Techniques in Quantification and Localization of Gene Expression

Bruce K. Patterson
Editor

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This book is lovingly dedicated to my wife, Carol Penfold, whose love and support drives me every day; to my parents, John and Chris, whose encouragement and love allowed me to choose my path; to my sister Marcy for being so understanding and caring; to my aunt Mary Christensen, Ph.D. and my grandfather Kermit Christensen, Ph.D., whose expert knowledge and teachings in virology and cell biology inspired me to combine the two; and to courageous friends and individuals afflicted with HIV, thoughts of you hearten my mind and effort.

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Preface

Can the son or daughter of a baseball pitcher or cricket bowler throw a ball 100 miles an hour? Is the son or daughter of an opera singer also an opera singer? Is a house with functional light switches lit? The line of thinking in these rhetorical questions also applies to human genetics. What do baseball pitchers, opera singers, light switches, and the Human Genome Project have in common? These questions address the issue of potential versus realization of function. Although sons and daughters of baseball pitchers and opera singers may have inherited the mechanical attributes to be baseball pitchers and opera singers, they may not, at any point in time, be baseball pitchers or opera singers. A house with functional light switches is not lit unless the light switches are on. Similarly, all of the genes discovered and sequenced as a result of the Human Genome Project are not expressed at the same time. Genome project information will allow us to determine the repertoire of genes in an individual, which is analogous to determining where the light switches in a house are located and whether they are functional (a mutation or deletion in the Genome Project Model). The pattern of "on" light switches in a house gives us functional information as to what the family inside is doing (e.g., eating, reading, sleeping). Similarly, the pattern of gene expression (RNA) gives us information on what our bodies are doing (e.g., breast cancer, fighting off a viral infection, depression). The pattern of gene expression has two major components—location and amount. Location is important in diseases that may be focal, such as the expression of *HER-2/c-erb/neu* oncogene in breast cancer. Gene quantity certainly has important implications in HIV disease progression and response to therapy. The focus of this book is to determine the pattern of gene expression through localization and quantification.

On the technical side, research involving human disease pathogenesis usually involves cell lines and animal models to ask multiple questions in a homogeneous or perturbable system while limiting multiple sampling. The necessity to address multiple questions is a technical issue and the need to perturb, in a controlled manner, can be an ethical issue when working with patients. In other words, we need to analyze DNA, RNA, and proteins simultaneously at a given point in time. This can be done in multiple experiments assuming the cells are equivalent in all experiments, or we could perform these analyses at the same time if the technology existed. Cells lines enable researchers to perform these analyses in multiple experiments and cell lines and animal models allow us to perturb the experimental system and perform additional analyses. Bearing in mind the welfare of our

courageous patients who volunteer for our studies, repeat sampling should be minimized. Minimization of patient sampling could be improved if we could answer multiple questions about a particular cell or tissue at one time. Our laboratory is committed to this goal by using multiparameter fluorescence analysis. Since fluorescence analysis is only limited by the number of colors in the spectrum and our ability to discriminate between those colors, we approach disease pathogenesis from the one color, one question strategy. All of the techniques in this book have already been, or can be, easily adapted to fluorescence detection. In addition, this book will describe how gene quantification and gene localization technologies can merge using common strategies.

Specialized technical chapters characterize the first section of this book. These chapters address the pertinent technical aspects needed to perform and interpret results from the application chapters. The detection of antigens and genes using fluorescently labeled antibodies and genes has become a powerful tool in diagnostics and disease pathogenesis research. The advantages of fluorescence applications include eliminating radioactivity from the laboratory, ease of use, and most importantly, multiparameter capabilities. The disadvantages of fluorescence include relative insensitivity when compared to radioactivity, expense, and autofluorescence in cells and tissues. In designing fluorescent probes for in situ hybridization, polymerase chain reaction (PCR) in situ hybridization, and fluorescence in situ 5′-nuclease assay (FISNA), many factors must be considered. Although probe design will depend somewhat on the particular application, we find that oligonucleotides are the most versatile for in situ applications. The advantages of oligonucleotides include size, ease of commercial or custom synthesis, purity, and labeling options. The disadvantages of oligonucleotides are relatively easy to overcome. Because oligonucleotides are short, the number of labels per probe is limited. Several strategies will be discussed to circumvent any disadvantages of using oligonucleotide chemistry. The choice of fluorescent labels on an oligonucleotide depends on the application, the use of other fluors on ligands or antibodies, and the availability of a particular fluor in a chemical configuration consistent with the synthesis. Fluors are available as deoxyribonucleotides (deoxy) or ribonucleotides (e.g., dUTP, dCTP), dideoxynucleotides, NHS esters, and phosphoramidites, for example. Oligonucleotides can be labeled during synthesis (e.g., phosphoramidites, deoxy- or ribonucleotides) or postsynthesis (e.g., NHS esters). Labeling options will be discussed in Chapter 6.

The most important determinant of a successful experiment involving fluorescence is signal-to-noise ratio (SNR). The SNR will govern the resolution of cells containing a particular gene from cells lacking that gene. In other words, the SNR determines the sensitivity of a particular assay. The signal can be increased by increasing the number of labels per probe, by increasing the number of probes containing a set number of labels, or by maximizing the intensity of dye fluorescence. Noise in fluorescence analysis of cells and tissue has been one of the major impediments in the widespread use of this technology. Specifically, in many of the common applications, autofluorescence in the fluorescein emission range is most troublesome. This problem can be avoided by using dyes that emit

outside the range of maximal autofluorescence or by using autofluorescence quenching dyes such as Evan's blue and trypan blue. Details on minimizing autofluorescence will be described in Chapter 5.

Quantification of gene expression, as described in this book, has two levels in biological systems. First, the number of cells expressing a particular gene in a heterogeneous cell population (sometimes called a tissue) can be quantified. Second, the number of gene copies in an individual cell under various conditions or in various milieus can be quantified. Quantitative techniques will be described in this book starting with techniques for quantifying cells expressing a particular gene in a heterogeneous cell population in chapters on quantification of cytokine-producing cells following immunostaining, reverse transcriptase PCR in situ hybridization (RT-PCR in situ hybridization) and reverse transcriptase fluorescence in situ 5′-nuclease assay (RT-FISNA). The next section of the book will describe methods to quantify the number of copies of a particular gene expressed in an individual cell in addition to quantifying the number of cells expressing the gene of interest. These chapters describe novel in situ hybridization strategies, some with extremely high sensitivity.

Finally, the use of fluorescence allows interfacing with other instruments to provide additional or confirmatory data. For example, we verified the intracellular amplification and detection of HIV-1 DNA by extensively washing cells to remove amplified product in the supernatant then lysing the cells and precipitating the fluorescence heteroduplex created in situ. This DNA was analyzed on a laser sequencer revealing a single peak of the appropriate size that was calculated using internal molecular weight markers labeled with another compatible fluorescent dye. The last chapter of this book will describe interfacing of instruments in our attempt to define disease at the single cell level.

If you don't know, make it glow!

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Basic Methods

1

Basic Flow Cytometry Instrumentation

Charles L. Goolsby and Cathy James

Over the last 20 years, flow cytometric analyses have emerged as an integral component of clinical pathology. The clinical applications of flow cytometry range from analysis of T cell subsets in immunodeficiency syndromes, immunophenotyping of hematological malignancies, enumeration of stem cell populations in bone marrow transplantation, DNA content, and cell cycle parameters in a wide range of malignancies to analysis of platelets and enumeration and assessment of maturation of reticulocytes. In AIDS, flow cytometric techniques have become the gold standard for the enumeration of T helper cells [1]. The use of flow cytometry as a tool for the detection of cells positive for a specific HIV DNA or RNA sequence following fluorescence in situ hybridization (FISH), polymerase chain reaction (PCR), or reverse transcriptase polymerase chain reaction (RT-PCR) procedures is becoming a commonly used tool for the investigation of the cellular characteristics of these cells, as well as the cellular heterogeneity within the positive cell population.

This review provides an introduction to the basics of flow cytometry instrumentation as well as a discussion of relevant issues of sample preparation and data analysis.

Instrumentation

Overview

In flow cytometric analyses, a single cell at a time is passed through a focused light beam, either from a laser or arc lamp source. The interaction of the cell (intrinsic measurements), or of dye molecules associated with specific cellular constituents (extrinsic measurements), and the focused light source is then measured on a number of light-sensitive detectors. It should be noted that an alternative design in which the cells are attached to a slide and a focused laser beam is scanned across the cells has also been developed and is commercially available (Compucyte, Inc., Cambridge, MA). Scattered light intensity, an intrinsic parameter, measures how the cell interacts or scatters light out of the incident light source

as the cell passes through the focal spot, either in a direction near the axis of the incident light (forward scatter) or centered at a right angle to the axis of the incident light (side scatter or 90° scatter). Extrinsic measurements are generally assessing the intensity of fluorescence light emitted by fluorescent molecules that have been added to the cells and that stain or associate with specific cellular proteins or molecules such as DNA or RNA, although absorption-based measurements have been employed [2]. The application that has spearheaded the clinical applications of flow cytometry has been the use of fluorescently labeled antibodies directed against cell surface antigens that are associated either with cell lineage or stage of cell differentiation [1, 3]. It should be noted that these analyses are not restricted to the detection of these cell surface antigens but include measurements of intracellular antigens [4, 5], DNA [6], and RNA [7], as well as fluorescent-based in situ hybridization [8–11], PCR [10, 12, 13], or RT-PCR [13] signals.

Delivery of Sample

The creation of a single-file stream of cells is accomplished by the delivery of the sample through a small-bore injection needle into a larger-diameter, rapidly flowing sheath stream. Acceleration of the sample stream as it enters the sheath stream leads to creation of a narrow, central, focused sample core stream within the sheath (hydrodynamic focusing; see Fig. 1.1, inset), which is on the order of a cell diameter or slightly larger. By adjusting the delivery velocity of the sample

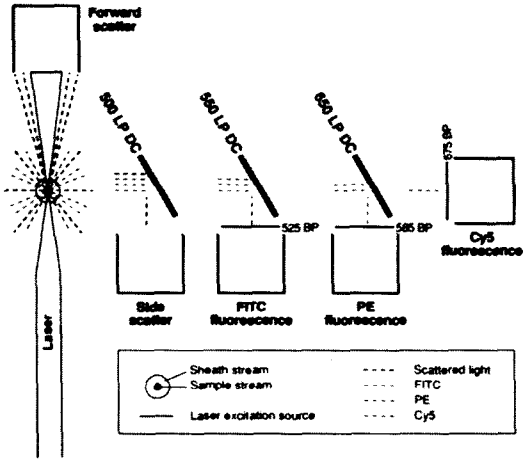

FIGURE 1.1. Schematic representation of a typical flow cytometer showing detection of forward and side scatter as well as FITC, PE, and PE-Cy5 (labeled Cy5 in figure) fluorescence emissions. LPDC = longpass dichroic interference filter; BP = bandpass interference filter.

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