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The History and Future of the Fluorescence Activated Cell Sorter and Flow Cytometry: A View from Stanford

Leonard A. Herzenberg,[1*] David Parks,[1] Bita Sahaf,[1] Omar Perez,[2] Mario Roederer[3] and Leonore A. Herzenberg[1]

The Fluorescence Activated Cell Sorter (FACS) was invented in the late 1960s by Bonner, Sweet, Hulett, Herzenberg, and others to do flow cytometry and cell sorting of viable cells. Becton Dickinson Immunocytometry Systems introduced the commercial machines in the early 1970s, using the Stanford patent and expertise supplied by the Herzenberg Laboratory and a Becton Dickinson engineering group under Bernie Shoor. Over the years, we have increased the number of measured FACS dimensions (parameters) and the speed of sorting to where we now simultaneously measure 12 fluorescent colors plus 2 scatter parameters. In this history, I illustrate the great utility of this state-of-the-art instrument, which allows us to simultaneously stain, analyze, and then sort cells from small samples of human blood cells from AIDS patients, infants, stem cell transplant patients, and others. I also illustrate analysis and sorting of multiple subpopulations of lymphocytes by use of 8–12 colors. In addition, I review single cell sorting used to clone and analyze hybridomas and discuss other applications of FACS developed over the past 30 years, as well as give our ideas on the future of FACS. These ideas are currently being implemented in new programs using the internet for data storage and analysis as well as developing new fluorochromes, e.g., green fluorescent protein and tandem dyes, with applications in such areas as apoptosis, gene expression, cytokine expression, cell biochemistry, redox regulation, and AIDS. Finally, I describe new FACS methods for measuring activated kinases and phosphatases and redox active enzymes in individual cells simultaneously with cell surface phenotyping. Thus, key functions can be studied in various subsets of cells without the need for prior sorting.

Preface

I want to thank the AACC for honoring me with the Ullman award and allowing me to present a lecture on the history and future of flow cytometry, which began when my colleagues Richard Sweet, Bill Bonner, Russ Hulett, and I invented the Fluorescence Activated Cell Sorter (FACS).[4] This honor acknowledges the now enormous field of flow cytometry and its many applications in immunology, cell biology, and molecular biology, and its clinical applications in AIDS, leukemia/lymphoma, and cancer detection and analysis, as well as clinical chemistry. Fittingly, the original support for work that led to the FACS came from the NIH under a grant program called "Automating the Clinical Laboratory" beginning in the late 1960s.

Introduction

I have attempted here to reproduce the Ullman Award lecture entitled "Past, Present and Future of Fluorescence Activated Cell Sorting (FACS)" that the AACC so graciously invited me to present.

In Fig. 1, I show a diagram of the FACS from our 1974 article in *Scientific American (1)*, which shows one laser and two light detectors, one for forward scatter (a measure of cell size) and one for fluorescence. The analysis

[1] Stanford University, Department of Genetics, Stanford, CA 94305.
[2] The Baxter Laboratory, Department of Microbiology and Immunology, Stanford University, Stanford, CA 94305.
[3] Vaccine Research Center, NIH, Bethesda, MD 20892.
*Author for correspondence. E-mail lenherz@darwin.stanford.edu.
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[4] Nonstandard abbreviations: FACS, fluorescence activated cell sorter; GFP, green fluorescent protein; EMA, ethidium monoazide; PBMC, peripheral blood mononuclear cell; GSH, glutathione; and GSB, glutathione *S*-bimane.

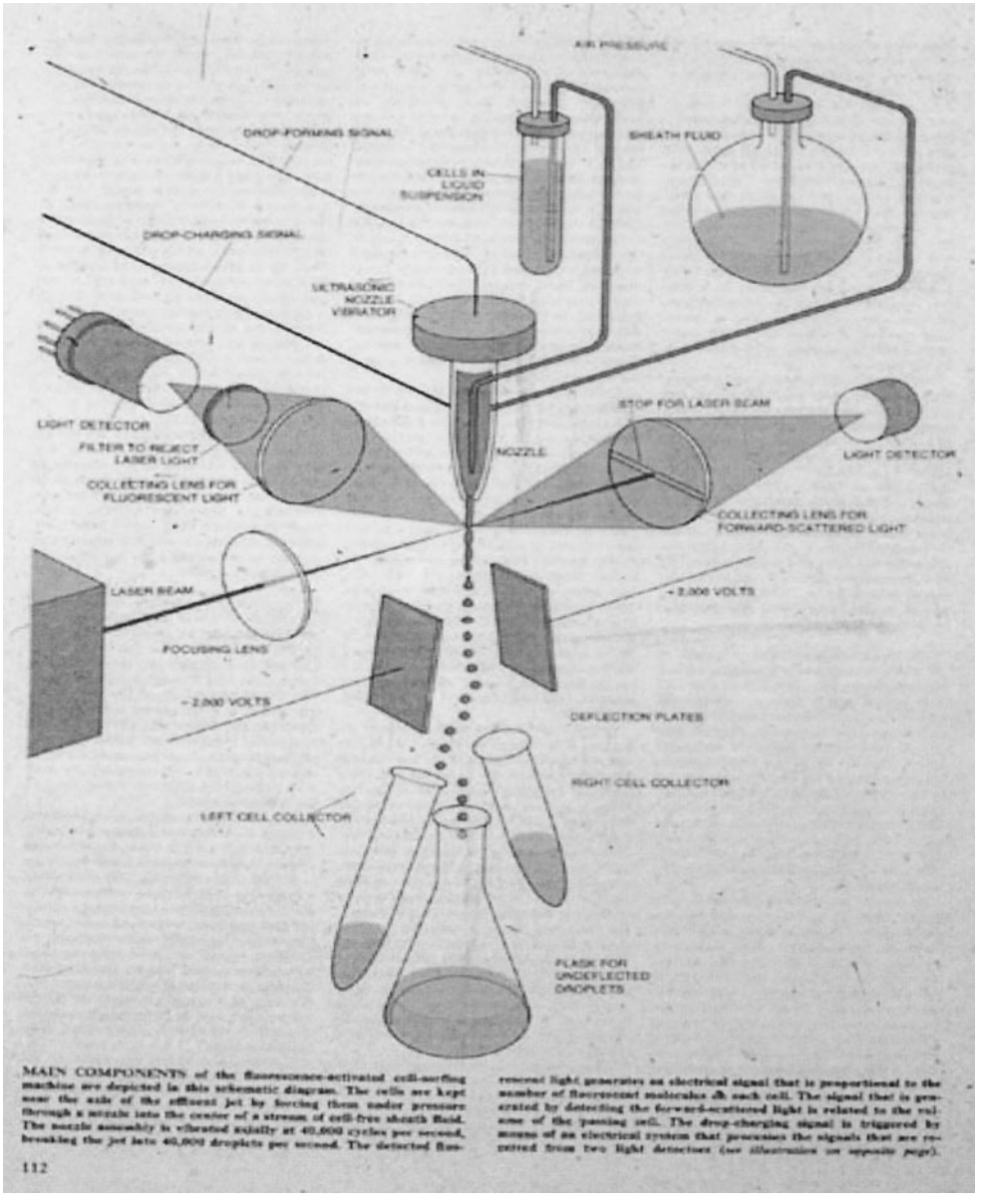

Fig. 1. Diagram of the FACS from our 1974 article in *Scientific American*, showing one laser and two light detectors, one for forward scatter (a measure of cell size) and one for fluorescence.

and sorting essentials of the FACS are unchanged to this day—quite unusual for a high-tech instrument, particularly one that is used in thousands of laboratories throughout the world. A recent estimate of the number of FACS instruments (generically defined) is 30 000 analyzers and sorters.

The Past

The ability to analyze and sort live cells was an important feature of our first machines. This made many of the applications developed in our laboratory and elsewhere possible. Particularly, it enabled determination of the functions of subsets of the cells that coexist in blood and various organs. I will show some illustrations of these studies later.

Shown in Fig. 2 is a diagram of a modern three-laser sorter with multiple detectors. I will show later that we now simultaneously detect 12 different fluorescent colors and have considerably increased the speed of analysis and sorting. Several of our collaborators have given different names to this modern sorter capability. We currently refer to it as "Hi-D FACS".

Stroboscopic images, photographed many years ago, of the stream and droplets coming from the FACS nozzle are shown in Fig. 3. Thousands of droplets are dramatically superimposed in each image, kept in place by the fantastic

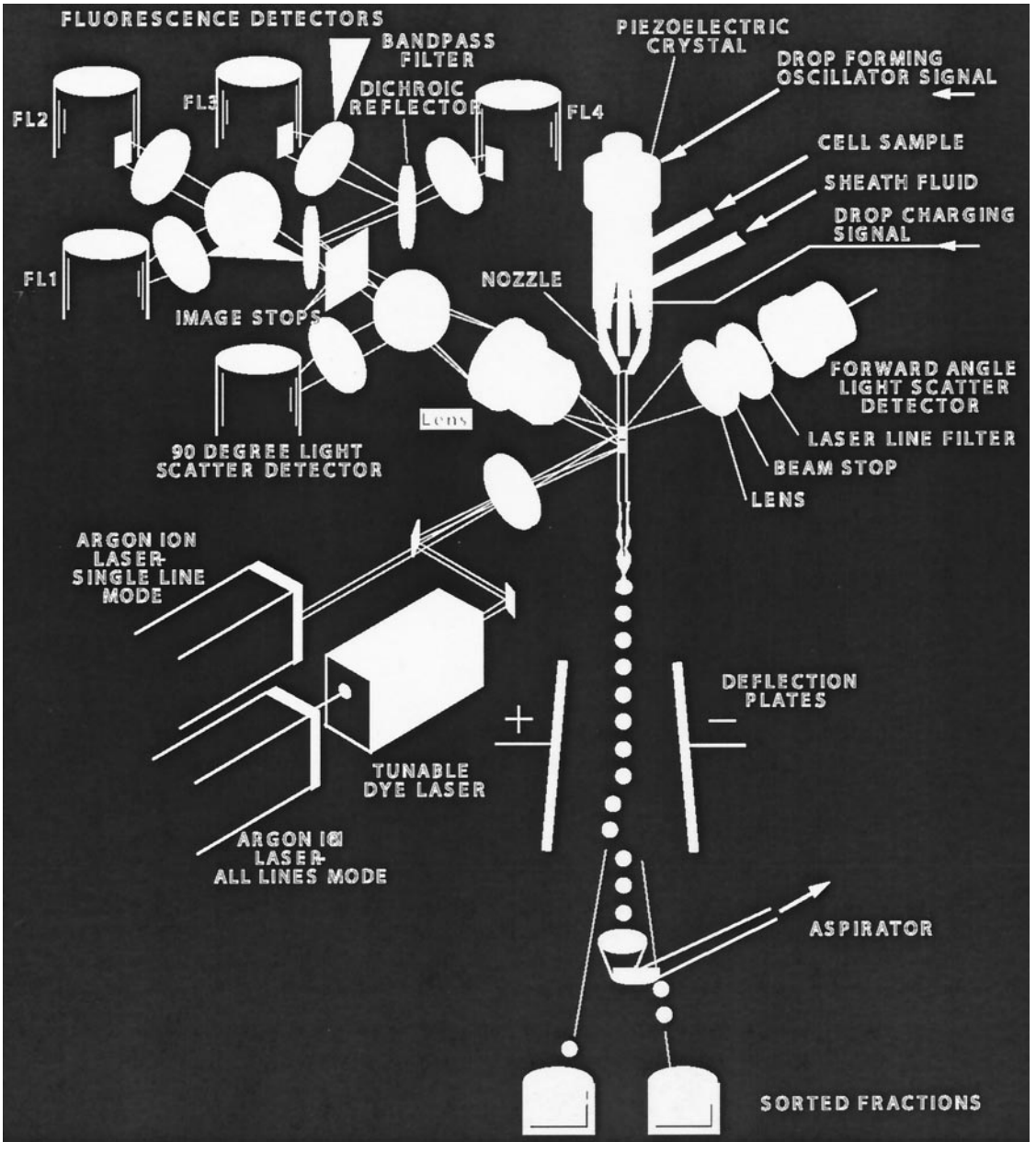

Fig. 2. Diagram of a modern three-laser sorter with three lasers and multiple detectors.

constancy of the stream, droplet generation, and droplet separation that characterizes the FACS operation. Individual cells, identified as they pass through the illumination/detection zone before the stream breaks up into droplets, are passively carried in only a minority of the droplets. Droplets containing cells that meet the sort criteria are charged at the moment of formation, and the charged droplets are sorted as they pass between constantly charged deflecting plates. This type of droplet separation was originally applied by Richard Sweet in his invention of the "ink-jet printer".

Shown in Fig. 4 is a beautiful old photo of laser beams under a nozzle and the deflection plates with an array of culture wells below.

More Fluorescence Colors

One of the early recognized needs was for more fluorescent colors so that we could use more reagents to simultaneously detect markers on cells. We started with two colors, fluorescein and rhodamine; later, Texas red was substituted for rhodamine. We found the first of the additional fluorescence colors in seaweeds off the local California coast. When you shine ultraviolet light on these seaweeds, you see a beautiful fluorescent picture (Fig. 5).

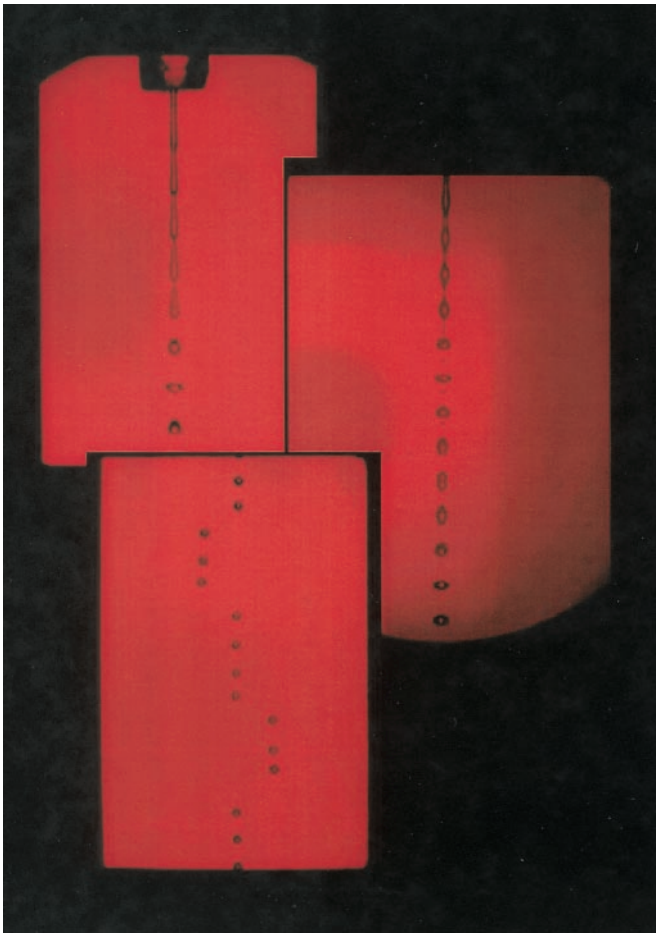

Fig. 3. Stroboscopic images, photographed many years ago, of the stream and droplets coming from the FACS nozzle.

In these time-lapse photographs, many droplets are superimposed, showing the constancy of drop formation.

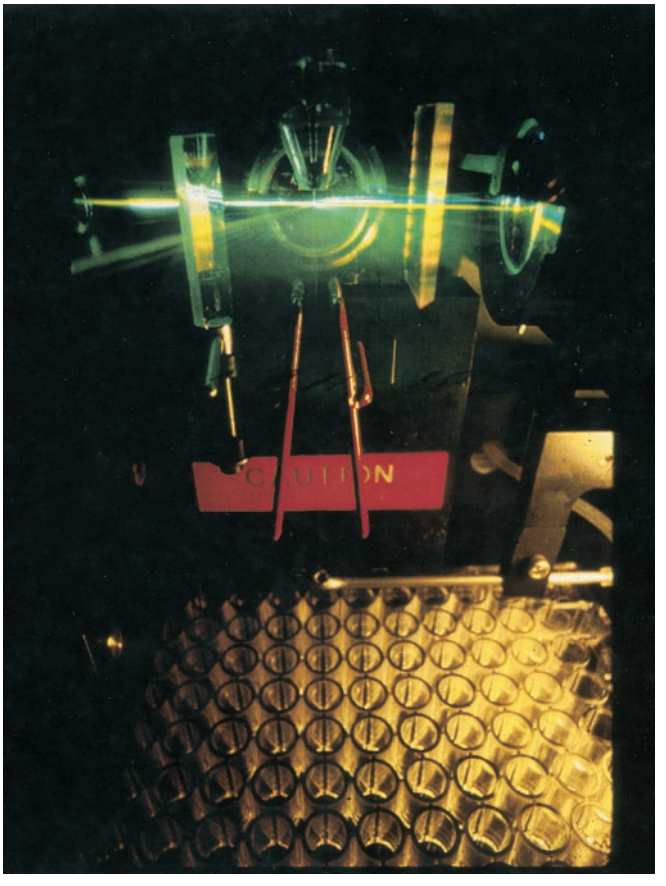

Fig. 4. Beautiful old photograph of laser beams under a nozzle and the deflection plates with an array of culture wells below.

We [Vernon Oi, Randy Hardy, and others *(2)*] separated the pigments by chromatography. The pigments are called phycobiliproteins. The patent for the phycobiliprotein uses in FACS and other applications was issued to Glaser, Oi, and Stryer *(3)*. Our laboratory pioneered the initial uses of these versatile and now widely used proteins in FACS studies.

Green fluorescent protein (GFP) is another example of a fluorescent protein that is widely used in FACS studies. GFP is made by the jelly fish *Aequoria victoria (4)* (Fig. 6) and is spontaneously fluorescent. The gene coding for GFP has been cloned and is now widely used as a reporter gene because the extent of its expression is readily measured by FACS.

We [Michael Anderson et al. *(5)*] mutated GFP to make two forms, which are excited at different wavelengths but emit at a common wavelength. In a multiple-laser FACS, in which the laser beams that excite each of the GFP forms intersect the cell stream one after the other, the signals emitted by each form are easily distinguished because they are directed to different detectors and arrive at different times. This effectively provides two reporter gene signals detectable from the same cell. Spectra of the

Fig. 5. Beautiful fluorescent picture produced when ultraviolet light is shown on seaweeds.

The phycobiliproteins are antenna molecules with tens of fluorochromes that transfer energy from wavelengths available underwater to chlorophyll so the plants can grow.

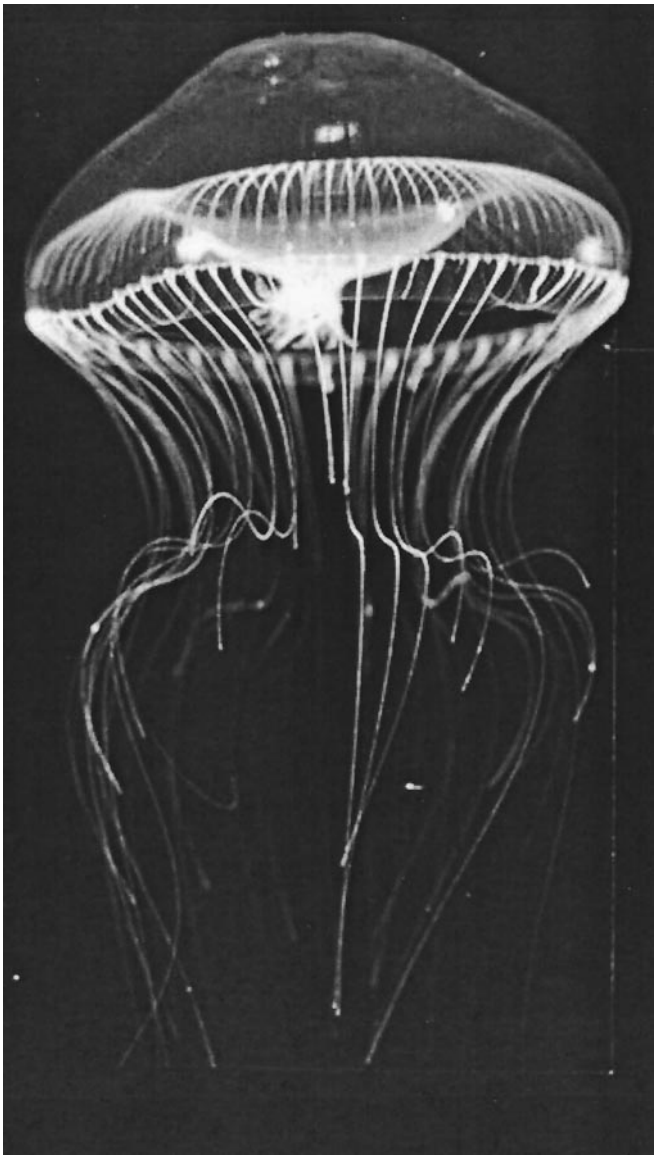

Fig. 6. GFP is made by the jellyfish *A. victoria* and is spontaneously fluorescent.

violet excited (Vex) and the blue excited (Bex) mutants, as well as wild-type GFP, are shown in Fig. 7.

FACS and Monoclonal Antibodies: Complementary Tools

The discovery that really made the FACS and flow cytometry a laboratory and clinical standard was the production of hybridomas by Kohler and Milstein *(6, 7)*. As is commonly known, hybridomas produce unlimited amounts of distinctive monoclonal antibodies, each of which is highly specific for its target antigens and can readily be coupled to fluorescein, phycobiliproteins, and other fluorochromes. The use of monoclonal antibodies as FACS reagents, which our laboratory pioneered, has enabled definition of hundreds of target antigens present on or in cells.

The FACS not only uses monoclonal antibodies pro-

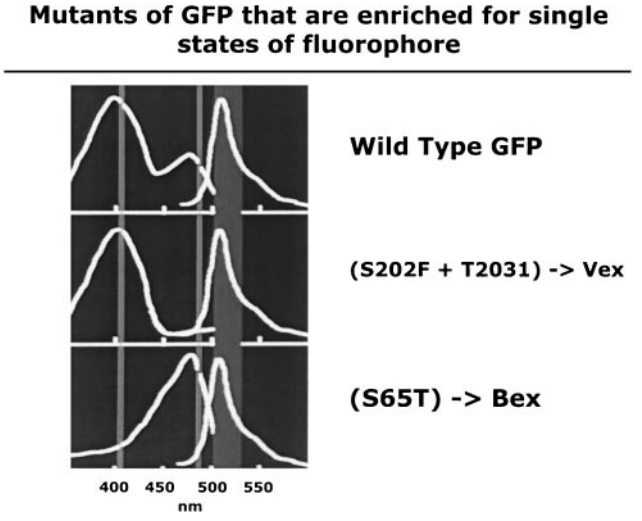

Fig. 7. Illustration of the violet excited (Vex) and the blue excited (Bex) mutants, as well as wild-type GFP.

duced by hybridomas, it is commonly used during hybridoma development to sort and clone individual cells from a hybridoma fusion. Single-cell sorting with the FACS is the most efficient way of cloning cells, especially when they are present at a very low frequency. In fact, many a hybridoma clone has been rescued by FACS from cultures where nonproducers were about to overgrow and a monoclonal antibody was about to be lost forever *(8)*.

The FACS Gal Reporter Gene System

An example of FACS use in functional assays is shown in Table 1. Some years ago, we (Garry Nolan and I) developed FACS Gal *(9)*, an intracellular β-galactosidase reporter gene assay system for studies of somatic cell genetics and gene regulation.

Hi-D FACS

With FACS Gal, phycobiliproteins, GFP, and monoclonal antibodies that could detect a wide variety of surface markers, we began to need and justify increasing the number of colors that could be simultaneously measured by FACS. Over the next few years, we published several reports on progressively increasing the number of colors and applying the progressively powerful FACS in new applications *(1, 5, 10–15)*.

Currently, we have a 12-color FACS that detects the colors shown in Fig. 8. Three lasers are needed to excite this many colors, indicated by the colored bars on the right for the krypton, argon, and dye laser, respectively. The different dyes are indicated on the left, whereas the excitation and emission spectra, as well as the laser line wavelengths, are given in the body of Fig. 8. The emission filters are indicated by the gray bars, which indicate both the central wavelength and the range of wavelengths passed by the collection filters. I summarize some of the advantages of using this instrument in Table 2 .

Table 1. FACS Gal.

- Quantifies gene expression in single cells. Data can be displayed as an expression distribution for a population
- Preserves cell viability throughout the procedure, allowing sorting and/or cloning of cells based on gene expression
- Provides a means of nonlethal genetic selection
- Has high sensitivity (as few as five molecules of enzyme per cell can be detected) with a wide dynamic range (to several million molecules per cell)
- Is compatible with multiparameter FACS analysis of surface phenotyping
- Is useful as a reporter gene in transgenic animal studies

Our present studies focusing on functional distinctions between subsets of human peripheral blood cells include studies of cytokine production by individual T-cell subsets, expression of activation markers, and apoptosis induction in such subsets *(16)*. Some of the methods we have developed for this work are outlined in Tables 3 and 4.

The importance of ethidium monoazide (EMA) staining for getting accurate cytokine measurements is illustrated in an example of CD4 cells making two cytokines (Fig. 9). Another example of the importance of EMA is staining for intracellular kinases, the protocol for which is given in Table 5. Fig. 10 shows color density and dot plots of intracellular staining, illustrating the importance of using EMA, which covalently and stably labels dead cells, to distinguish the dead cells before fixation and perme-

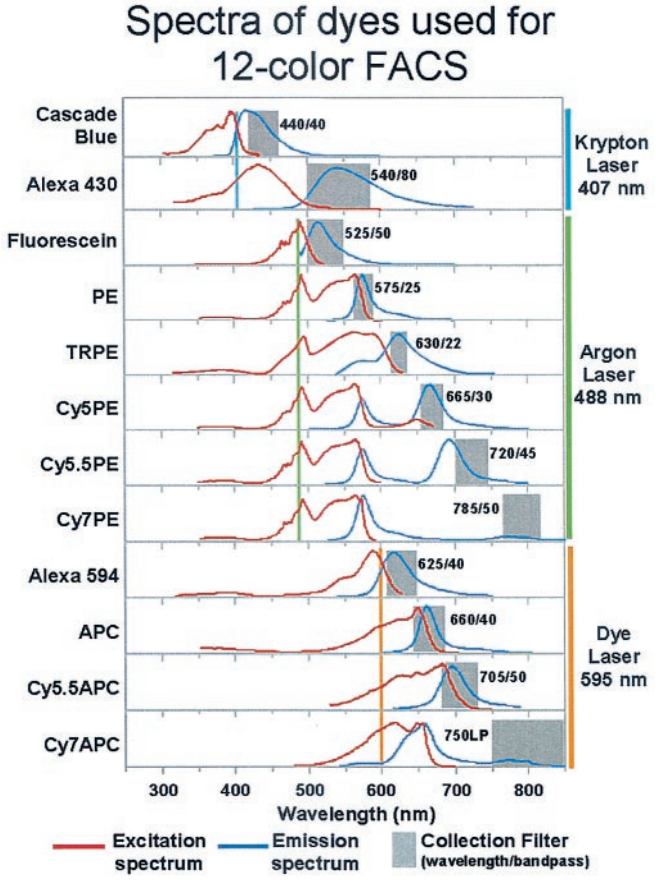

Fig. 8. Twelve-color FACS that detects the colors shown.

Three lasers are needed to excite this many colors, indicated by the *colored bars* on the *right* for the krypton, argon, and dye lasers, respectively. The different dyes are indicated on the *left*, whereas the excitation and emission spectra, as well as the laser line wavelengths, are given in the *body* of the figure. The emission filters are indicated by the *gray bars*, which indicate both the central wavelength and the range of wavelengths passed by the collection filters. *PE*, phycoerythrin; *TRPE*, Texas red phycoerythrin; *APC*, allophycocyanin.

Table 2. Hi-D FACS: Advantages of multiple fluorescence parameters.

- Economy of reagents and labor: combining multiple reagents reduces the number of total tests (tubes) and/or allows more tests per sample, e.g., with 12 colors, a leukemia/lymphoma panel can be done in 1–3 tubes instead of 10–20
- Information content increases more than geometrically with the number of parameters
- More parameters enable unambiguous identification of more subsets
- More reagents per sample reduces the number of separate stains and therefore the amount of sample required
- Subset identification can be combined with intracellular metabolic measurements, e.g., GSH, calcium, DNA, mitochondrial indices
  - Measurement of internal proteins, e.g., cytokines, enzymes (kinases, phosphatases, caspases), redox measurements (thioredoxin, TRR,[a] PDI).
  - Other surface molecules, e.g., surface thiols, other modifications on surface
- Detailed phenotyping of precious samples with small numbers of cells
  - Tumor infiltrating lymphocytes (TILs)
  - Residual tumor cells
  - Neonatal blood
  - Rare event analysis
  - Fetal cells in maternal blood
- Antigen-specific T or B cells

[a] TRR, thioredoxin reductase; PDI, protein disulfide isomerase.

Table 3. Analysis of intracellular cytokines.[a]

- Culture cells with antigen-specific or nonspecific (PMA[b]/ionomycin, CD3/CD28) stimuli
- After an appropriate stimulation period, culture cells with an inhibitor of Golgi transport (secretion) to retain newly synthesized cytokines in the cell
- Stain for surface markers
- Fix and permeabilize cells
- Stain for intracellular cytokines

[a] Very important: for accurately counting cells that are producing cytokines, it is crucial to eliminate cells that died before the staining steps are initiated. The procedure for doing this is described in Table 4 *(23)*.

[b] PMA, phorbol myristate acetate.

Table 4. Eliminating dead cells for accurate internal staining of cytokines.

- Cells are incubated with EMA, produced by Molecular Probes *(24)*
- EMA enters dead cells (membranes not intact) and intercalates into the DNA
- Exposure to light from a fluorescent lamp causes covalent linkage of EMA to DNA
- EMA fluorescence remains associated with the dead cells through washing, permeabilization, and antibody staining of cytokines
- EMA-stained cells are gated out before analysis of the FACS data

Table 5. Intracellular staining of kinases and activated kinases.[a]

- Stimulate and harvest cells
- Block Fc receptors
- Stain cell surface antigens
- Covalently label dead cells by EMA
- Fix and permeabilize cells
- Stain intracellular antigens

[a] The buffers are modified as in two reports by Perez and coworkers *(25, 26)*.

Table 6. The technical future of FACS.

- Improving detection capabilities
- Speeding up hardware
- Increasing software power
- Automation

Table 7. The future also includes . . . .

- Hi-D FACS kinase assays, which are powerful tools to assess signal transduction and functional capabilities at the single-cell level
- Biochemical access to rare cells: naïve/memory T cells, stem cells, patient-derived cells
- Clinical applications: pharmacodynamic monitoring, disease/activation state correlation
- High-throughput screening on a multiparameter platform
- Multidimensional assessment of cell signaling networks to develop a mechanistic understanding of cell function

abilization when staining for intracellular kinase activity. Fully 23% of the stimulated cells harvested for staining were dead, as judged by EMA staining. The lower left plot shows the percentages of cells in which three kinases are detected after gating out the dead cells.

Redox Studies by FACS

We now turn to some Hi-D studies that focus on redox, a major current interest of our laboratory. We have used Hi-D FACS to examine intracellular redox status in subsets of peripheral blood mononuclear cells (PBMCs) from individuals with HIV infection, inflammation, sepsis, and other disease *(16–20)*. In the first example, shown in Fig. 11, we measured intracellular glutathione (GSH) concentrations in CD4 T cells and other PBMC subsets in HIV-infected individuals. Results showed that GSH concentrations, detected by FACS as glutathione *S*-bimane (GSB), are correlated in PBMC subsets *(20)*.

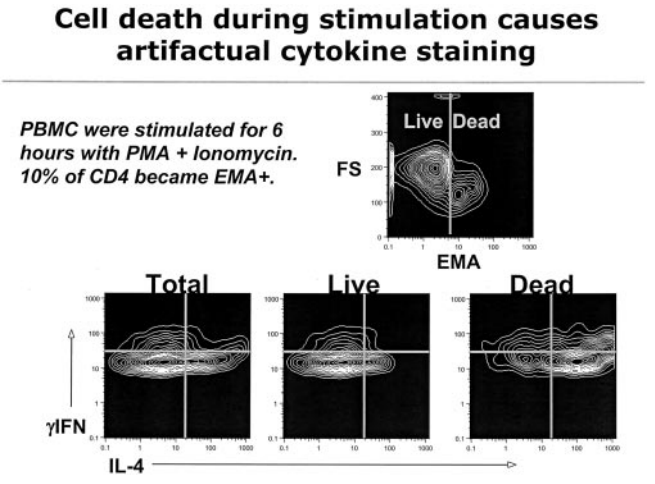

Fig. 9. The importance of EMA staining for getting accurate cytokine measurements in an example of CD4 cells making two cytokines.

PMA, phorbol myristate acetate; FS, forward scatter; *γIFN*, interferon-γ; *IL-4*, interleukin-4.

Two recent examples, not yet published, are shown in Figs. 12 and 13. In one, we used another fluorescent Molecular Probes reagent, Alexa594 maleimide (ALM), which covalently binds to −SH groups and thus provides a measure of cell surface thiols on viable cells. In the second, we used a monoclonal antibody to a well-known redox molecule, thioredoxin, to measure intracellular thioredoxin concentrations. Fig. 13 shows the positive correlation between surface thiols and intracellular thiore-

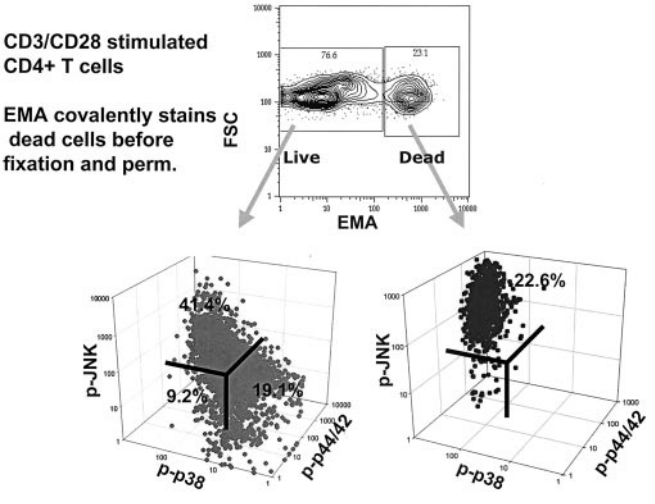

Fig. 10. Color density and plots of intracellular staining.

This figure illustrates the importance of using EMA, which covalently and stably labels dead cells, to distinguish them before fixation and permeabilization in staining procedures for intracellular kinase activities. *FSC*, forward scatter.

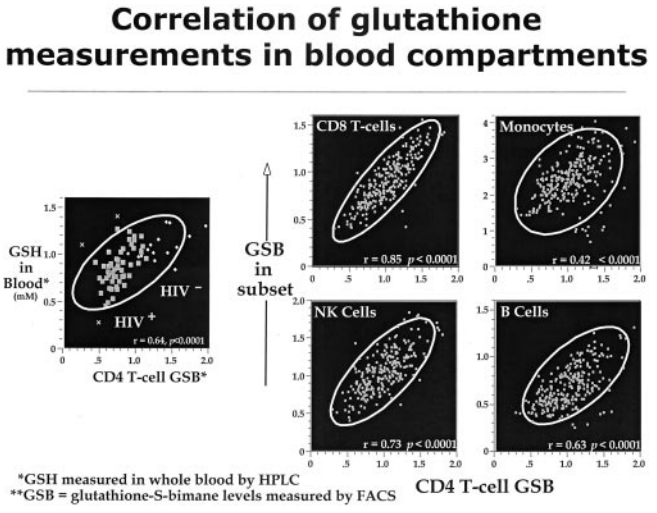

Fig. 11. Measured intracellular GSH concentrations in CD4 T cells and other PBMC subsets in HIV-infected individuals.

Results show that GSH concentrations, detected by FACS as GSB, are correlated in PBMC subsets.

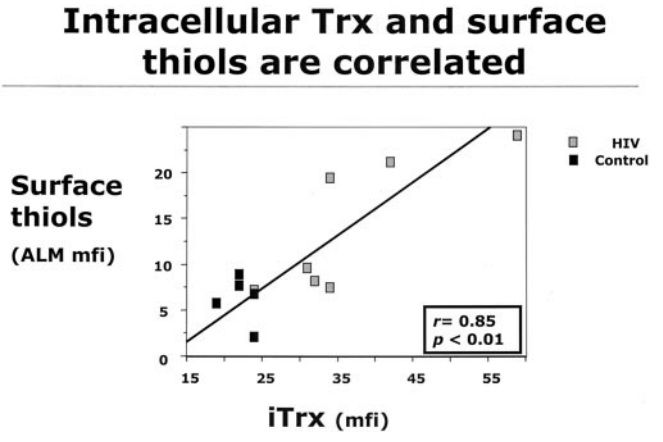

Fig. 13. Positive correlation between surface thiols and intracellular thioredoxin (*iTrx*).

Both measures show high concentrations in HIV-infected individuals and lower concentrations in healthy controls. *mfi*, median fluorescence intensity.

doxin: both measures show high concentrations in HIV-infected individuals and lower concentrations in healthy controls. When we complete this study, we hope to understand more about redox regulation and the relationship of intracellular redox and surface thiols.

Where to Now?

This leads us to what I think we are all interested in: where is FACS going? This broadly useful technology, which started in the late 1960s and became more and more widely used through the 1990s, has been evolving slowly throughout this period. We plan to see that it continues to evolve in this new century and perhaps to help speed up evolution to the benefit of all FACS users. Tables 6 and 7 outline some of the goals for the improvement of FACS and the application of new FACS methods for cell analysis and disease diagnosis and monitoring.

In this short review of work primarily from the Herzenberg Laboratory, students and postdoctorate fellows from the laboratory, and collaborators, we have not mentioned many of the people who have made important contributions to flow cytometry. Early contributors to the field include Mack Fulwyer and Marvin van Dilla from Los Alamos National Laboratory *(21)*. A very important documenter and innovator is Howard Shapiro with his several editions of *Practical Flow Cytometry (22)*.

This work has clearly been the labor and ideas of many students, postdoctorate fellows, engineers, and others who have passed through the Herzenberg Laboratory in the last three to four decades. Some are included by name, but many are not listed. In Fig. 14 those whose work is specifically mentioned are shown more clearly.

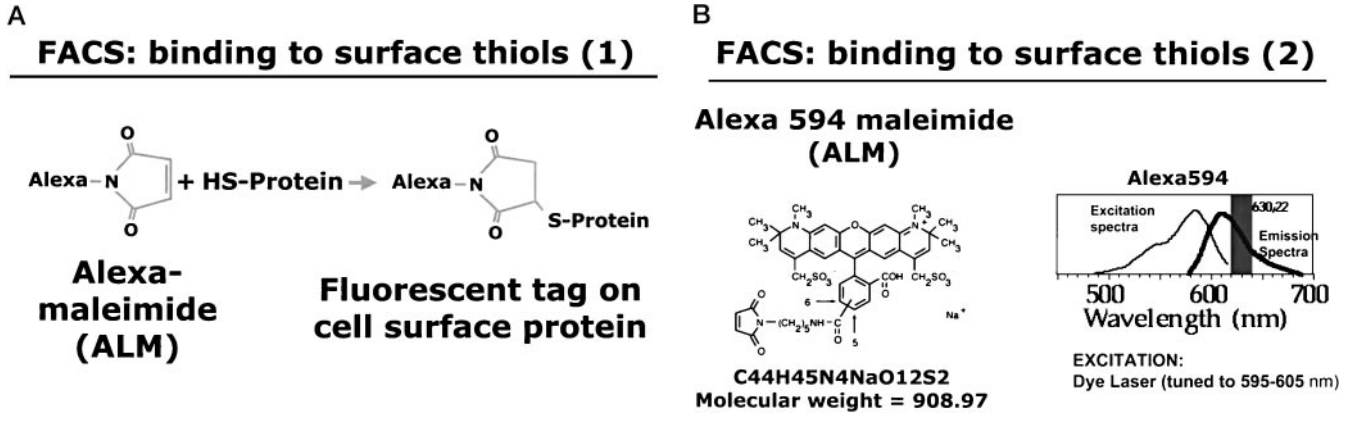

Fig. 12. Use of a fluorescent Molecular Probes reagent, Alexa maleimide (*ALM*), which covalently binds to −SH groups and therefore provides a measure of cell surface thiols on viable cells.

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Fig. 14. Acknowledgments.

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