

The Evolution of Cytometers

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Microscopy is difficult when cells are on the fly;
It's lucky the cytometer's now quicker than the eye.
This hasn't been the case throughout the gadget's evolution,
But lasers and computers have provided a solution
To problems of illuminating cells, at least a myriad
Per second, and collecting ample data, in that period,
To pick out some to keep, and destine others for rejection.
For modern labs, the instrument's a natural selection!

The first microscopes were as likely to be gentlemen's toys as scientists' tools. By the beginning of the 20th century, the professionals had largely taken over from the amateurs, and it had become possible to draw at least some quantitative conclusions from observations made using microscopes. Hemocytometers allowed an observer to derive a reasonably accurate count of the number of cells or other particles in a unit volume of specimen, although precision of counts was limited by both counting statistics and dilution errors. Using eyepiece reticles, grids, etc., one could also measure the size of microscopic objects, at least in two dimensions. It was not, however, until some time later that the tools developed by chemists and physicists for spectroscopy and photometry or radiometry were adapted for use with the microscope, thus producing the first true optical cytometers.

THE DAWN OF CYTOMETRY

I have written at length about the development of cytometers on numerous occasions, most recently in the 4th edition of *Practical Flow Cytometry* (1); in this briefer exposition, influenced by the title suggested by the Editor of this journal, I will emphasize the lineage of the instruments. I will apologize in advance to the many colleagues whose contributions I cannot discuss in the allotted space, and thus hope to avoid possible derogatory comments about my own lineage. I am pleased that Leonard Ornstein, a pioneer in the development of both static and flow cytometric apparatus and techniques, appears to share my perspective on the early history of cytometry, as evidenced by an informative and entertaining reminiscence he published in 1987 (2).

It is probably fair to say that the evolution of cytometers from microscopes began in the 1930s in Stockholm. By this time, conventional histologic staining techniques of light microscopy had suggested that tumors might have abnormalities in DNA and RNA content. Torbjörn Caspers-

son (3), working at the Karolinska Institute (Stockholm, Sweden), began to study cellular nucleic acids and their relation to cell growth and function. He developed a series of progressively more sophisticated microspectrophotometers, which made fairly precise measurements of nucleic acid and protein content based on the intrinsic ultraviolet (UV) absorption of these substances near 260 and 280 nm.

Caspersson's early apparatus now seems hopelessly primitive. Cadmium spark sources were used for ultraviolet illumination; photocurrent measurements were done with string electrometers, unless the signal was strong enough to permit use of a vacuum-tube amplifier. However, even this primitive apparatus got results, and attracted the attention of other researchers; many of the advances in analytical cytology from the 1940s on were made by people who had made the pilgrimage to Stockholm. Ornstein (2) documents the influence of Caspersson's work in establishing the role of DNA as the genetic material.

It was during the 1950s that analytical cytology acquired its name, coined by Francis O. Schmitt of the Massachusetts Institute of Technology (MIT; Cambridge, MA); the first and second editions of a book entitled *Analytical Cytology*, edited by Robert Mellors, of the Memorial Sloan-Kettering Cancer Institute (New York, NY), appeared in 1955 and 1959 (4). The book included chapters on the fluorescent antibody method, on histochemistry, and on phase, interference, and polarizing microscopy; Arthur Pollister, Ornstein's mentor at Columbia University (New York, NY), and Ornstein contributed several chapters, including one on the theory and practice

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of absorption measurements that is well worth reading even today.

Early Microspectrophotometry and Image Cytometry

Microspectrophotometers were first made by putting a small "pinhole" aperture, technically known as a field stop, in the image plane of a microscope, restricting the field of view to the area of a single cell, and placing a photodetector behind the field stop. If a 40 \times objective lens is used, measuring the transmission through, or the absorption of, a cell 10 μm in diameter requires a 400 μm diameter field stop; with a smaller field stop, it becomes possible to measure the transmission through a correspondingly smaller area of the specimen. A 40 μm field stop allows measurement of a 1 μm diameter area of the specimen, and. By moving the specimen in precise incremental steps in the x and y directions (i.e., in the plane of the slide) in a raster pattern, and recording the information, it becomes possible to measure the integrated absorption of a cell, and/or to make an image of the cell with each pixel corresponding in intensity to the transmission or absorption value. This was the first, and, until the 1950s, the only approach to scanning cytometry. By the 1960s, Zeiss (Oberkochen, Germany) had commercialized a current version of Caspersson's apparatus, and others had begun to build high-resolution scanning microscopes incorporating a variety of technologies.

Motivation for Progress: Cancer Cytology and Hematology Automation

By the mid-1950s, it had become apparent that malignant cells were likely to contain more nucleic acid than normal cells, and Mellors proposed construction of an automatic scanning instrument for screening cervical cytology (Papanicolaou or "Pap") smears. Tolles et al. (5), at Airborne Instruments Laboratory (Mineola, NY), described the "Cytoanalyzer" built for this purpose. A "Nipkow disc" containing a series of apertures rotated in the image plane of a microscope, producing a raster scan of a specimen with approximately 5 μm resolution. A hard-wired analyzer extracted nuclear size and density information; cells were then classified as normal or malignant using these parameters. The Cytoanalyzer was, to make a long story short, right more of the time than it was wrong, but its false-positive and false-negative rates were too high for it to be suitable for clinical use. The results were encouraging enough for the American Cancer Society and the National Cancer Institute to continue funding research on cytology automation.

Recording and storing cell images was a nontrivial task in the 1960s, when mainframe computers occupied entire rooms, required kilowatts of power and heavy-duty air conditioning, and cost millions of dollars, which bought a processor with 160 kb of memory and a 6.4 μs instruction cycle (equivalent clock speed 160 kHz). Input and output and data storage typically used tape drives; large random access storage media had not yet arrived on the scene. However, when minicomputers became available in the

middle of the decade, there were at least a few groups of analytical cytologists ready to use them. The TICAS system, assembled in the late 1960s by George Wied of the University of Chicago, Gunter Bahr of the Armed Forces Institute of Pathology, and Peter Bartels of the University of Arizona (Tucson, AZ), interfaced Zeiss's commercial version of the Caspersson microspectrophotometer to a minicomputer, with the aim of automating interpretation of Pap smears (6).

The use of stage motion for scanning made operation extremely slow; it could take many minutes to produce a high-resolution scanned image of a single cell, and there were no computers available to capture the data. Somewhat higher speed could be achieved by using Nipkow discs or galvanometer-driven moving mirrors for image scanning, and limiting the tasks of the motorized stage to bringing a new field of the specimen into view and into focus; this required some electronic storage capability, and made measurements susceptible to errors due to uneven illumination across the field, although this could be compensated for. My colleagues and I at National Institutes of Health (NIH) built "Spectre II" (7), which incorporated a galvanometer mirror scanning system (8) developed by Kendall Preston, an Airborne Instruments alumnus then working at Perkin-Elmer (Norwalk, CT), and a Digital Equipment Corporation (Maynard, MA) LINC-8 computer. While this system had sufficient computer power to capture high-resolution cell images (0.2 μm pixels), data were recorded on 9-track tape and transported (by "sneakernet") to a mainframe elsewhere on the NIH campus for analysis (9).

Since the late 1940s and early 1950s had already given us Howdy Doody, Milton Berle, and the Ricardos, it might be expected that, somewhere around that time, someone would have tried to automate the process of looking down the microscope and counting cells using video technology. Most of the imaging cytometers developed then were not based on video cameras, for a number of reasons, not the least of which was the variable light sensitivity of different regions of a camera tube, which made quantitative measurements difficult. However, it was recognized that the raster scan mechanism of a cathode ray tube could be used on the illumination side of an image analysis system, with the "flying spot" illuminating only a small segment of the specimen plane at any given time (10). The CYDAC system, a flying spot scanner built at Airborne Instruments Laboratory, was used in studies of the automation of differential leukocyte counting (11) and chromosome analysis (12) by Mortimer Mendelsohn (later the first president of the Society for Analytical Cytology), Brian Mayall (later the founding editor of this journal), Judith Prewitt, and their colleagues, then at the University of Pennsylvania.

FLOW CYTOMETRY AND SORTING: WHY AND HOW

Somewhat simpler tasks of cell or particle identification, characterization, and counting than those involved in Pap smear analysis and differential white cell counting had

attracted the attention of other groups of researchers at least since the 1930s. During World War II, the U.S. Army became interested in developing devices for rapid detection bacterial biowarfare agents in aerosols (a continuing preoccupation); this would require processing a relatively large volume of sample in substantially less time than would have been possible using even a low-resolution scanning system. The apparatus built in the Chemistry Department of Northwestern University (Evanston, IL) by Gucker et al. (13) in support of this project achieved the necessary rapid specimen transport by injecting the air stream containing the sample into the center of a larger sheath stream of flowing air that passed through the focal point of a dark-field microscope. Particles passing through the system scattered light into a collection lens, eventually producing electrical signals from a photodetector. The instrument could detect objects on the order of 0.5 μm in diameter, and is generally recognized as having been the first flow cytometer used for observation of biological cells.

By the late 1940s and early 1950s, the same principles, including the use of sheath flow, were applied to the detection and counting of red blood cells in saline solutions (14), providing effective automation for a diagnostic test notorious for its imprecision when performed by a human observer using a hemocytometer and a microscope. Neither the bacterial counter nor the early red cell counters had any significant capacity either for discriminating different types of cells or for making quantitative measurements. Both types of instrument were measuring what we would now recognize as side scatter signals; although larger particles, in general, produced larger signals than smaller ones, correlations between particle sizes and signal amplitudes were not particularly strong.

An alternative flow-based method for cell counting was developed in the 1950s by Wallace Coulter (15). Recognizing that cells, which are surrounded by a lipid membrane, are relatively poor conductors of electricity as compared to saline, he devised an apparatus in which cells passed one by one through a small (<100 μm) orifice between two chambers filled with saline. When a cell passed through, the electrical impedance of the orifice increased in proportion to the volume of the cell, producing a voltage pulse. The Coulter counter was widely adopted in clinical laboratories for blood cell counting; it was soon established that it could provide more accurate measurements of cell size than had previously been available (16,17).

In the early 1960s, investigators working with Leitz (Wetzlar, Germany) (18) conceived a hematology counter that added a fluorescence measurement to the light scattering measurement used in red cell counting. If a fluorescent dye such as acridine orange were added to the blood sample, white cells would be stained much more brightly than red cells; the white cell count could then be derived from the fluorescence signal, and the red cell count from the scatter signal. It was also noted that acridine orange fluorescence could be used to discriminate mononuclear cells from granulocytes. However, it is not clear that the

device, which would have represented a new level of sophistication in flow cytometry, was ever actually built.

Around the same time, the promising results obtained with the Cytoanalyzer in attempts to automate reading of Pap smears (5) encouraged executives at the International Business Machines Corporation (IBM) (Armonk, NY) to look into producing an improved instrument. Assuming this would be some kind of image analyzer, IBM gave technical responsibility for the program to Louis Kametsky, who had developed a successful optical character reader. He did some calculations of what would be required in the way of light sources, scanning rates, and computer storage and processing speeds to solve the problem using image analysis, and concluded that a different approach would be required.

Having learned from pathologists in New York that cell size and nucleic acid content could provide a good indicator of whether cervical cells were normal or abnormal, Kametsky traveled to Caspersson's laboratory in Stockholm and learned microspectrophotometry. He then built a microscope-based flow cytometer that used a transmission measurement at visible wavelengths to estimate cell size and a 260 nm UV absorption measurement to estimate nucleic acid content (19,20). Subsequent versions of this instrument, which incorporated a dedicated computer system, could measure as many as four cellular parameters (21). A brief trial on cervical cytology specimens indicated the system had some ability to discriminate normal from abnormal cells (22); it could also produce distinguishable signals from different types of cells in blood samples stained with a combination of acidic and basic dyes, suggesting that flow cytometry might be usable for differential leukocyte counting.

The first commercial flow cytometric differential counter, introduced in the early 1970s, was Technicon's (Tarrytown, NY) Hemalog D (23,24); Ornstein was a prime mover in its development, having interacted with Kametsky's group along the way (2). The Hemalog D used light scattering and absorption measurements made at different wavelengths in three different flow cytometers to classify leukocytes. Chromogenic enzyme substrates were used to identify neutrophils and eosinophils by the presence of moderate to high and very high concentrations of peroxidase, while another channel identified monocytes by their esterase content. Basophil identification was based on detection of glycosaminoglycans in basophil granules using Alcian blue. A single tungsten-halogen lamp served as light source for all three flow systems.

Although the Hemalog D employed cytochemical staining procedures that were well regarded by hematologists for such purposes as determination of lineage of leukemic cells, the apparatus, which worked pretty well, was initially regarded with a great deal of suspicion, at least in part due to the novelty of flow cytometry. The developers and manufacturers of image analyzing differential counters, which certainly didn't perform much better than did the Hemalog D, did what they could to keep potential users suspicious of flow cytometry for as long as

possible; the technology would eventually be legitimized by its dramatic impact on immunology, which was facilitated by the introduction of cell sorting and immunofluorescence measurements.

Although impedance (Coulter) counters and optical flow cytometers could analyze hundreds of cells per second, providing a high enough data acquisition rate to be useful for clinical use, microscope-based static cytometers offered a significant advantage. A system with computer-controlled stage motion could be programmed to reposition a cell on a slide within the field of view of the objective (7), allowing the cell to be identified or otherwise characterized by visual observation; it was, initially, not possible to extract cells with known measured characteristics from a flow cytometer. Until this could be done, it would be difficult to verify any cell classification arrived at using a flow cytometer, especially where the diagnosis of cervical cancer or leukemia might be involved.

This problem was solved in the mid-1960s, when both Mack Fulwyler (25), working at the Los Alamos National Laboratory (Los Alamos, NM), and Kamensky, at IBM (26), demonstrated cell sorters built as adjuncts to their flow cytometers. Kamensky's system used a syringe pump to extract selected cells from its relatively slow-flowing sample stream. Fulwyler's was based on ink jet printer technology then recently developed by Richard Sweet (27) at Stanford University (Stanford, CA); following passage through the cytometer's measurement system (originally a Coulter orifice), the saline sample stream was broken into droplets, and those droplets that contained cells with selected measurement values were electrically charged at the droplet break-off point. The selected charged droplets were then deflected into a collection vessel by an electric field; uncharged droplets went, as it were, down the drain.

In the early 1970s, the group at Los Alamos led the way in implementation of practical multiparameter flow cytometers; their larger instruments, with droplet sorting capability, combined two-color fluorescence measurements with measurements of Coulter volume and (thanks to the contributions of Paul Mullaney, Gary Salzman, and others) light scattering at several angles (28-31). The cytometers were interfaced to Digital Equipment Corporation minicomputers. Several instruments made at Los Alamos were delivered to the NIH; other institutions copied most or all of the Los Alamos design in their own laboratory-built apparatus.

FLUORESCENCE AND FLOW: MADE FOR EACH OTHER

Fluorescence measurement was introduced to flow cytometry in the late 1960s as a means of improving both quantitative and qualitative analyses. By that time, Van Dilla et al. (32) at Los Alamos and Dittrich and Göhde (33) in Germany had built fluorescence flow cytometers to measure cellular DNA content, facilitating analysis of abnormalities in tumor cells and of cell cycle kinetics in both neoplastic and normal cells. The Los Alamos instrument

incorporated the orthogonal "body plan" now standard in laser-source instruments, with the optical axes of illumination and light collection at right angles to each other and to the direction of sample flow. Kamensky, who had left IBM to found Bio/Physics Systems (Mahopac, NY), produced the Cytofluorograf, an orthogonal geometry fluorescence flow cytometer that was the first commercial product to incorporate an argon ion laser; Göhde's Partec (Münster, Germany) Impulscytophotometer (ICP) instrument, built around a fluorescence microscope with arc lamp illumination, was distributed commercially by Phywe (Göttingen, Germany).

Leonard Herzenberg and his colleagues (34), at Stanford University, realizing that fluorescence flow cytometry and subsequent cell sorting could provide a useful and novel method for purifying living cells for further study, developed a series of instruments after exposure to a Kamensky prototype lent to them by IBM (35). Although their original apparatus (36), with arc lamp illumination, was not sufficiently sensitive to permit them to achieve their objective of sorting cells from the immune system, based on the presence and intensity of staining by fluorescently labeled antibodies, the second version (37), which used a water-cooled argon laser, was more than adequate. This was commercialized as the Fluorescence-Activated Cell Sorter (FACS) in 1974 by a group at Becton-Dickinson (B-D, now BD Biosciences, San Jose, CA), led by Bernard Shoor.

Coulter Electronics (now Beckman Coulter, Fullerton, CA), which by 1970 had become a very large and successful manufacturer of laboratory hematology counters, pursued the development of fluorescence flow cytometers through a subsidiary, Particle Technology, under Mack Fulwyler's direction in Los Alamos. The Two Parameter Sorter (TPS-1), Coulter's first product in this area, reached the market in 1975. It used an air-cooled 35 mW argon ion laser source and could measure forward scatter and fluorescence.

Multiple wavelength fluorescence excitation was introduced to flow cytometry in apparatus built at Block Engineering (Cambridge, MA) during an abortive attempt to develop a hematology instrument. The first instrument (38) derived five illuminating beams from a single arc lamp; the second (39) used three laser beams; both could analyze over 30,000 cells per second and, using hardwired preprocessors and integral minicomputers, identify cells comprising less than 1/100,000 of the total sample. The laser source system incorporated forward and side scatter measurements, which permitted lymphocyte gating (40), influenced by work done at Los Alamos (31). Block also built a slow flow system intended for detection of hepatitis B virus and antigen in serum; it could discriminate scatter signals from large viruses (41) and could theoretically detect a few dozen fluorescein molecules above background. The Block cytometers were never sold commercially, but influenced the optical, electronic, and systems design of later instruments.

By the time the Society for Analytical Cytology (now ISAC) came into being in 1978, B-D, Coulter, and Ortho (a

division of Johnson & Johnson (Raritan, NJ) that bought Bio/Physics Systems) were producing flow cytometers that could measure small- (forward scatter) and large- (side scatter) angle light scattering and fluorescence in at least two wavelength regions, analyzing several thousand cells per second, and with droplet deflection cell sorting capability. Ortho was also distributing the ICP, which, by virtue of its optical design, could make higher precision measurements of DNA content than could laser-based flow cytometers. DNA content analysis was receiving considerable attention as a means of characterizing the aggressiveness of breast cancer and other malignancies, and, at least in part due to the results of a Herzenberg sabbatical in Cesar Milstein's lab at the University of Cambridge (UK), monoclonal antibodies had begun to emerge as practical reagents for dissecting the stages of development of cells of the blood and immune system. Loken, Parks, and Herzenberg had successfully performed a two-color immunofluorescence experiment, introducing fluorescence compensation in the process (42), although it was clear that a great deal needed to be done in the area of fluorescent label development to realize the potential of monoclonal antibodies.

STILL IN THE PICTURE: STATIC CYTOMETERS

The late 1960s and early 1970s saw the development of a number of image analyzing automated differential leukocyte counters. Corning (Corning, NY) produced the LARC, based on work by Bacus (43); Geometric Data (Wayne, PA) introduced the Hematrak (44), and Coulter offered a system based on research by Young (45). Other manufacturers subsequently entered the market. The instruments all incorporated bright field microscopes and examined cells stained with Wright's, Giemsa's, or similar stains in smears on slides. Bacus later founded a company called Cell Analysis Systems, (Lombard, IL) which sold bright field image analyzing systems that were applied to DNA content measurement in tumor cells, using Feulgen staining, and detection of hormone receptors, using (immuno)cytochemistry.

Image cytometers incorporating both bright field and fluorescence microscopes existed in research laboratories, but were much slower, usually somewhat less precise and much less sensitive (not easily adapted for immunofluorescence analysis), and almost always even less user-friendly than the early flow cytometers. In general, these instruments couldn't sort, although Meridian Instruments' (Okemos, MI) ACAS system, which incorporated both lamp illumination and laser illumination for fluorescence measurements, could destroy unwanted cells with high-intensity laser light (46). This technique of photodamage cell selection, or "cell zapping," was also envisioned as an adjunct to flow cytometry (47-49).

However, by far the most exciting development in static cytometry in the 1980s was the adaptation of the technique of confocal microscopy to fluorescence imaging. Historical details can be found in books by Inoué and Spring (50) and Pawley (51). In a conventional fluorescence microscope, a fairly large area (and volume) of the

specimen is/are illuminated at any given time. Although a field stop can restrict the area being measured in a microspectrofluorometer or scanning fluorescence microscope, the image resolution is adversely affected by the collection of fluorescence emission from planes above and below the focal plane. Various means of eliminating this light are employed in different types of confocal microscopes, with the end result that it has become possible to create high-resolution images of very thin sections of a specimen, and to reconstruct three-dimensional detail from a succession of such images. Although confocal microscopes are far outnumbered by flow cytometers at present, they are becoming essential components of the cytometric armamentarium.

FLOW CYTOMETRY IN AND OUT OF THE MAINSTREAM

From the early 1970s on, commercial production of instruments allowed researchers who had not developed and built their own apparatus to pursue applications of fluorescence flow cytometry and sorting. Advances in the technology itself continued to occur primarily in a relatively small community of academic, government, and industrial labs. What got done in any one lab was determined by the biological problems and/or clinical applications under investigation, and also by the migration of instruments and/or investigators from one place to another. This process has gotten some attention from real historians of science, resulting in publications by Peter Keating and Alberto Cambrosio (52) and in a video history by Ramunas Kondratas, which was funded and otherwise heavily influenced by B-D; a summary is available online from the Smithsonian Institution Archives (www.si.edu/archives/ihd/videocatalog/9554.htm).

At Stanford University, the emphasis remained on sorting on the basis of immunofluorescence signals with the aim of isolating morphologically indistinguishable viable lymphocytes with differences in functional characteristics. Placing the observation point in a jet in air, rather than in a flow chamber, shortened the distance between this point and the droplet break-off point, making faster sorting possible. One notable descendant of the Stanford instrument was the computer-controlled, multiparameter cytometer/sorter built by the Jovins at the Max Planck Institute for Biophysical Chemistry in Göttingen, Germany (53). This apparatus could operate at short ultraviolet wavelengths; it was used to measure such parameters as intrinsic protein fluorescence, membrane fluidity (using fluorescence polarization), and receptor proximity (using energy transfer), and to establish the utility of Hoechst (Frankfurt-am-Main, Germany) 33342 as a vital DNA stain and thioflavin T as an RNA stain.

The multiparameter flow cytometers developed at Los Alamos were copied by investigators at other institutions, e.g., the Salk Institute (La Jolla, CA), Colorado State University (Ft. Collins, CO), the University of California at Los Angeles (Los Angeles, CA), and the University of Houston (Houston, TX), where an instrument was applied to multiparameter flow cytometric analysis of bacteria (54). Los

Alamos also provided the inoculum for the subsequent growth of another major center for flow cytometer development, that at Lawrence Livermore National Laboratory (Livermore, CA), where high-speed flow sorting was perfected as a means for separating human chromosomes (55,56). The MoFlo high-speed sorter developed by Ger van den Engh and others at Livermore was subsequently refined by Cytomation (now DakoCytomation, Ft. Collins, CO), and has been produced commercially by them since 1994.

The digital pulse processing recently introduced into commercial instruments from Luminex Corporation (Austin, TX) and BD Biosciences mirrors work done beginning in the 1970s by Leon Wheelless et al. (57) at the University of Rochester (Rochester, NY) on slit-scanning flow systems, leading to the development of progressively more elaborate apparatus for processing pulse waveforms and for imaging cells in flow, intended for use in cancer cytology. Digital pulse processing was used in bench-top instruments produced in the early 1990s by RATCOM (Miami, FL) (58), but, because analog-to-digital converters (ADCs) with sufficient speed and resolution were not available, neither these systems nor those built at Rochester could achieve a large enough dynamic range to eliminate the need for the logarithmic amplifiers that became commonplace in flow cytometers during the 1980s.

An arc source instrument first described by Lindmo and Steen in 1979 (59,60) observed cells in sheath flow after a jet in air intersected the flat surface of a cover slip, making multiangle scatter and fluorescence measurements with sufficient sensitivity to characterize bacteria (61). An early commercial version of this apparatus was produced by Leitz; a later version was made by Skatron (Lier, Norway), and an even later one, formerly available from Bio-Rad (Hercules, CA) as the Bryte HS™, is now being produced by Apogee (Northwood, Middlesex) in the U.K.

Flow Cytometer Domestication: From Behemoths to Bench-tops

Relatively small arc source systems such as the Impulscytometer and the Bryte HS managed to achieve reasonably high measurement precision and sensitivity, due at least in part to their use of high-numerical aperture (NA) microscope optics, which provided more efficient light collection than was available in laser source flow cytometers. In the mid and late 1970s, Kamensky's Bio/Physics Systems and its successor, Ortho Diagnostics Systems (Westwood, MA), introduced flow cytometers and sorters in which measurements were made in flat-sided quartz flow cuvettes, and in which "high-dry" microscope objectives were used to increase light collection. This made it possible to use air-cooled rather than water-cooled lasers for immunofluorescence measurements, decreasing the size, cost, and power consumption of instruments and making them easier to introduce into an emerging clinical market. Ortho's Spectrum III, a highly automated system, without sorting, was aimed at clinical users, as were B-D's FACS analyzer, a small but sensitive analytical apparatus employing an arc lamp source, and Coulter's EPICS C (62),

an ergonomically designed, computer-controlled "knobless" instrument that included sorting capability. By the mid-1980s, with the emergence of AIDS, increasing demand for clinical instruments, B-D had brought out the FACScan, a three-color bench-top analyzer using a rectangular cuvette with a gel-coupled lens for highly efficient light collection, which could make more sensitive immunofluorescence measurements using a 15 mW air-cooled argon laser source than were possible using ten times more laser power in stream-in-air sorters. The FACScan was followed by the FACSsort, which included a relatively slow fluidic sorter; both were succeeded by the FACSCalibur, which offered both a fluidic sorting option and a fourth fluorescence channel with excitation from a red (635–640 nm) diode laser.

From the mid-1990s on, there has been a proliferation of diode and solid-state lasers, and these small, energy-efficient, and (usually) relatively inexpensive sources have increasingly been incorporated into flow cytometers. The use of violet (395–415 nm) diode lasers in cytometry was first described at the 2000 ISAC meeting (63); by the 2002 meeting, at least three manufacturers had incorporated such sources into instruments. Frequency-doubled diode-pumped YAG lasers, emitting green light at 532 nm, have also come into use, as have doubled semiconductor lasers emitting at 488–492 nm, now available as replacements for both air- and water-cooled argon lasers. Both diode and solid-state lasers are now available as ultraviolet (350–375 nm) sources.

Although the instruments Kamensky built at IBM were computer controlled, computers were expensive options for most flow cytometers until the early 1980s, by which time microprocessor-based systems could do the work of an earlier generation of minicomputers. The FACScan was equipped with a desktop computer system built by Hewlett-Packard (Palo Alto, CA); within a few years, personal computer systems were integrated into almost all commercial flow cytometers, with B-D switching to Apple (Cupertino, CA) Macintosh systems for its product line and Coulter and Cytomation favoring IBM PC-compatible products and Microsoft (Redmond, WA) software, initially running under DOS and later under Windows. An increasing amount of the internal electronics of flow cytometers has become computer-based, with the latest systems incorporating special-purpose large-scale integrated circuits, microprocessors, microcontrollers, and digital signal processing chips.

The development of digital audio, telephony, and video has resulted in large increases in the performance, and decreases in the price, of ADCs, which are critical elements in data acquisition systems for any type of instrumentation, flow cytometers included. The ADCs originally used with flow cytometers had only 8- or 10-bit resolution, making it necessary to use logarithmic amplifiers to process signals with a large dynamic range. This necessitated the use of hardware for fluorescence compensation. While this approach is feasible when three or four colors are measured, it is essentially impossible to implement for modern multibeam instruments in which measurements

of 12 or more colors may be made. The alternative is software compensation (63), which is best applied to linear data digitized to a resolution of at least 16 bits. In the early 1990s, Auer et al. (64) implemented software compensation in the Beckman Coulter EPICS XL analyzer, which captures 20-bit linear data, eliminating the need for logarithmic amplifiers. Other manufacturers, e.g., BD Biosciences, DakoCytomation, and Partec, have developed their own approaches to high-resolution digital data analysis, and we can expect that, as has been the case for audio and video, digital techniques in flow cytometry will drive their predecessors to extinction.

FUTURE DIRECTIONS IN CYTOMETRY

Speaking of extinction, we may see renewed interest in this measurement parameter in the near future. Extinction measurements, which were favored for cell sizing in older Ortho flow cytometers, require low-noise light sources; air-cooled argon and helium-neon lasers are too noisy, but diode and solid-state lasers (and light emitting diodes [LEDs]) can readily meet the requirements.

With the introduction of the FACSAria sorter in late 2002, BD Biosciences successfully hybridized the behemoth high-speed cell sorter and the bench-top analyzer; other manufacturers will almost certainly be demonstrating similar progeny at the 2004 ISAC meeting. Most hybrids are sterile, but this should be an advantage as far as cell sorters are concerned.

We can expect flow cytometers to continue to decrease in size and energy consumption, and hope that they will eventually decrease in cost. It is widely known that flow cytometry makes it possible to analyze and sort over 100,000 cells per second, to identify rare cells that represent only one of every 10 million cells in mixed populations, to simultaneously measure light scattering at two or three angles and fluorescence in 12 or more spectral regions, to measure fluorescence with a precision better than one percent, and to detect and quantify a few hundred molecules of fluorescent antibody bound to a cell surface. It is less widely appreciated that it may be difficult or impossible to accomplish two or three of these amazing feats at once, but it is anticipated that newer systems will be taught new tricks.

Slow flow systems, with millisecond rather than microsecond observation times, have already been used to detect single fluorescent molecules (65), and, if they are ever made available to the right users, may prove useful for high-precision analysis of bacteria (66), viruses, and macromolecules (67,68). The microfluidic technology incorporated in some of these systems (66,68) makes it possible to stop and reverse flow. Large-scale microfluidic integration (69) should allow thousands of individual cells to be placed on an individual "chip," subjected to various manipulations, and studied over time. Looking at the same cells over time is a tall order for a flow cytometer, but is easily accomplished using instruments ranging down in complexity and cost from multiphoton confocal microscopes through laser scanning cytometers, such as those developed by Kamensky's CompuCyte Corporation

(Cambridge, MA) (70-72), to simple fluorescence microscope cytometers using inexpensive charge-coupled device (CCD) detectors and even less expensive LED light sources.

The Society for Analytical Cytology was founded in 1978, and became the International Society for Analytical Cytology in 1991. In the interval, AIDS provided flow cytometry with what the computer people call a "killer application," in both figurative and literal senses. As ISAC passes its 25th Anniversary, it is gratifying to see at least some of its members working toward simple, inexpensive cytometric apparatus to aid in the fight against AIDS and other health problems in both developed and developing countries. May we thus provide "a light unto the nations," even if it is only at the milliwatt level.

LITERATURE CITED

1. Shapiro HM. Practical flow cytometry. 4th edition. Hoboken, NJ: Wiley-Liss; 2003. 681 p.
2. Ornstein L. Tenuous but contingent connections. *Electrophoresis* 1987;8:3-13.
3. Caspersson TO. Cell growth and cell function. New York: Norton; 1950, 185 pp.
4. Mellors RC, editor. Analytical cytology. 2nd edition. New York: McGraw-Hill; 1959, 534 pp.
5. Tolles WE. The cytoanalyzer: an example of physics in medical research. *Trans N Y Acad Sci* 1955;17:250-256.
6. Wied GL, Bahr GF, editors. Automated cell identification and cell sorting. New York: Academic Press; 1970, 403 pp.
7. Stein PG, Lipkin LE, Shapiro HM. Spectre II: general-purpose microscope input for a computer. *Science* 1969;166:328-333.
8. Ingram M, Preston K, Jr. Automatic analysis of blood cells. *Sci Am* 1970;223:72-78.
9. Shapiro HM, Bryan SD, Lipkin LE, Stein PG, Lemkin PF. Computer-aided microspectrophotometry of biological specimens. *Exp Cell Res* 1971;67:81-85.
10. Young JZ. A flying-spot microscope. *Nature* 1951;167:231.
11. Prewitt JMS, Mendelsohn ML. The analysis of cell images. *Ann NY Acad Sci* 1966;128:1035-1053.
12. Mendelsohn ML, editor. Automation of cytogenetics. Asilomar Workshop, November 30-December 2, 1975. CONF-751158. Livermore, CA: Lawrence Livermore Laboratory; 1976, 187 p.
13. Gucker FT, Jr, O'Konski CT, Pickard HB, Pitts JN, Jr. A photoelectric counter for colloidal particles. *J Am Chem Soc* 1947;69:2422-2431.
14. Crosland-Taylor PJ. A device for counting small particles suspended in fluid through a tube. *Nature* 1953;171:37-38.
15. Coulter WH. High speed automatic blood cell counter and cell size analyzer. *Proc Natl Electronics Conf* 1956;12:1034-1042.
16. Brecher G, Schneiderman M, Williams GZ. Evaluation of electronic red blood cell counter. *Am J Clin Pathol* 1956;26:1439-1449.
17. Mattern CFT, Brackett FS, Olson BJ. Determination of number and size of particles by electrical gating: blood cells. *J Appl Physiol* 1957;10:56-70.
18. Hallermann L, Thom R, Gerhartz H. Elektronische Differentialzählung von Granulocyten und Lymphocyten nach intravitaler Fluochromierung mit Acridinorange. *Verh Deutsch Ges Inn Med* 1964;70:217-219.
19. Kamensky LA. Cytology automation. *Adv Biol Med Phys* 1973;14:93-161.
20. Kamensky LA, Melamed MR, Derman H. Spectrophotometer: new instrument for ultrarapid cell analysis. *Science* 1965;150:630-631.
21. Kamensky LA, Melamed MR. Instrumentation for automated examinations of cellular specimens. *Proc IEEE* 1969;57:2007-2016.
22. Koenig SH, Brown RD, Kamensky LA, Sedlis A, Melamed MR. Efficacy of a rapid cell spectrophotometer in screening for cervical cancer. *Cancer* 1968;21:1019-1026.
23. Ornstein L, Ansley HR. Spectral matching of classical cytochemistry to automated cytology. *J Histochem Cytochem* 1974;22:453-469.
24. Mansberg HP, Saunders AM, Groner W. The Hemalog D white cell differential system. *J Histochem Cytochem* 1974;22:711-724.
25. Fulwyler MJ. Electronic separation of biological cells by volume. *Science* 1965;150:910-911.

26. Kamensky LA, Melamed MR. Spectrophotometric cell sorter. *Science* 1967;156:1364-1365.
27. Sweet RG. High frequency recording with electrostatically deflected ink jets. *Rev Sci Instrum* 1965;36:131-136.
28. Mullaney PF, Van Dilla MA, Coulter JR, Dean PN. Cell sizing: a light scattering photometer for rapid volume determination. *Rev Sci Instrum* 1969;40:1029-1032.
29. Steinkamp JA, Fulwyler MJ, Coulter JR, Hiebert RD, Horney JL, Mullaney PF. A new multiparameter separator for microscopic particles and biological cells. *Rev Sci Instrum* 1973;44:1301-1310.
30. Salzman GC, Crowell JM, Goad CA, Hansen KM, Hiebert RD, LaBauve PM, Martin JC, Ingram M, Mullaney PF. A flow-system multiangle light-scattering instrument for cell characterization. *Clin Chem* 1975;21:1297-1304.
31. Salzman GC, Crowell JM, Martin JC, Trujillo TT, Romero A, Mullaney PF, LaBauve PM. Cell identification by laser light scattering: identification and separation of unstained leukocytes. *Acta Cytol* 1975;19:374-377.
32. Van Dilla MA, Trujillo TT, Mullaney PF, Coulter JR. Cell microfluorometry: a method for rapid fluorescence measurement. *Science* 1969;163:1213-1214.
33. Dittrich W, Göhde W. Impulsfluorometrie bei Einzelzellen in Suspensionen. *Z Naturforsch* 1969;24b:360-361.
34. Herzenberg LA, Sweet RG, Herzenberg LA. Fluorescence activated cell sorting. *Sci Am* 1976;234:108-117.
35. Saunders AM, Hulett HR. Microfluorometry: comparison of single measurements to a rapid flow system. *J Histochem Cytochem* 1969;17:188.
36. Hulett HR, Bonner WA, Barrett J, Herzenberg LA. Automated separation of mammalian cells as a function of intracellular fluorescence. *Science* 1969;166:747-749.
37. Bonner WA, Hulett HR, Sweet RG, Herzenberg LA. Fluorescence activated cell sorting. *Rev Sci Instrum* 1972;43:404-409.
38. Curbelo R, Schildkraut ER, Hirschfeld T, Webb RH, Block MJ, Shapiro HM. A generalized machine for automated flow cytology system design. *J Histochem Cytochem* 1976;24:388-395.
39. Shapiro HM, Schildkraut ER, Curbelo R, Turner RB, Webb RH, Brown DC, Block MJ. Cytomat-R: a computer-controlled multiple laser source multiparameter flow cytophotometer system. *J Histochem Cytochem* 1977;25:836-844.
40. Shapiro HM. Fluorescent dyes for differential counts by flow cytometry: does histochemistry tell us much more than cell geometry? *J Histochem Cytochem* 1977;25:976-989.
41. Hercher M, Mueller W, Shapiro HM. Detection and discrimination of individual viruses by flow cytometry. *J Histochem Cytochem* 1979;27:350-352.
42. Loken MR, Parks DR, Herzenberg LA. Two-color immunofluorescence using a fluorescence-activated cell sorter. *J Histochem Cytochem* 1977;25:899-907.
43. Bacus JW, Gose EE. Leukocyte pattern recognition. *IEEE Trans Sys Man Cybernet* 1972;SMC-2:513-525.
44. Miller MN. Design and clinical results of HEMATRAK: an automated differential counter. *IEEE Trans Biomed Eng* 1976;BME-23:400-405.
45. Young IT. The classification of white blood cells. *IEEE Trans Biomed Eng* 1972;BME-19:291-298.
46. Schindler ML, Olinger MR, Holland JF. Automated analysis and survival selection of anchorage-dependent cells under normal growth conditions. *Cytometry* 1985;6:368-374.
47. Martin JC, Jett JH. Photodamage, a basis for super high speed cell selection. *Cytometry* 1981;2:114.
48. Shapiro HM. Apparatus and method for killing unwanted cells. United States Patent 4,395,397, 1983.
49. Herweijer H, Stokdijk W, Visser JWM. High speed photodamage cell selection using bromodeoxyuridine/Hoechst 33342 photosensitized cell killing. *Cytometry* 1988;9:143-149.
50. Inoué S, Spring KR. Video microscopy. The fundamentals. 2nd edition. New York: Plenum Press; 1997. 742 p.
51. Pawley JB, editor. Handbook of biological confocal microscopy. 2nd edition. New York: Plenum Press; 1995. 632 p.
52. Keating P, Cambrosio A. Biomedical platforms. realigning the normal and the pathological in late twentieth-century medicine. Cambridge, MA: MIT Press; 2003. 544 p.
53. Arndt-Jovin DJ, Jovin TM. Computer-controlled multiparameter analysis and sorting of cells and particles. *J Histochem Cytochem* 1974;22:622-625.
54. Bailey JE, Fazel-Madjlessi J, McQuitty DN, Lee YN, Allred JC, Oro JA. Characterization of bacterial growth by means of flow microfluorometry. *Science* 1977;198:1175-1176.
55. Peters D, Branscomb E, Dean P, Merrill T, Pintel D, Van Dilla M, Gray JW. The LLNL high-speed sorter: design features, operational characteristics, and biological utility. *Cytometry* 1985;6:290-301.
56. Gray JW, Dean PN, Fuscoe JC, Peters DC, Trask BJ, van den Engh GJ, Van Dilla MA. High-speed chromosome sorting. *Science* 1987;238:323-329.
57. Wheeless LL, Jr. Slit-Scanning. In: Melamed MR, Lindmo T, Mendelsohn ML, editors. Flow cytometry and sorting. 2nd edition. New York: John Wiley and Sons, Inc.; 1990. p 109-125.
58. Cram LS. Spin-offs from the NASA space program for tumor diagnosis. *Cytometry* 2001;43:1.
59. Steen HB, Lindmo T. Flow cytometry: a high resolution instrument for everyone. *Science* 1979;204:403-404.
60. Steen HB. Further developments of a microscope-based flow cytometer: light scatter detection and excitation intensity compensation. *Cytometry* 1980;1:26-31.
61. Steen HB, Boye E, Skarstad K, Bloom B, Godal T, Mustafa S. Applications of flow cytometry on bacteria: cell cycle kinetics, drug effects, and quantitation of antibody binding. *Cytometry* 1982;2:249-257.
62. Leif SB, Leif RC, Auer R. The EPICS C analyzer: an ergonomically designed flow cytometer computer system. *Anal Quant Cytol Histol* 1985;7:187-191.
63. Shapiro HM, Perlmutter NG. Violet laser diodes as light sources for flow cytometry. *Cytometry* 2001;44:133-136.
64. Bagwell CB, Adams EG. Fluorescence spectral overlap compensation for any number of flow cytometry parameters. *Ann NY Acad Sci* 1993;677:167-184.
65. Nguyen DC, Keller RA, Jett JH, Martin JC. Detection of single molecules of phycoerythrin in hydrodynamically focused flows by laser-induced fluorescence. *Anal Chem* 1987;59:2158-2161.
66. Fu AY, Chou HP, Spence C, Arnold FH, Quake SR. An integrated microfabricated cell sorter. *Anal Chem* 2002;74:2451-2457.
67. Goodwin PM, Johnson ME, Martin JC, Ambrose WP, Marrone BL, Jett JH, Keller RA. Rapid sizing of individual fluorescently stained DNA fragments by flow cytometry. *Nucleic Acids Res* 1993;21:803-806.
68. Chou H-P, Spence C, Scherer A, Quake S. A microfabricated device for sizing and sorting DNA molecules. *Proc Natl Acad Sci USA* 1999;96:11-13.
69. Thorsen T, Maerkl SJ, Quake SR. Microfluidic large scale integration. *Science* 2002;298:580-584.
70. Kamensky LA, Kamensky LD. Microscope-based multiparameter laser scanning cytometer yielding data comparable to flow cytometry data. *Cytometry* 1991;12:381-387.
71. Kamensky LA, Burger DE, Gershman RJ, Kamensky LD, Luther E. Slide-based laser scanning cytometry. *Acta Cytol* 1997;41:123-143.
72. Darzynkiewicz Z, Bedner E, Li X, Gorczyca W, Melamed MR. Laser-scanning cytometry: a new instrumentation with many applications. *Exp Cell Res* 1999;249:1-12.