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I magine sitting in a dark room for hours staring into a fluorescence microscope manually counting green cells in a field, changing the filter pack, and now recounting red cells in the field. And then moving the field and repeating the process, again, again, and again to score at least a hundred cells. In a complex cell population, such as bone marrow, was that cell positive? Or did it just have higher autofluorescence? Should I score that cell as negative, +, ++, +++, or ++++? The eye is a sensitive light detector, but very poor at quantitation of relative intensity. That was life before FACS.

Los Alamos National Laboratory is best known for creating the atomic bomb, but it is also the birthplace of flow cytometry and cell sorting. Using the technology that Dick Sweet (1) at Stanford University had invented to develop the ink jet printer, Mack Fulwyler (2) at Los Alamos modified a Coulter counter to sort cells based on differences in volume. Wallace Coulter had previously developed an instrument, routinely used in clinical laboratories, to rapidly count cells or particles that were discriminated on the basis of electrical resistance, which was proportional to their volume, as they passed through a detector in a liquid stream. In clinical laboratories, this allows accurate counting of platelets, erythrocytes, and leukocytes. By incorporating a piezocrystal that broke the stream into droplets and by applying an electrical charge to the droplets, it was possible to sort the cells, left and right, at a rate of up to 1000 per second and collect them as they exited the nozzle (2). Learning of this development, Leonard (Len) Herzenberg, a geneticist and immunologist who had himself spent too many hours in a dark room counting immunofluorescence-stained cells, assembled an outstanding team of engineers at Stanford University to replicate Fulwyler's cell sorter, but with an important improvement: the addition of a light source, a mercury arc lamp, and photomultiplier tube detectors so that cells stained with fluorochrome-coupled Abs or ligands could be identified based on their fluorescence emission rather than just cell volume. Thus was born the instrument Len named the "fluorescence-activated cell sorter" or FACS.

As a preface to his 1969 *Science* paper (reprinted here and comprised of one figure, one table, and no supplementary figures) describing this new technology (3), he noted, "Separation of large numbers of functionally different cell types from the complex mixtures found in such organs as spleen, bone marrow, lymph nodes, liver, or kidney would be useful in biological and biochemical investigations." As proof of concept, splenocytes from mice that were immunized with sheep erythrocytes were mixed with Chinese hamster ovary (CHO) cells and the cells were labeled with fluorescein diacetate, a dye that penetrates intact cell membranes and, after cleavage by intracellular esterases, covalently labels proteins. Based on the differential uptake and hence distinct fluorescent emission of the CHO cells and splenocytes, the cells were sorted and assayed for their ability to secrete Abs using a "Jerne plaque assay" (4). This technique involved adding splenocytes from an immunized mouse to a lawn of sheep erythrocytes in agarose, incubating, and then adding rabbit complement to lyse the RBCs that had been coated with Abs secreted by B cells in the splenic population, visualized as a clear zone in the lawn of RBCs surrounding the Ab-producing B cell. Cell sorting based on differential fluorescence had allowed for the separation of viable and functional Ab-producing splenocytes from CHO cells. Len concluded the paper with this sentence: "The possibility of substituting other fluorogenic substrates for [fluorescein diacetate] should be considered as well as the use of other fluorescent dyes and of fluorescent antibody techniques."

By 1972, the FACS had been updated to incorporate an argon ion laser for more efficient illumination, and Michael Julius, Tohru Masuda, and Len (5) now demonstrated that it was possible to sort Ag-specific B cells from the spleen of an immunized mouse using fluorescein-conjugated keyhole limpet hemocyanin (KLH). Enrichment of the Ag-specific B cells was on the order of 500-fold, but purity postsorting was only 40–52%, unacceptable by today's standards but remarkable in 1972! Importantly, Len and colleagues showed that when these B cells were adoptively transferred into irradiated congenic mice, the B cells were able to mount a recall response when challenged with KLH, but that the response was greatly enhanced when cells from the negative-sorted fraction of splenocytes or naive thymocytes were cotransferred. In the Discussion of their *Proceedings of the National Academy of Sciences of the United States of America* article (reprinted here) (5), they concluded that it seemed likely that naive thymocytes or "primed cooperators" (the term helper T cell had not yet been coined and CD Ags had not been discovered) were required for optimal production of Ab by B cells. They were intrigued by the finding that Ag-specific B cells could bind fluorescent-labeled KLH and be sorted, but that "the amount of antigen bound to cooperators (if any) is very small compared to the amount bound to precursors of antibody-forming cells," and that "Our data should not be taken as indication that T-cells do not bind antigen, but merely that there is not

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Abbreviations used in this article: CHO, Chinese hamster ovary; KLH, keyhole limpet hemocyanin.

enough antigen bound to T-cells to allow their deflection with the current sensitivity of the machine." Appreciate that this was before Zinkernagel and Doherty (6) discovered the concept of MHC restriction of T cells and long before we knew that T cells recognize MHC-bound peptide complexes rather than intact protein (7, 8). Len and colleagues further predicted, "our separation technique is specifically designed to detect and separate rare cells, and we ultimately hope to investigate the primary response by use of a virgin spleen-cell population enriched with antigen-binding cells." Today fluorochrome-labeled MHC–peptide tetrameric complexes and FACS are routinely used to quantitate and isolate T cells from both naive and immunized mice and humans (9).

Len realized that all immunologists and many other scientists would benefit greatly by making these technologies available to the general scientific community. Obtaining a National Institutes of Health grant to fund the project, Len convinced his friend and colleague Bernie Shoor, who directed a medical device division of Becton Dickinson (BD) nearby in Sunnyvale, CA, to commercialize the FACS II, and the instruments were delivered to Stanford and the National Institutes of Health in Bethesda, MD. The FACS II is now in the collection of the Smithsonian Institution. Bernie's initial estimate that the commercial market for FACS was perhaps a few dozen machines worldwide turned out to be off by many logs.

After completing my graduate studies, spending too many hours in a dark room counting fluorescent cells under a microscope and having read Len's seminal papers, I headed to ground zero of flow cytometry: for a postdoctoral fellowship at the University of New Mexico and the Los Alamos Laboratory with Noel Warner, who was a prior postdoctoral scholar of Len Herzenberg. Using the newly minted FACS III and the home-built machine at Los Alamos, I learned the power and joy of using flow cytometry to explore the remarkable complexity of the immune system. The FACS III had no computer. We documented our results by taking Polaroid photographs of the dots on the oscilloscope screen and recorded percentages by directly manipulating the knobs on the FACS to count the events above a threshold based on running control-stained cells before the stained samples.

During this time, Len realized that a significant limitation to flow cytometry was the availability of high-quality, standardized staining reagents. Polyclonal Abs had the disadvantage of variability between lots, and the complex mixture of Abs was reactive and cross-reactive with numerous epitopes and Ags. Again, Len perceived a need and found a solution by taking a sabbatical year at Cambridge University in the laboratory of César Milstein, who had recently invented hybridoma technology (10). Mastering the technique and generating some of the first mAbs against mouse B and T cells (11), Len returned to Stanford and convinced Bernie Shoor to establish the BD Monoclonal Center, marrying the FACS with a source of reliable and reproducible reagents. Innovations and improvements of the FACS continued in the laboratory of Len and Lee

Herzenberg and their dedicated and skilled team of engineers, computer scientists, chemists, and cell biologists. Computers were designed to handle the multiparameter data, amplifiers were converted from linear to log to accommodate the huge dynamic range of fluorescent signals, lasers and fluorescent detectors were added to permit the simultaneous excitation and emission of more dyes, compensation networks were devised to correct for spectral overlap, and new fluorescent reagents were developed to allow for identification of new cell populations in the immune systems of mice and humans.

I joined the BD Monoclonal Center as a junior scientist in 1981 and with my then girlfriend, now wife, Linda Lloyd (who worked with Len at Stanford) was adopted into the Herzenberg extended scientific family. It has been remarkable to experience the impact of the FACS on so many disciplines, from the initial separation of mouse spleen cells from CHO cells to applications in medical diagnostics, AIDS research, vaccine development, stem cell research, transplantation, cell biology, and microbial and plant biology. Today when we routinely use FACS—with up to 20-parameter detection (12)—I am amazed at the scientific discoveries made possible by flow cytometry. Similar to another visionary from Silicon Valley, Steve Jobs, Len Herzenberg changed the world. Borrowing a line from detective Joe Friday, it is "just the FACS" that Len's monumental contributions revolutionized the biological and medical sciences.

Disclosures

The author has no financial conflicts of interest.

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