

The Past as Prologue

Flow cytometry, like most scientific developments, has roots firmly grounded in history. In particular, flow technology finds intellectual antecedents in microscopy, blood cell counting instruments, and the inkjet technology that was, in the 1960s, being developed for computer printers. It was the coming together of these three strands of endeavor that provided the basis for the development of the first flow cytometers. Because thorough accounts of the history of flow cytometry have been written elsewhere (and make a fascinating story for those interested in the history of science), we cover history here in just enough detail to give readers a perspective as to why current instruments have developed as they have.

Microscopes have, since the 17th century, been used to examine tissue sections and cells. Particularly since the end of the 19th century, stains have been developed that make various cellular constituents visible; in the 1940s and 1950s, fluorescence microscopy began to be used in conjunction with fluorescent stains for nucleic acids in order to detect malignant cells. With the advent of antibody technology and the work of Albert Coons in linking antibodies with fluorescent tags, the use of fluorescent stains gained wider and more specific applications. In particular, cell suspensions or tissue sections are now routinely stained with antibodies specific for antigenic markers of cell

Flow Cytometry: First Principles, Third Edition. Alice Longobardi Givan and Paul K. Wallace.

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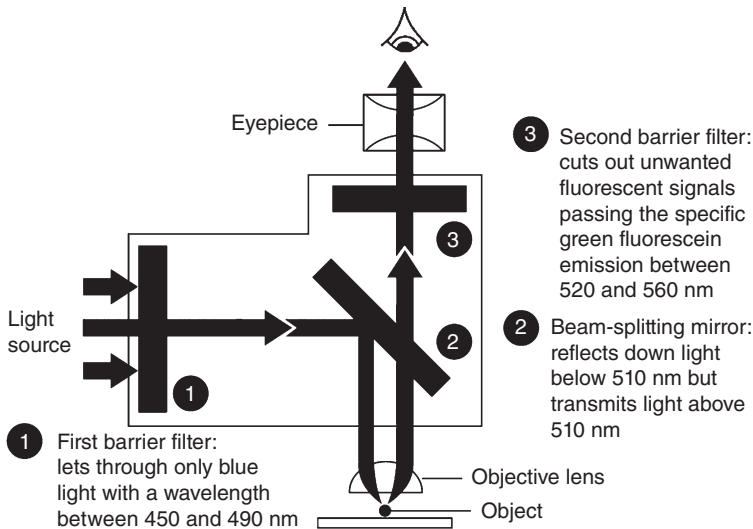


Fig. 1.1. The optical path of a fluorescence microscope. In this example, the filters and mirrors are set for detection of fluorescein fluorescence. Modified from Alberts et al. (1989).

type or function. The antibodies are either directly or indirectly conjugated to fluorescent molecules (most commonly fluorescein). The cellular material can then be examined on a glass slide under a microscope, and the fluorescence of the cells can be excited and observed (Fig. 1.1). The fluorescence microscope allows us to see cells, identify them in terms of both their physical structure and their orientation within tissues, and then determine whether and in what pattern the cells fluoresce when stained with one or another of the specific stains available. In addition, a microscope can also be fitted with a camera or photodetector, which will then record the intensity of fluorescence arising from the field in view. The logical extension of this technique is image analysis cytometry, which digitizes the output to allow precise quantitation of fluorescence intensity patterns in detail (pixel by pixel) within that field of view.

The development of hybridoma cultures for the production of monoclonal antibodies (for which César Milstein and Georges J.F. Köhler were awarded the Nobel Prize in 1984) led to a vast increase in the number of cellular components that can be specifically stained and used to classify cells. Whereas monoclonal antibody techniques are not

directly related to the development of flow technology, their invention was a serendipitous event that had a great impact on the potential utility of flow cytometric systems.

In 1934, Andrew Moldavan in Montreal took a first step from static microscopy toward a flowing system. He suggested the development of an apparatus to count red blood cells and neutral-red-stained yeast cells as they were forced through a capillary on a microscope stage. A photodetector attached to the microscope eyepiece would register each passing cell. Although it is unclear from Moldavan's paper whether he actually ever built this cytometer, the development of staining procedures over the next 30 years made it obvious that the technique he suggested could be useful not simply for counting the number of cells but also for comparing their characteristics.

In the mid-1960s, Louis Kamensky took his background in optical character recognition and applied it to the problem of automated cervical cytology screening. He developed a microscope-based spectrophotometer (on the pattern of the one suggested by Moldavan) that measured and recorded ultraviolet absorption and the scatter of blue light ("as an alternative to mimicking the complex scanning methods of the human microscopist") from cells flowing "at rates exceeding 500 cells per second" past a microscope objective (Fig. 1.2). Then, in 1967, Kamensky and Myron Melamed elaborated this design into a sorting instrument that provided for the electronic actuation of a syringe to pull cells with high absorption/scatter ratios out of the flow stream. These "suspicious" cells could then be subjected to detailed microscopic analysis. In 1969, Wolfgang Dittrich and Wolfgang Göhde in Münster, Germany, described a flow chamber for a microscope-based system with which fluorescence intensity histograms could be generated based on the ethidium bromide fluorescence from the DNA of alcohol-fixed cells.

During this period of advances in flow microscopy, so-called Coulter technology had been developed by Wallace Coulter for analysis of blood cells. In the 1950s, instruments were produced that counted cells as they flowed in a liquid stream; analysis was based on the amount by which the cells increased electrical resistance as they displaced an isotonic saline solution while flowing through an orifice. Cells were thereby classified more or less on the basis of their volume because larger cells have greater electrical resistance. These Coulter counters soon became essential equipment in hospital hematology laboratories, allowing the rapid

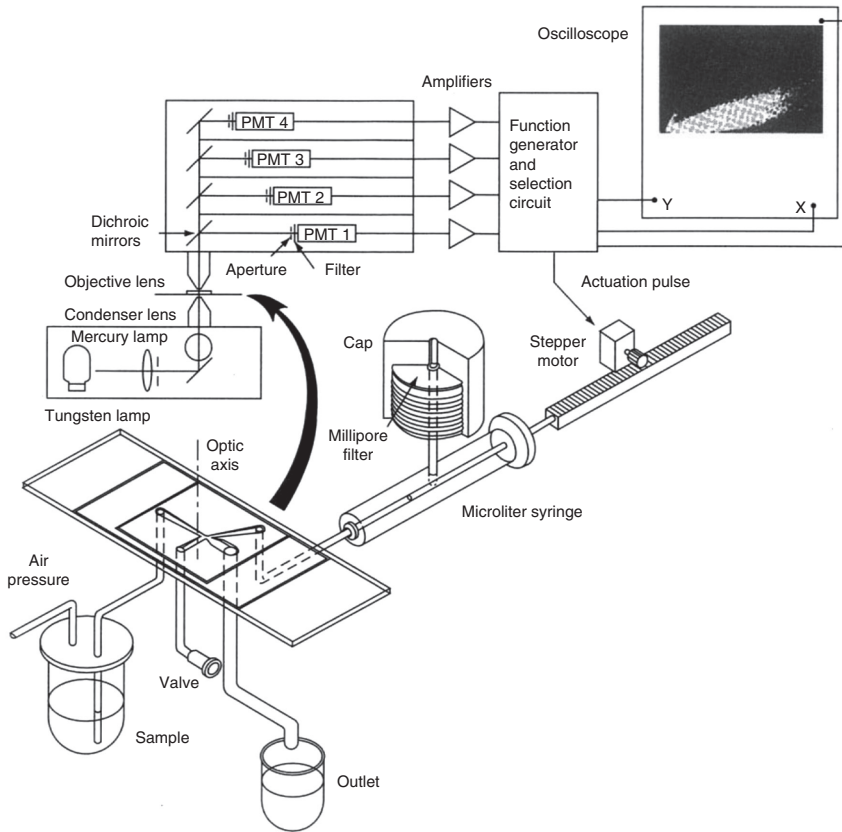


Fig. 1.2. A diagram of Kamentsky's original flow sorter. From Kamentsky and Melamed (1967)/American Association for the Advancement of Science - AAAS.

and automated counting of white and red blood cells. They actually incorporated many of the features of analysis that we now think of as being typical of flow cytometry: the rapid flow of single cells in file through an orifice, the electronic detection of signals from those cells, and the automated analysis of those signals.

At the same time as Kamentsky was working on cervical screening, Mack Fulwyler at the Los Alamos Laboratory in New Mexico was in need of a new project. He had been studying the contamination of food by radioactive fallout from nuclear bombs. This project had become less urgent because, in 1963, the United States, the Soviet

Union, and Great Britain signed the Nuclear Test Ban Treaty; this Treaty limited the testing of nuclear weapons under water, in the atmosphere, and in outer space. For a new project, Fulwyler decided to investigate an issue well known to everyone looking at red blood cells in Coulter counters: Red cells show a bimodal distribution of their electrical resistance (“Coulter volume”). Anyone looking at erythrocytes under the microscope cannot help but be impressed by the remarkable structural uniformity of these cells. Some biologists had hypothesized that the bimodal distribution of the electrical resistance of erythrocytes resulted from the existence of two classes of these cells. Fulwyler doubted that the bimodal Coulter volume distribution represented two classes of these microscopically very uniform cells. He felt that the bimodal profile might simply be an artifact based on some quirky aspect of the electronic resistance measurements. The most direct way of testing these two alternatives was to separate red blood cells according to their electronic resistance signals and then to determine whether the separated classes remained distinct (i.e., unimodal) when they were reanalyzed.

The technique that Fulwyler developed for sorting the erythrocytes combined Coulter methodology with the inkjet technology being developed at Stanford University by Richard G. Sweet for running computer printers. Inkjet technology involves the vibration of a nozzle so as to generate a stream that breaks up into discrete drops—followed by the charging and grounding of that stream at appropriate times so as to leave indicated drops, as they break off, carrying an electrical charge. For purposes of printing, those charged drops of ink can then be deflected to positions on the paper as required by computer print messages. Fulwyler took the intellectual leap of combining this methodology with Coulter flow technology; he developed an instrument that would charge drops containing suspended cells, thereby allowing deflection of the cells (within the drops) as dictated by signals based on the cell’s measured Coulter volume.

The data from this limited but pioneering experiment led to a conclusion that with hindsight seems obvious: Erythrocytes are indeed uniform. When they are sorted according to their electrical resistance, the resulting cells from one class or the other still show a bimodal distribution when reanalyzed for their electrical resistance profile. The bimodal “volume” signal from erythrocytes was therefore artifactual—resulting in part from the discoid (nonspherical) shape of the cells.

The technology developed for this landmark experiment is the essence of all the technology required for flow sorting as we now know it. That experiment also, unwittingly, emphasized an aspect of flow cytometry that has remained with us to this day: Flow cytometrists still need to be continually vigilant (and know how to use a microscope) because signals from cells (particularly signals that are assumed to be related to cell volume) are subject to artifactual influences and may not be what they seem. (Fulwyler's 1965 paper actually describes the separation of mouse from human erythrocytes and the separation of a large component from a population of mouse lymphoma cells; the experiments on the bimodal signals from red cells have been relegated to flow folk history.) It should also be noted that the Nuclear Test Ban Treaty, while intended to limit fallout, also indirectly resulted in the invention of flow cytometry.

In 1953, P.J. Crosland-Taylor, working at the Middlesex Hospital in London, noted that attempts to count small particles suspended in fluid flowing through a tube had not hitherto been very successful. With particles such as red blood cells, the experimenter must choose between a wide tube that allows particles to pass two or more abreast or a narrow tube that makes microscopical observation of the contents of the tube difficult due to the different refractive indices of the tube and the suspending fluid. In addition, narrow tubes tend to block easily. In response to this dilemma, Crosland-Taylor applied the principles of laminar flow to the design of a flow system. A suspension of red blood cells was injected into the center of a faster-flowing stream, thus allowing the cells to be aligned in a narrow central file within the core of the wider stream (preparatory to electronic counting). This principle of *hydrodynamic focusing* was pivotal for the further development of the field of flow cytometry.

Antibodies bind with great specificity to their antigens. Antibodies can be conjugated to fluorescent chemicals. These fluorescent antibodies can be used as stains that distinguish different classes of cells from each other according to their fluorescence. With the development of hybridomas that produce monoclonal antibodies, a large selection of antibodies became available to distinguish and classify different cells. If flow cytometers could be engineered to detect the fluorescence of cells (in addition to their scattered light), then they would be much more useful instruments. In 1969, Marvin Van Dilla and other members of the Los Alamos (New Mexico) Laboratory group reported

development of the first fluorescence-detection cytometer that utilized the principle of hydrodynamic focusing and, unlike microscope-based systems, had the axes of flow, illumination, and detection all orthogonal to each other; it also used an argon ion laser as a light source (Fig. 1.3). Indeed, the configuration of this instrument provided a framework that could support both the illumination and detection electronics of Kamentsky's device as well as the rapid flow and vibrating fluid jet of Fulwyler's sorter. In Van Dilla's initial report, the instrument was used for the detection of fluorescence from the Feulgen-DNA staining of Chinese hamster ovary cells and of leukocytes (as well as detection of their Coulter volume). The authors also "anticipated that extension of this method is possible and of potential value." Indeed, shortly thereafter, the Leonard Herzenberg group at Stanford University in California published a paper demonstrating the use of a similar cytometer to sort mouse spleen and Chinese hamster ovary cells on the basis of their fluorescence (due to accumulation of fluorescein diacetate). These instruments thus led to systems for combining multiparameter fluorescence, light scatter, and "Coulter volume" detection with cell sorting.

These sorting cytometers began to be used to look at ways of distinguishing and separating white blood cells. By the end of the 1960s,

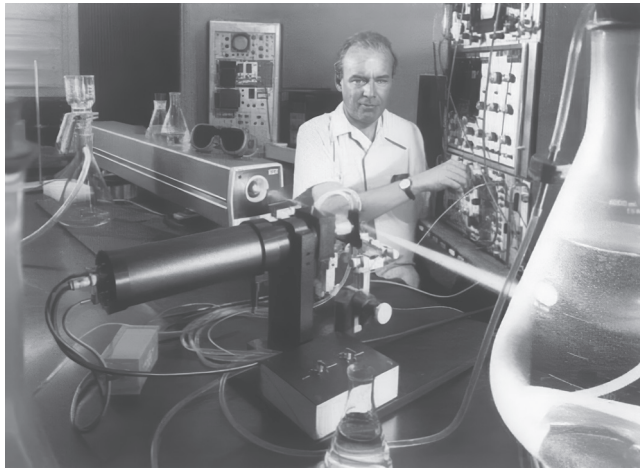


Fig. 1.3. Marvin Van Dilla and the Livermore flow sorter in 1973. Lawrence Livermore National Laboratory / Public Domain.

they were able to sort lymphocytes and granulocytes into highly purified states. The remaining history of flow cytometry involves the elaboration of this technology, the exploitation of flow cytometers for varied applications, and the collaboration between scientists and industry for the commercial production (beginning in the 1970s) of cytometers as user-friendly tools (Figs. 1.4–1.6). At the same time, as these instruments began to be seen as commercially-marketable products, research and development continued at medical technology companies, small “start-ups,” government laboratories, and academic institutions around the world. At these centers, innovative instruments continue to indicate the leading edge of flow technology. At the present time, this technology is moving simultaneously in two directions: On the one hand, increasingly sophisticated instruments are being developed that can measure and analyze more aspects of more varied types of particles more and more sensitively and that can sort particles on the basis of these aspects at faster and faster rates. On the other hand, a different type of sophistication has streamlined instruments (Fig. 1.7) so that they have become user-friendly and essential equipment on many laboratory benches.

One further note on the history of flow cytometry: When we were in the process of writing this chapter—and wanting to give credit where

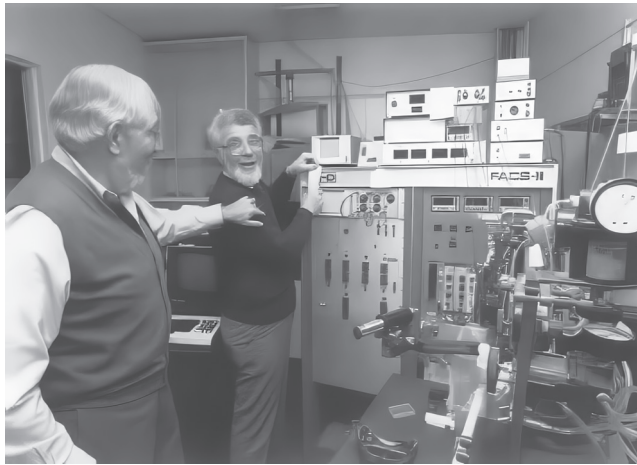


Fig. 1.4. Bernard Shoor (left) and Leonard Herzenberg at Stanford University with one of the original Becton Dickinson flow cytometers as it was packed for shipment to the Smithsonian Museum. Courtesy of Edward Souza, Stanford News Service.



Fig. 1.5. Paul Wallace (left) and Bob Frescatore running patient AIDS samples on a Beckman Coulter EPICS V in 1984.



Fig. 1.6. Alice Givan operating a Becton Dickinson FACS 420 c.1985.

credit is due—we were startled to come across an obituary in the *New York Times* dated 27 October 2021, with the headline “Mihaly Csikszentmihalyi, the Father of ‘Flow,’ Dies at 87.” How had we missed the person who seemed from the headline to have been an essential



Fig. 1.7. Six user-friendly benchtop cytometers (in alphabetical order). Top: Beckman Coulter CytoFLEX LX, BD Bioscience FACS Lyric; middle: Bio-Rad ZE5, Cytek Aurora; bottom: Miltenyi MACSQuant, Thermo Fisher Attune NxT. Beckman Coulter, Becton, Dickinson and Company, Bio-Rad Laboratories, Inc, Cytek Biosciences, MBC Biolabs, Thermo Fisher Scientific Inc.

contributor to the development of flow cytometers? As it turns out, “flow” has more than one meaning. In the case of Dr. Csikszentmihalyi, as stated in the *New York Times* obituary, flow “was a state of mind, a level of concentration in which outside stimuli, even time itself, seem to fall away.” In 1990, he wrote a book entitled “*Flow: The Psychology of Optimal Experience.*” This book has been praised by U.S. President Bill Clinton, U.K. Prime Minister Tony Blair, and Jimmy Johnson, the coach

of the Dallas Cowboys. If the book has been useful to presidents, prime ministers, and football coaches, it might indeed be useful to those of us who are concerned with the other kind of flow.

FURTHER READING

Throughout this book, the “Further Reading” references at the end of each chapter, while not exhaustive, are intended to point the way into the specific literature related to the chapter in question. At the end of the book, “Selected General Resources” directs readers to globally useful literature. Titles in bold at the end of each chapter are texts that are fully cited in the General Resources at the end.

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Chapter 1 in **Melamed et al.** and Chapter 3 in **Shapiro** are good historical reviews of flow cytometry. Alberto Cambrosio and Peter Keating (2000) have used flow cytometry as a model for looking at historical changes in the way scientists use instrumentation to view the world: “Of lymphocytes and pixels: The techno-visual production of cell populations.” *Studies in History and Philosophy of Biological and Biomedical Sciences* 31: 233–270.