LAH 407

# Chapter 46
# Overview: Flow Cytometry and FACS

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This section is devoted to the application of flow cytometry to the study of immunology. Fluorescence-activated cell sorting (FACS) has made an inestimable impact on both basic and clinical research over the last two decades. This impact is a direct result of the unique technical power of the methodology and the ever-increasing availability of instruments. In 1975 there were less than 300 flow cytometers in the world and use was largely restricted to a small set of experts; by 1985 there were perhaps 1000, and by 1995 there were over 7000 instruments in both research and clinical labs, and this number continues to rise [1]. Detailed historical perspectives of FACS technology can be found in several references [1–3].

Since the last edition of this handbook in 1986, flow cytometry has come to play a much larger role in both breadth and depth in immunological research. Major factors responsible for the widespread expansion in use have been the introduction of moderate priced laser-based benchtop analyzers, the development of reagents for three color immunofluorescence analysis with a single laser excitation, and the commercial availability of an array of fluorochrome-conjugated monoclonal antibody reagents. Together these developments have made high quality multiparameter flow analysis widely accessible and suitable for routine use. At the more sophisticated end of the spectrum, commercial two and three laser sorters now make it possible to work with five or six fluorescence measurements on each cell, opening new levels of subtlety in subset and functional analysis.

In flow cytometry, cells in suspension are moved through an interrogation region where they are illuminated by one or more focused laser beams. Light scatter and multiple fluorescence signals are collected, processed, and recorded in a computer for individual cells as they flow one-by-one past the interrogation point. Cell types can be identified and purified based on these measurements.

FACS provides the means to perform two extremely important tasks for basic research. First, FACS uniquely facilitates the rapid collection of quantitative, multidimensional measurements on large numbers of individual cells (i.e., statistically meaningful). Cell subpopulations can be precisely defined by specific levels of a combination of individual phenotypic markers. Importantly, many different samples can be analyzed in a short period of time. Second, FACS uniquely facilitates the physical separation (sorting) of desired cells, based on complex phenotypes from heterogeneous cell mixtures. Any combination of measured properties can be used and the outcome is viable cells at purities approaching 100%.

The immune system is comprised of dozens of functionally distinct cell types. Typically, these cell types have been identified by the presence or absence of a variety of different molecules at the cell surface (or even on the basis of a quantitative measurement of the number of molecules per cell); more recently, measurements that probe the internal state of cells have become available. Most of these cell types cannot be identified on the basis of only one or even two simultaneous measurements; the unique identification of subsets of T and B cells in the periphery often requires 3 or more simultaneous markers to be quantitatively measured. Hence the necessity for multiparameter FACS analysis to enumerate subsets of such populations. Such enumeration is now the basis for clinical diagnoses and prognoses in many pathological conditions, including cancer and AIDS.

In order to study all of these different subsets of cells, it is often desirable to isolate and test them separately. This makes it possible to investigate the functionality of these cells in the absence of the ubiquitous communication systems (such as cytokines or integrins) that exist among different cell types. FACS is the only method currently in existence that can separate populations of viable cells on the basis of quantitative measurements of several parameters.

This section is comprised of nine chapters that detail the utility of flow cytometry to the experimental immunologist. They cover the gamut from instrumentation to experimental methods. The focus is on general principles and research applications. Information on specific applications for clinical flow cytometry can be found in several excellent texts [4–7].

The first two chapters provide the groundwork for the FACS experiment. Parks describes the instrument itself, covering optical, fluidic, and electronic aspects. The chapter provides discussions of light scatter and fluorescence measurements, including dye saturation, fluorescence compensation, autofluorescence, and use of standard particles. The theoretical and practical aspects of cell sorting are discussed, with details on carrying out routine and reliably accurate sorting. In the second chapter, Waggoner and Seadler provide a comprehensive overview of the prerequisite fluorescent molecules needed for any FACS experiment. This chapter introduces the basic principles underlying fluorescence and its detection. It provides information about all of the probes that are commonly used for detection of biomolecules (such as DNA, RNA, protein), in addition to those that are commonly conjugated to immunoglobulins to make fluorescent detection reagents. Finally, protocols are provided that can be used to synthesize these reagents in the laboratory.

The next chapter (Kantor and Roederer) lays the groundwork for a majority of immunophenotyping experiments. This chapter describes how to optimize FACS reagents (such as titrating antibody reagents) and experiments (such as using appropriate reagent combinations), and provides suggestions for the design of complex multi-color experiments. It shows examples of some of the pitfalls of these complex experiments, while demonstrating the unique power of multiparameter FACS.

In the fourth chapter, Parks and Bigos discuss data analysis, emphasizing the requirements for annotation, standardization,

and instrument monitoring to assure that reasonably analyzable data are obtained. Display and numerical analysis methods are presented with an emphasis on the desirability of using robust statistical measures whenever they are appropriate.

The fifth and sixth chapters describe important techniques for the isolation and analysis of rare subpopulations by flow cytometric sorting. Esser et al describe pre-separation techniques that are used to pre-enrich cell populations, usually on the basis of a single biophysical parameter. These techniques range from the commonly-used Ficoll density gradients to separate PBMC from whole blood, to magnetic bead sorting of cells expressing certain surface markers. Gross and colleagues give a brief overview of the use of FACS to separate extremely rare cells (i.e., $< 10^{-4}$ frequency).

The last three chapters in this section describe some techniques for functional characterization of cells. Poot et al provide a comprehensive overview of the methods that can be utilized to quantitate a variety of intracellular components, including DNA and RNA, and to determine pH, calcium flux, etc. Anderson et al describe a FACS methodology for the measurement of intracellular glutathione, the major antioxidant of all cells, which has a critical influence on a wide variety of immunological signal transduction pathways and functionalities. Finally, Katsikis et al evaluate several methods for the determination of apoptosis in lymphocytes. Apoptosis has become a focus of increasing interest in the last several years, as the research community has begun to appreciate its critical role in the regulation of the immune response and the maintenance of homeostasis.

Our goal for this section was to provide the reader with a basic understanding of the principles underlying flow cytometry, and to provide information that will aid researchers in the design, execution, and analysis of successful experiments. However, this section is by no means complete. There are several comprehensive texts devoted to flow cytometry (such as [1, 3, 8–10]), and we encourage those researchers undertaking extensive FACS experiments to read them. In particular, Shapiro's book [1] provides a readable, single-authored account including history, underlying physics, a comprehensive survey of the available measurements, probes, techniques and applications, comparisons of commercial instruments, and a 2300-reference bibliography. Darzynkiewicz, Robinson, and Crissman [9] offer 71 chapters by a variety of experts giving detailed discussions and recipes for flow cytometry techniques, measurement procedures, and interpretation of results; detailed recipes are provided by Robinson et al [10]. The newest developments in flow cytometry are often published in the journal *Cytometry*. Finally, there is an internet site that currently serves an electronic mail listserver as well as a World Wide Web site (which can currently be accessed at "http://www.cyto.purdue.edu") for an internet-based discussion group. This forum provides new as well as experienced users a way to ask questions, exchange data, pose hypotheses, or simply start discussions about any cytometry-related topic and receive feedback in a matter of hours to days.

## References


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