Preface

Flow cytometry is a method to conduct a multiparameter analysis of cells suspended in liquid and passing through a laser beam. As cells pass through the flow chamber they are hit with the laser light beam which is scattered in different directions and recorded as forward light scatter and side light scatter. In this book, the authors discuss the principles, methodology and applications of flow cytometry. Topics include flow cytometry and epifluorescence analyses of freshwater bacterio- and virioplankton communities; application of flow cytometry in phenotyping, analysis and functional characterization of T regulatory and immune suppressor cells; multicolor panel design strategies in rare event analysis of dendritic cells and regulatory T cells; advanced characterization of individual particle morphology light-scattering flow cytometry; and application of flow cytometry in environmental science.

Chapter 1 – Concerns about obtaining accurate determinations of the concentrations of viruses and bacteria in freshwater samples led us to examine a broad battery of counting and storage procedures for use in flow cytometry (FCM) and epifluorescence microscopy (EFM) analyses. Sample preparations were done so as to optimize counts and preservation by using different types and concentrations of aldehyde-based fixatives, stains belonging to the SYBR family, dilution media, temperature and storage conditions. Whenever possible, FCM and EFM counts were compared. Results obtained using FCM supported the addition of fixative for bacteria, preferably glutaraldehyde at a final concentration of 2%, dilution in 0.2-µm or 0.02-µm filtered Tris-EDTA buffer (TE, pH = 7.5), staining with SYBR Green I at a final concentration of 10^{-4} and incubating at ambient temperature for at least 15 minutes. For viruses, there was no need to add fixative, whereas dilution in recently-autoclaved and 0.02-µm filtered TE and incubation with SYBR Gold at a final concentration of 2×10^{-5} at 75°C for 10 minutes is recommended. If possible, FCM samples should be counted on day = 0, although the authors do show that bacterioplankton samples, at least, may be stored at 4°C and counted at 24 h later but not beyond if samples cannot be frozen in liquid nitrogen. The conditions required for optimum EFM counts of both bacteria and viruses involved were to stain filters with SYBR Gold at a final concentration of 10^{-3}. Slides could be counted for up to 1 month if rapidly frozen and stored at –20°C.

The authors results performed on lake samples clearly demonstrate the importance of defining the best conditions in order to get reliable counts of microbial communities such as viruses and bacteria. Each research laboratory should undertake such tests according to the equipment available, and the needs and area of their research.

Chapter 2 – Flow cytometry is an inevitable tool in clinical diagnosis of various pathological conditions. Recently, its application plays a significant role in monitoring of several immune suppressor cells in patients with cancer and autoimmune disorders. Regulatory T cells (Treg cells) are immune cells which help in maintaining immune homeostasis in physiological conditions by patrolling unwanted immune responses; however, in cancers and autoimmune diseases, Treg cell numbers and function are significantly altered. In this chapter, the authors focus and update the information regarding Treg cells and their subsets, and application of flow cytometry in phenotyping and functional characterization of various immune regulatory and suppressor cells in multiple myeloma patients.

Chapter 3 – The need for multidimensional analysis using complex stain combinations has been expanding considerably, especially to discriminate, classify and/or isolate rare cell populations, such as dendritic cells (DCs) and regulatory T cells (Treg). Several principles need to be taken into account when setting up a multicolor flow cytometry experiment for rare event analysis.

The frequency of the event of interest in a sample dictates how many cells must be processed. The lower the frequency of the events of interest, the more events need to be acquired, which might result in large data files. However, the more events one counts, the better the precision will be, as the minimal counting error is equal to the square root of the actual counted events of interest.

It is crucial to understand that reliably counting a rare event is like finding a needle in a haystack. Moreover, the spurious events (``noise``) need to be recognized as well, in order to exclude them, determine what causes them and minimize their occurrence. The signal-to noise ratio can be increased in several ways. Firstly, it is very important to carefully select the reagents to improve the discrimination of different populations in multicolor flow cytometry, which depends on the instrument configuration. Furthermore, the rare event should be positively identified by multiple fluorescence parameters, because the random noise is diluted out by a power function of the number of parameters. Secondly, a critical step in panel design is to match the fluorochromes by brightness according to antigen density. The golden rule is to define the antigens with the lowest expression by the brightest chromophore and vice versa. Thirdly, fluorescence spillover can have a huge impact on the resolution sensitivity of dim markers in multicolor flow cytometry experiments. The more colors you have in your tube, the more spillover you have, resulting in a higher background. Also, if multiple antigens are present on a given cell ("coexpression"), it is better to spread them across as many lasers as possible. Finally, the total noise can be characterized by using an appropriate negative gating control, such as isotype-matched fluorochrome-conjugated antibodies or the fluorescence minus one method.

Here, the authors will discuss all the principles that need to be taken into consideration for a successful multicolor panel design to analyze rare events. They will also illustrate multicolor panel design strategies by specifying the flow cytometric identification and quantification of two rare populations: DC and Treg.

Chapter 4 – Light scattering has played an important role in flow cytometry since the beginning of its technological development. Most of the significant results were demonstrated with analyses of individual particles from light scattering. However the progress in fluorescent biomarkers reduced the role of light scattering in flow-cytometer analyses substantially. Nevertheless the light scattering is still alive and the authors are presenting results from a new stage in the development of light-scattering analysis in flow cytometry.

These new advances stem from angle-resolved methodology. With this review the authors show how the angle-resolved light scattering provides determination of physical characteristics of individual particles. Moreover this approach allows one to retrieve particle dimensions with a precision ranging from 10 nm to 100 nm depending on the shape complexity of a particle. This is subdiffraction precision that cannot be realized with conventional optical microscopy. Moreover the flow-cytometer analysis provides statistically proven results for characterization of cell populations because of the high rate of measurements. In order to demonstrate the advantages of angle-resolved measurement of light scattering the authors describe the principles of scanning flow cytometry that allows us to measure the light-scattering profiles (LSPs) of individual particles. In the development of this approach, they introduce the latest improvement that relates to simultaneous measurement of regular and polarized LSPs for one particle. The methodological goal of the angle-resolved technique is the determination of morphological characteristics of particles from a solution of the inverse light-scattering (ILS) problem. The authors introduce a few methods to solve the ILS problem utilizing the numerical algorithm in global optimization. The bisphere characteristics, sizes, and refractive indices of each sphere composing the bisphere were successfully retrieved by processing the regular and polarized LSPs. The LSPs showed a near-perfect agreement with T-matrix simulations, resulting in a 50-nm precision for sizing bispheres. They rigorously characterized individual blood microparticles, determining their sizes and refractive indices from measured LSPs. The authors introduce a novel approach for determination of the volume and shape of individual blood platelets modeled as oblate spheroids from angle-resolved light scattering using the flow-cytometer technique. Accurate measurement of the spheroidal aspect ratio of the platelets allowed us to discover the substantial differences between native and activated 10 μM adenosine diphosphate samples of platelets. They demonstrate a flow-cytometer method to measure the morphology of single *Escherichia coli* cells with a precision ranging from 50 nm to 250 nm and from 5 nm to 25 nm for bacteria length and diameter, respectively. High precision in the determination of cell morphology allows us to measure kinetics of intracellular processes like red-blood-cell lysis and lymphocyte apoptosis. The authors discuss further applications of angle-resolved light scattering using flow cytometry in the conclusion.

Chapter 5 – Flow cytometry (FCM) is a powerful technology which is routinely used in the medical and veterinary diagnostics. Nowadays it is also an emerging research tool applied in the different fields of environmental studies. This short communication provides the insight to the interesting applications of FCM developed recently in order to study microorganisms, plants and animals. Examples of possible implementation of FCM in environmental monitoring are also given. Major advantages of FCM include automatism and precision of measurements; FCM analyses are time-saving and require small volumes of sample. The authors believe that an increase in FCM availability on the market and simultaneous decrease of technology costs will firmly establish FCM in the different fields of environmental studies and help to improve already existing methods.