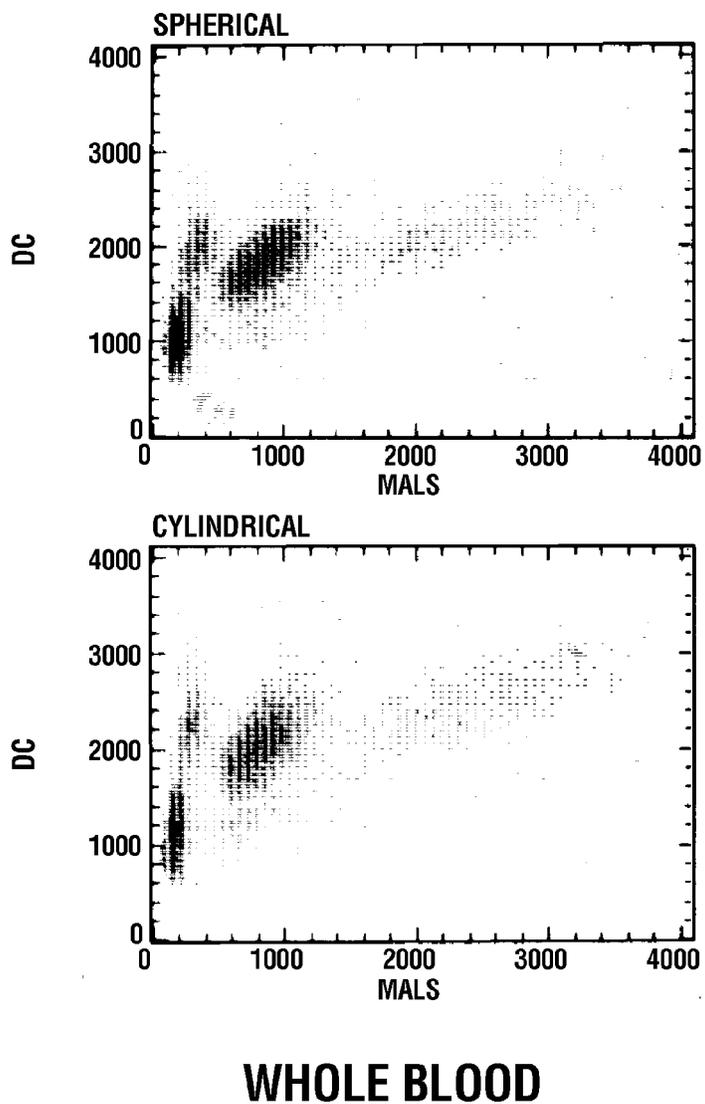**CYLINDRICAL**

WHOLE BLOOD

Figure 6. Whole blood preparation. Coulter electronic cell volume (DC) plotted against median angle light scattering (MALS).
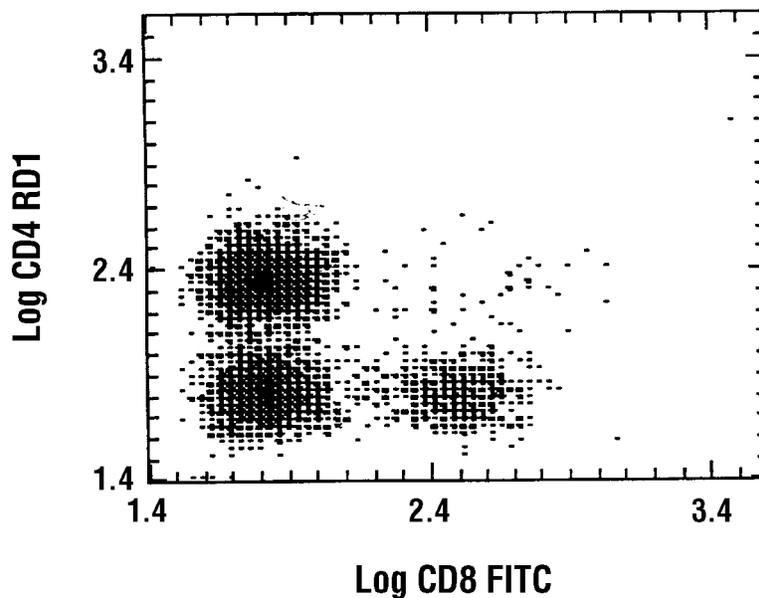
Figure 7. Two parameter fluorescence distribution gated on the lymphocyte distribution shown in Figure 6. The cells are stained with a COULTER CYTO-STAT preparation. Log CD8 (T8 FITC) is the abscissa; log CD4 (T4 RD1) is the ordinate.

Table 1.   Fluorescence Intensity Measurements with Immuno-Brite Beads

Peak	Sphere	Cylinder	Sphere/ Cylinder
Low	197	101	1.95
Medium	749	375	2.00
High	2,902	1,511	1.92

Table 2. Electronic Impedance Studies with Latron Beads

Parameter	Sphere		Cylinder		Sphere/ Cylinder
	Peak	CV in %	Peak	CV in %	Peak
DC	377	2.8	405	3.0	0.93
RF	467	3.5	386	10.4	1.21
MALS	707	4.6	767	3.3	0.92

Table 3. Studies with DNA-Check

Parameter	Sphere		Cylinder		Sphere/ Cylinder
	Peak	CV in %	Peak	CV in %	Peak
DC	2,546	0.93	2,402	0.56	1.06
RF	3,071	1.98	1,835	4.80	1.67
MLS	2,152	2.88	2,236	2.51	0.96
90° Scatter	2,411	4.3	2,213	3.33	1.09
Fluorescence	2,320	3.14	1,342	3.72	1.73