Cytometry 20:185–190 (1995)

Use of a Spherical Multiparameter Transducer for Flow Cytometry

R.C. Leif, M.L. Cayer, W. Dailey, T. Stribling, and K. Gordon
Coulter Corporation, Miami, Florida

Received for publication June 28, 1993; accepted August 25, 1994

Fused silicon dioxide, multiparameter flow transducers with 50 μm internal square cross section and ~60 μm length can simultaneously measure DC and RF impedance as well as fluorescence and multiple-angle light scattering. A spherical version of such a transducer was mounted in an EPICS CVA flow-cell housing and was installed on a research prototype equipped with an argon-ion laser. The signal that was produced by the spherical transducer with EPICS DNA-Check beads was 1.73 times greater than that produced with the standard cylindrical flow cell. Similarly, with EPICS 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 optimal design for combined electrooptical, multiparameter flow cytometry analyzers. © 1995 Wiley-Liss, Inc.

Key terms: Automated analysis, fluorescence, Coulter, light scatter, automated multiparameter analyzer for cells, complete cell analysis, cell volume, focused flow, immunohematology, electrooptical transducers

The possibility of an "Automated Multiparameter Analyzer for Cells" (AMAC) based on the simultaneous acquisition of multiparameter, electrooptical 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 electrooptical transducers to measure simultaneously 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. Because 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 electrooptical transducer for the AMAC is a sphere with an inner square flow channel (6). Leif and Wells (8) 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 acquired through a flat wall. They also described the increase in spherical aberration due to the flat wall, which significantly limits the optical resolution. The minimal spot size for a spherical flow cell was calculated to be 1.8 μm. Other flow transducers, including electrooptical units, have been described by Steinkamp (14). Watson (16,18) described the addition of two spherical lenses to a square-cross-section flow cell. One of these was mirrored, and the other was constructed of a higher-refractive-index glass and served as a focusing lens. Watson claimed a theoretical sixfold increase in light-gathering efficiency vs. the square-walled flow cell. However, the addition of the two lenses has two disadvantages. First, fabrication from three elements rather than one element results in a larger transducer that, in turn, increases the working distance and the size of the collection optics. Thus, the use of some microscope objectives can be precluded. Second, 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

This study was presented in part at the XVI Congress of the International Society for Analytical Cytology, Colorado Springs, Colorado.

Address reprint requests to Robert C. Leif, PhD, Vice President and Research Director, Newport Instruments, 5648 Toyon Road, San Diego, CA, 92115. E-mail rleif@crash.cts.com

transducers (13) has been optimized by Watson (17,18). His design employs a highly efficient ellipsoidal-spherical flow cell with an internal 250 × 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 (personal communication) has described and marketed another combined electrooptical 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 that could extend the capability of a commercially available, totally optical, four-part differential leukocyte analyzer to include an immunofluorescence measurement has been recently published (3).

Figure 1 is a drawing of an idealized 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 impedance (Coulter volume) and radiofrequency (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 (8). 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 (8). 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 not commercially available. However, an expensive lens with a 0.90 numerical aperture and a working distance of 1.3 mm in air, which, in fused silica, is less than 1 mm, is available from Mitutoyo. A 1 mm 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.

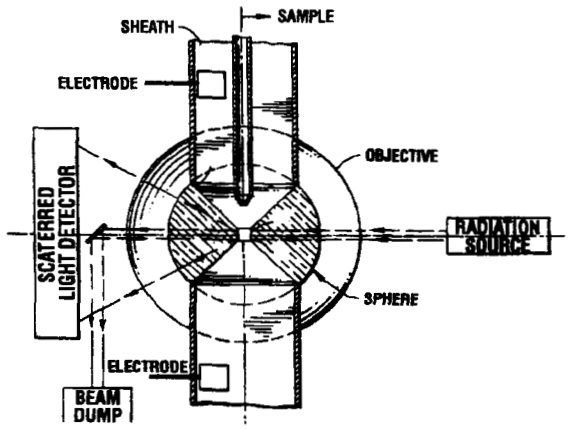

FIG. 1. Drawing of a spherical flow transducer. The light enters the sphere through the flat on the right. The objective is shown in back of the sphere.

MATERIALS AND METHODS

A fused-silica, spherical flow cell with a radius of 1.7 mm and a 50-μm-square aperture and without mirroring was compared to the standard Coulter cylindrical flow cell, which has a comparable aperture diameter and comparable outside dimensions. Both had cup-shaped inlets and outlets. The standard flow cell is used in the Coulter VCS, STKS, and MAXM clinical hematology instruments. Measurements were made on a prototype equipped with a 20 mW 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 Elite assembly. The spherical and cylindrical transducers were mounted in EPICS CVA flow-cell housings (Fig. 2).

EPICS Immuno-Brite (PN 6603473; 10 μm diameter), Coulter Latron Control (PN 7546914; 5.1 μm diameter), and EPICS DNA-Check beads (PN 6603488; 10 μm diameter) were employed to compare the spherical flow cell to the conventional flow cell. Thirty-three microliters of whole blood were reacted for 2 min with 10 μl of T8(CD8)-FITC/T4(CD4)-RD1 (phycoerythrin; Coulter Cyto-Stat; PN 6603802) and were then lysed and quenched with STKS reagents (PN 7546917). Ten thousand events were taken in list mode and were 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 Figure 3. The ratios of the modes of the peaks of linear fluorescence from the spherical and cy-

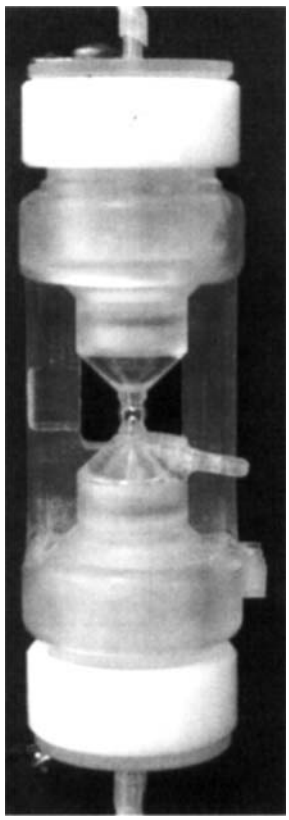

FIG. 2. Electronic photograph of a CVA with spherical flow cell. Both the sample and the sheath flow upward.

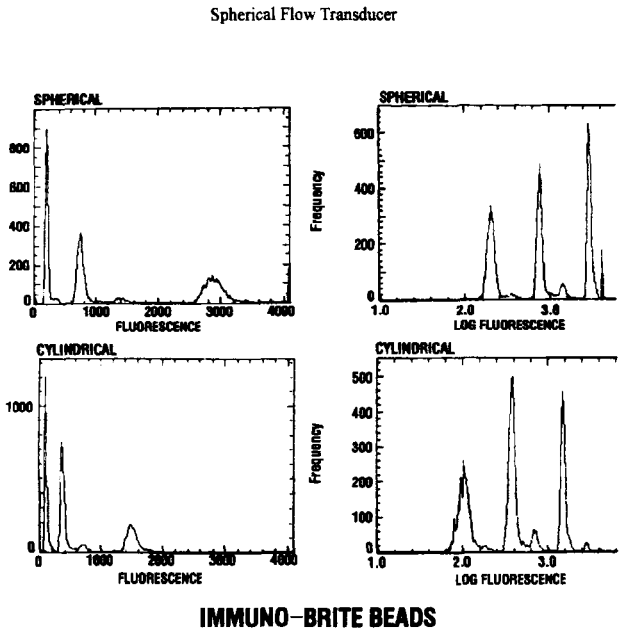

FIG. 3. Single-parameter fluorescence distributions of EPICS Immuno-Brite beads. **Top:** Results obtained with the spherical transducer. **Bottom:** Results obtained with the conventional cylindrical flow cell.

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

	Sphere		Cylinder		Sphere/
Parameter	Peak	CV (%)	Peak	CV (%)	cylinder 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

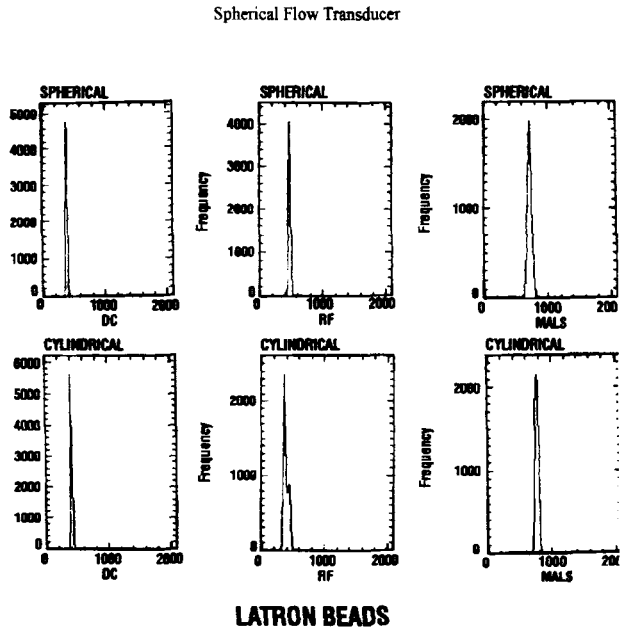

FIG. 4. Coulter Latron bead study. **Left:** DC impedance (Coulter volume). **Middle:** Radio frequency impedance. **Right:** Median-angle light scatter. **Top:** Spherical flow cell. **Bottom:** Cylindrical flow cell.

lindrical 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 (Fig. 4) was slightly lower (2.8 vs. 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 (MALS) CV for the cylindrical flow cell was lower (3.3 vs. 4.6). The MALS detectors were standard for the STKS and MAXM instruments and, therefore, were 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

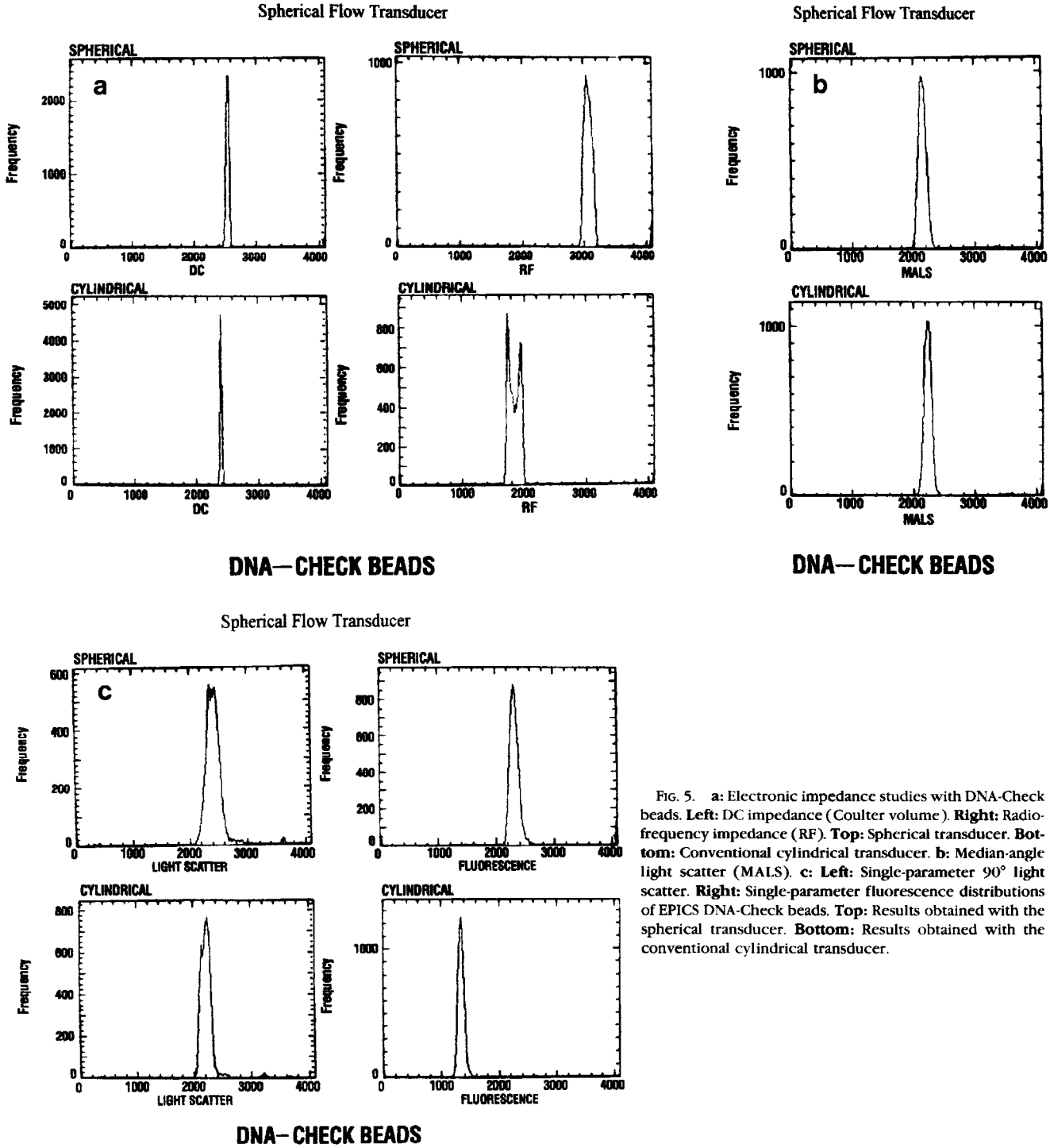

FIG. 5. **a:** Electronic impedance studies with DNA-Check beads. **Left:** DC impedance (Coulter volume). **Right:** Radio-frequency impedance (RF). **Top:** Spherical transducer. **Bottom:** Conventional cylindrical transducer. **b:** Median-angle light scatter (MALS). **c: Left:** Single-parameter 90° light scatter. **Right:** Single-parameter fluorescence distributions of EPICS DNA-Check beads. **Top:** Results obtained with the spherical transducer. **Bottom:** Results obtained with the conventional cylindrical transducer.

spherical flow cell and 0.56 for the cylindrical flow cell. The RF was better on the spherical than on the cylindrical flow cell, which, presumably, was an artifact. MALS was comparable with the sphere CV (2.88) and was 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 MALS (Fig. 6) were essentially the same for the two flow cells. The spherical flow cell produced (Fig. 7) a well-defined dual-label flu-

Table 3
Studies With DNA-Check Beads

Parameter	Sphere Peak	Sphere CV (%)	Cylinder Peak	Cylinder CV (%)	Sphere/ cylinder 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

orescence distribution of lymphocytes. Thus, we have demonstrated that, to produce a five-part differential count, a spherical flow cell makes the same measurements as the conventional cylindrical flow cell; but it does so with greater sensitivity for immunofluorescence.

DISCUSSION

The CVs of the physical parameters (DC, RF, and MALS) obtained with both transducers were smaller for the DNA-Check beads compared to the Latron beads. This was expected, because 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 the capacity to mirror the opposite side. A comparison of the electronic-impedance studies demonstrates that the larger area of a square orifice compared to a circular orifice does not result in a decrease in 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 that is capable of simultaneous fluorescence and physical measurements previously described in Leif et al. (9) has finally been completed. The use of this technology will permit rapid, inexpensive complete cell analysis of leukocyte classes and subclasses.

ACKNOWLEDGMENTS

We thank Mark Wells and Theresa Daly for their editorial assistance and Raoul Fernandez for preparing the figures. The authors also thank Coulter Corporation for its kindness, support, and generosity in permitting us to publish the results of our research. We also appreciate the information on microscope objectives that was supplied by Howard Shapiro. This paper is dedicated to our late collaborator, Tom Stribling.

Spherical Flow Transducer

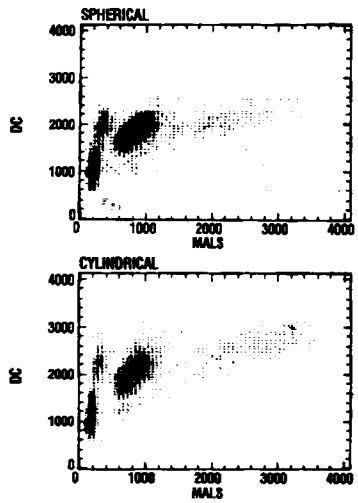

FIG. 6. Whole blood preparation. Coulter electronic cell volume (DC) plotted against median angle light scattering (MALS).

Spherical Flow Transducer

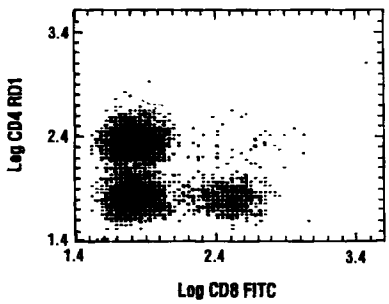

FIG. 7. Two-parameter fluorescence distribution gated on the lymphocyte distribution shown in Figure 6. The cells are stained with a Coulter Cyto-Stat preparation. Log CD8 (T8 FITC) is the abscissa; log CD4 (T4 RD1) is the ordinate.

LITERATURE CITED

1. Condrau MA, Schwendener RA, Zimmermann M, Muser MH, Graf U, Niederer P, Anliker M: Time-resolved flow cytometry for the measurement of lanthanide chelate fluorescence: II. Instrument design and experimental results. Cytometry 16:195–205, 1994.
2. Dean PN: Commercial instruments. In: Flow Cytometry and Sorting, 2d Ed, Melamed MR, Lindmo T, and Mendelsohn ML (eds). Wiley-Liss, New York, 1990, pp 171–186.
3. Doorndos RMP, Hennink EJ, Putman CAJ, De Groth BG, Greve J: White blood cell differentiation using a solid state flow cytometer. Cytometry 14:589–594, 1993.
4. Kachel V: Electrical resistance pulse sizing: Coulter sizing. In: Flow Cytometry and Sorting, 2nd Ed, Melamed MR, Lindmo T, and Mendelsohn ML (eds). Wiley-Liss, New York, 1990, pp 45–80.
5. Leif RC: A proposal for an automated multiparameter analyzer for cells (AMAC). In: Automated Cell Identification and Sorting, Wied GL, Bahr GF (eds). Academic Press, New York, 1970, pp 131–159.
6. Leif RC: Orifice Inside Optical Elements. U.S. Patent 4,348,107, 1982.
7. Leif RC: The AMAC IIIS transducer. In: Advances in Microscopy, Cowden R (ed). Alan R. Liss, Inc., New York, 1985, pp 213–222.
8. Leif RC, Wells M: Optical analysis of the AMAC IIIS transducer. Appl Optics 26:3244–3248, 1987.
9. Leif RC, Thomas RA, Yopp TA, Watson BD, Guarino VR, Hindman DHK, Lefkove N, Vallarino LM: Development of instrumentation and fluorochromes for automated multiparameter analysis of cells. Clin Chem 23:1492–1498, 1977.
10. Rectenwald D, Warner N, Fulwyler M, Unterleitner F: Highly sensitive dual fluorescence flow-cytometer based on mercury-arc illumination. Soc Anal Cytol Meet, Automated Cytology IX, Schloss Elmau, Bavaria, Oct. 1982.

11. Rodriguez CM, Coulter WH: Multi-part differential analyzing apparatus utilizing light scatter techniques. U.S. Patent 5,125,737, 1992.
12. Shapiro HM: Buying flow cytometers. In: Practical Flow Cytometry, 2nd Ed. Alan R. Liss, Inc., New York, 1988, pp 199–210.
13. Skogen-Hagenson MJ, Salzman GC, Mullaney PF, Brockman WH: A high efficiency flow cytometer. J Histochem Cytochem 25:784–789, 1977.
14. Steinkamp JA: Flow cytometry. Rev Sci Instrum 55:1375–1400, 1984.
15. Thomas RA, Yopp TA, Watson BD, Hindman DHK, Cameron BF, Leif SB, Leif RC, Roque L, Britt W: Combined optical and electronic analysis of cells with the AMAC transducers. J Histochem Cytochem 25:827–835, 1977.
16. Watson JV: A method for improving light collection by 600% from square cross section flow cytometry chambers. Br J Cancer 51:433–35, 1985.
17. Watson JV: Flow cytometry chamber with 4π light collection suitable for epifluorescence microscopes. Cytometry 10:681–688, 1989.
18. Watson JV: Light and optics. In: Introduction to Flow Cytometry. Cambridge University Press, Cambridge, 1991, pp 18–64.