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Critical Review

The Relevance of Flow Cytometry for Biochemical Analysis

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Summary

Flow cytometry (FCM) allows the simultaneous measurement of multiple fluorescences and light scatter induced by illumination of single cells or microscopic particles in suspension, as they flow rapidly through a sensing area. In some systems, individual cells or particles may be sorted according to the properties exhibited. By using appropriate fluorescent markers, FCM is unique in that multiple structural and functional parameters can be quantified simultaneously on a single-particle basis, whereas up to thousands of biological particles per second may be examined. FCM is increasingly used for basic, clinical, biotechnological, and environmental studies of biochemical relevance. In this critical review, we summarize the main advantages and limitations of FCM for biochemical studies and discuss briefly the most relevant parameters and analytical strategies. Graphical examples of the biological information provided by multiparametric FCM are presented. Also, this review contains specific sections on flow cytoenzymology, FCM analysis of isolated subcellular organelles, and cell-free FCM.

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Keywords Cell function; cell sorting; flow cytometry; fluorescence; fluorochromes; isolated subcellular elements; multiplexed assay.

INTRODUCTION

Flow cytometry (FCM) allows the simultaneous measurement of multiple fluorescences and light scatter induced by illumination of single cells or microscopic particles in suspension, as they flow rapidly through a sensing area (*1*, *2*). In some systems (cell sorters), individual cells or particles may be physically separated according to their properties (Fig. 1). Thus, FCM is unique in that multiple biological parameters can be quantified simultaneously on a single-particle basis, while up to thousands of events per second may be examined. As a result, large and heterogeneous cell populations are described based on the biometric properties of their individuals (Table 1).

Because of its historical development (*1*) and its important clinical implications, the largest body of current applications is diagnostic/prognostic, based on immunophenotyping and DNA content assays (*3*, *4*). However, FCM is now a choice methodology in basic and applied studies, including cellular and molecular biology (*5*, *6*), biotechnology (*7*), toxicology (*8*), microbiology (*9*), plant physiology (*10*), and oceanography/limnology (*11*). On the other hand, clinical FCM increasingly implements biochemical assays to improve sensitivity of abnormal cell identification (*12*) and to provide functional information about pathogenetic mechanisms involved in disease conditions (*13*).

FCM Analysis of Cell Biochemistry: Parameters and Probes

Individual cells, bearing multiple markers on their surface, contain intracellular compartments with their own metabolic environment. Functional integrity of membranes is necessary for the regulation of such compartments that, in turn, condition metabolic pathways within them. In many cases, homeostasis of cell compartments requires regulated transport across cell membranes. On the other hand, plasma membrane is deeply involved in biochemical responses that mediate cell activation triggered by multiple stimuli. Many of these responses are dependent on specific receptors and trigger consistent changes in ionic status and enzyme activities in signal transduction pathways, leading

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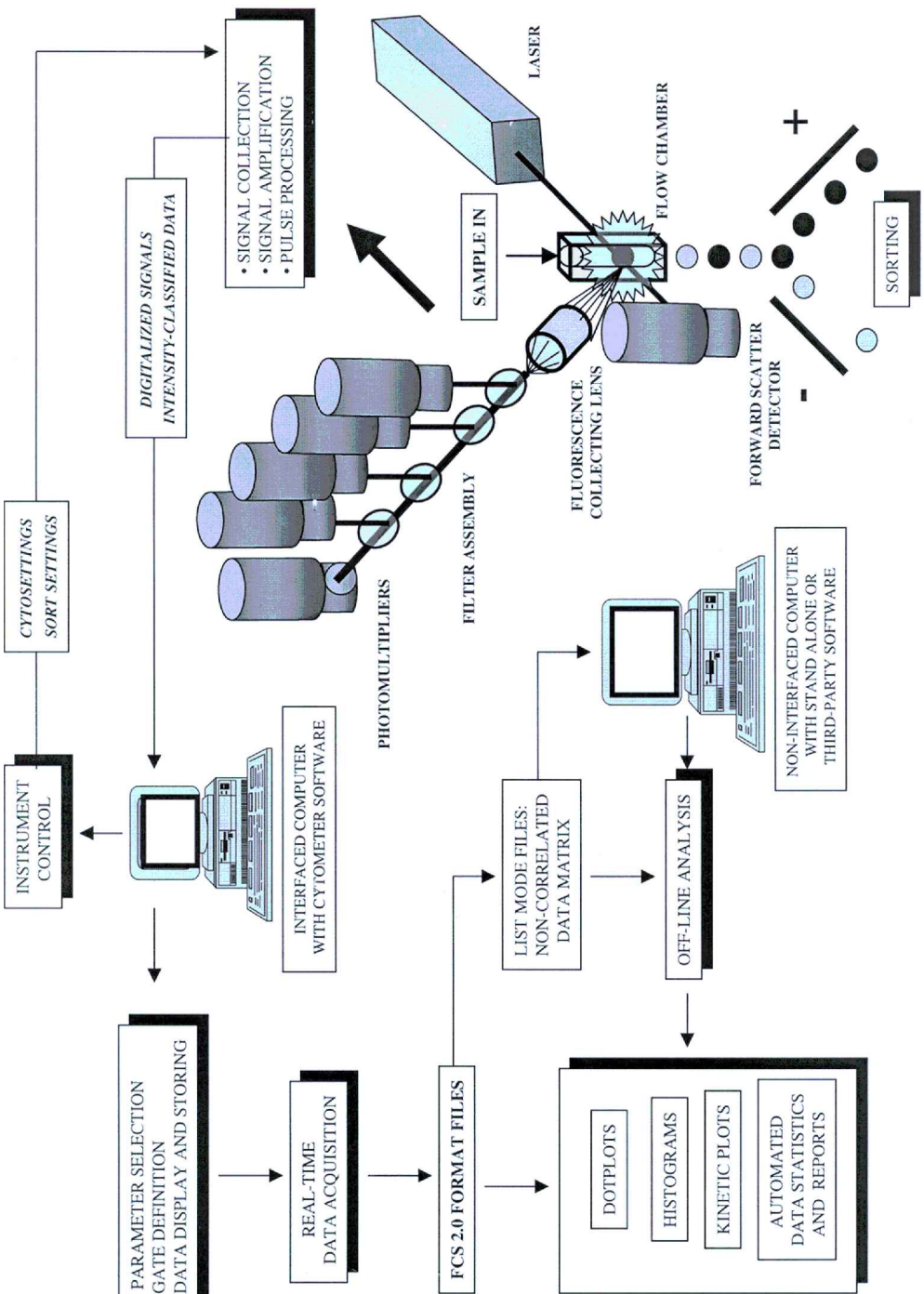

Figure 1. Schematics of a typical flow cytometry/cell sorting instrument. The scheme shows how cells are forced to cross a laser beam at the flow chamber. Multiple fluorescence and light scatter signals are collected and directed through the fluorescence collecting lens and filter assembly. Separate photomultipliers or photodiodes amplify the signals accordingly and the instrument electronics processes and classifies the pulses generated by single cells or particles. In some systems (Cell sorters) cells may be separated by electrostatic charging devices after breaking the flow into microscopic individual droplets containing single cells. The interfaced computer receives the processed signals in real time and allows the control of instrumental settings for analysis (cytosettings) and cell sorting (sortsettings), as well as predefinition of the protocol for multiparameter data acquisition. The results of analysis are formatted as FCS files (Flow Cytometry Standard) and may be displayed as different graphic representations of cell population distribution (univariate histograms or multivariate dotplots) where user-defined numerical and statistical analysis is performed by dedicated software. Data may be stored as noncorrelated data matrix for each single cell (listmode files) in a way permitting to reproduce virtually and instantly the data acquisition process, while keeping or changing as desired some features of the analysis, such as gate definition, parameter correlation, type of display, and so on. FCS files may be shared and transfered by physical support systems (floppy disks, CD disks) or by local or global networks to other independent computers to proceed to further data analysis, including the use of third-party software for FCM applications.

Table 1
Biochemical assays by flow cytometry: samples and probes

Type of biological samples	
• Pluricellular organisms	• Isolated nuclei
• Cell spheroids	• Subcellular elements
• Hybridomas	• Chromosomes
• Cell fusions	• Liposomes
• Human cells	• Mallory bodies
• Animal cells	• Amyloid plaque fibers
• Plant protoplasts	• Membrane fractions
• Prokaryotic cells	• Viral particles
• Yeasts	• Soluble antigens[1]
• Microalgae	• DNA sequences[1]
Type of fluorochromes and fluorescent markers	
• Fluorochromes reacting with specific chemical groups	• Fluorescent pH indicators
• Fluorochrome pairs for resonance energy transfer	• Fluorescent ion chelators
• Fluorescent antibodies	• Membrane-potential sensitive distribution fluorescent dyes
• Fluorescent lectins	• Fluorogenic substrates of intracellular enzymes
• Fluorescent nucleic acid sequences	• Fluorescent macromolecules
• Fluorescent lipids	• Fluorescent synthetic particles
	• Endogenous fluorescent molecules

[1]Using fluorescent microspheres as capture reagents and fluorescent ligands as reporter molecules.

ultimately to regulation of gene expression, cell diferentiation, and/or proliferation.

FCM is applied successfully to study each step of this vast complexity of cellular biochemistry. For most applications, cells must be stained with fluorescent markers of defined optical and biological properties (Table 1), but FCM takes also advantage of endogenous fluorochromes related to intracellular functions (*1*). In this way, as summarized in Table 2, the range of parameters available for the FCM evaluation of cell biochemistry has been extended from broad assessment of cell behaviour to quantification of single molecules undergoing or regulating specific biochemical reactions.

A significant part of FCM studies involves analysis of these parameters in relation to cell activation (*14, 15*) and proliferation (*16*), cell sensitivity (*17*, *18*) or resistance to drug action (*19*), and cell death by apoptosis or necrosis in a wide range of experimental settings (*20–22*). Although most of these studies fall within the scope of basic research, the development of simple assays for these parameters has allowed their application to different clinical situations (*3*, *23*).

Specific Features, Strategies and Limitations of Functional FCM

Because of the unique feature of FCM, i.e., the multiparametric examination (and physical separation) of single cells or particles at very fast rate, this particular technique of biochemical analysis has evident advantages over other conventional methodologies. Thus, the large number of cells analyzed and the instrumental settings of current cytometers provide multiple strategies to obtain primary information, and allow a large number of general applications, as Table 3 attempts to cover. From a practical point of view, the main assets of FCM can be summarized as follows:

Multiparametric Data Acquisition. Most standard biochemical procedures determine a single parameter per assay and are not sensitive enough for single-cell analysis. FCM instruments allow routinely two morphology-related parameters (forward- and side-light scatter) and 3–5 fluorescence signals per single particle. In this way, in a single-tube assay, one or more parameters may be used to identify and select ("gated analysis") cell subsets in heterogeneous populations (e.g., live, apoptotic, or necrotic cells; cells of different origin or lineage; cells in different cell cycle stage and so on), whereas other signals may be assigned to analyze specific structures or functions in these selected populations. An example of this concept is illustrated in the single-tube assay shown in Fig. 2. The number of available parameters per single cell increases when multiple-laser cytometers are used. Obviously, the analysis of multiple aliquots per sample allows to expand indefinitely the number of parameters by combining separately fluorescent markers of different biological properties but similar optical properties. An example of this concept is illustrated in the integrated analysis shown in Fig. 3. This type of FCM analysis (panel analysis) is the hallmark of immunohematology, where typically more than 20 fluorescent monoclonal antibodies against epitopes in leukocyte plasma membrane may be used for typing leukemias and lymphomas (*4, 24*).

Multivariate Data Analysis. Due to the hardware and software design of modern cytometers, multiparametric acquisition is interfaced to multivariate data analysis. In this way, a cell population is not described by mere enumeration of the individual properties measured but by their correlation on a single-cell basis, thus increasing the discriminating power. Moreover, the possibility of storing FCM data as an uncorrelated data matrix for each analyzed cell ("list mode files") allows one to define, if necessary, new parametric correlations and population selection by replaying (off-line) those electronic files. This is an invaluable tool especially when small or infrequent samples are studied.

Fast Analysis of Large Number of Live Cells. FCM may be performed on a large variety of biological material in different conditions of vitality (e.g., intact fresh cells, fixed and/or permeabilized cells), as indicated in Table 3. The use of live cells allows one to probe multiple biochemical parameters in minimally perturbed intracellular environments, as well as in near-physiological extracellular conditions.

Table 2
Biochemical assays by flow cytometry: parameters

Cell surface parameters	Cytosolic parameters	Nuclear parameters	Subcellular elements
Membrane integrity	General protein	DNA content	Normal mitochondria
Membrane potential	Mitochondrial activity	RNA content	Megamitochondria
Membrane recycling	Mitochondria content	Nuclear total proteins	*Cis*-Golgi vesicles
Receptor expression	Cytosolic pH	Nuclear specific proteins	*Trans*-Golgi vesicles
Receptor interactions	Lysosomal pH	Chromatin conformation	Endosomes
Receptor modulation	Tyrosine phosphorylation	Cyclins and CDks	Phagosomes
Surface glycoconjugates	Cytosolic Ca^{2+}	Proliferation-related antigens	Chloroplasts
Ligand binding to surface receptors	ROS and NOS	DNA synthesis	Thylakoids
Cell-cell adhesion	Enzyme activity:	DNA strand breaks	Extracellular analytes
Membrane fluidity	Oxidases	DNA oxidation	
Cholesterol content	Dehydrogenases	DNA repair	
Loss of lipid assimetry	Esterases	Nuclear receptors	
Permeability to fluorescent probes	Proteases	Gene expression	
Membrane peroxidation	Transferases	Gene reporting	
Membrane shedding	Protein modification		
Endocytosis	Free soluble thiols		
Phagocytosis	Glutathione		
Pynocytosis	Protein thiols		
Efflux pumps	Nonpolar lipids		
Bacterial cell wall	Polar lipids		
Yeast cell wall	Cytoskeletal proteins		
	Granule content		

The fast rate of data acquisition and the possibility of examining millions of individual particles in a reasonable time allows the detection and accurate analysis of infrequent or rare cells, down to 1 event per 10^8 cells (*25*). Such a possibility is in contrast with bulk standard fluorimetric determinations in which millions of cells (or their extracts) are analyzed at the same time, yielding a single mean concentration value.

Individual Cell Sorting. Some FCM systems are able to separate physically individual cell or particles according to their cytometric properties. The most advanced cell sorters are based on electromagnetic deflection of individual droplets generated by high-frequency vibration of the flow chamber (*1*). In such systems, up to four different subpopulations can be sorted simultaneously or, on the other hand, one single cell can be deposited in a given position of a microwell array. Cell sorting allows the combination of the intrinsic capabilities of FCM results with information obtained by image (conventional and confocal microscopy) and molecular (polymerase chain reaction, in situ hybridization) techniques, and provides a preparative tool for rapid isolation of living rare cells of biochemical relevance, such as stem cells (*26*), transfectants (*27*), or hybridomas producing a given antibody (*1*). It is worth mentioning the contribution of flow sorting of chromosomes to the sequencing of the human genome (*28*, *29*).

As indicated in Table 4, there are also critical points and difficulties when performing adequate functional analysis by FCM, which mostly depend on the maintenance of adequate viability or metabolic capacity of cells and subcellular elements as well as avoiding the interference of fluorescent probes with cellular functions.

FCM Approach to Classic Biochemistry: Flow Cytoenzymology

Currently, a wide range of fluorescent substrates or their fluorogenic precursors are available for FCM analysis (*1*, *2*, *30*). On the other hand, flow cytometers incorporate time as a parameter to follow the kinetics of fluorescence variations on the specific modification of substrates (*14*, *31*). For these reasons, flow cytoenzymology (*30*) appears as a promising application of FCM for analysis of metabolism. Flow cytoenzymology is applied to a growing number of enzymatic activities in multiple biochemical pathways (*32–34*) and these studies may have a direct clinical impact (*35*, *36*). Thus, they are currently applied to assess leukocyte function, to correlate cell metabolism and malignant capacity in fresh tumor cells, and to evaluate drug metabolism and therapy monitorization in pharmacological studies (*37*).

Flow Cytometric Analysis of Isolated Subcellular Compartments

The use of flow cytometers to analyze functional properties of isolated subcellular particles is less frequent than its application

Table 3
Biochemical assays by flow cytometry: strategies, information, and applications

Assay strategies	Primary information	Main general applications
a) *According to the biological material*: • Assays using fresh cells • Assays using fixed cells • Assays using subcellular elements • Multiplexed assays b) *According to specific cell selection*: • Nongated assays • Gated assays c) *According to assay duration*: • Single end-point assays • Sequential end-point assays • Kinetic assays with unperturbed cells • Kinetic assays following cell stimulation with ligands d) *According to data analysis*: • On-line analysis (real time) • Off-line (Listmode analysis)	• Intensity of expression of multiple parameters within homogeneous cell populations • Heterogeneity of expression of multiple parameters in cell subpopulations • Correlation between different parameters in cell populations • Ratio between multiple parameters in single cells • Evolution of fast and/or transient dynamic parameters • Evolution of slow and/or sustained dynamic parameters • Detection and analysis of rare cells/particles • Correlation with parameters analyzed with other techniques following cell sorting	• Identification/characterization of cells based upon multiple biochemical parameters • Diagnostic applications, including detection of rare pathological cells • Analysis of cell activation, including receptor biology and signal transduction • Analysis of gene expression, including gene engineering • Analysis of cell cycle and proliferation-related events • Analysis of differentiation • Flow cytoenzymology • Analysis of cell viability and cell death, including apoptosis and necrosis • Analysis of microbial biochemistry, including sensitivity to drugs • Control of biotechnological processes, including growth conditions and productivity • Environmental biochemistry

in whole cell studies. However, most current instruments are adequately sensitive for subcellular analyses, which have always been a hallmark of biochemistry.

Some methodological aspects become critical when analyzing single subcellular particles by FCM because of their small size, the different permeability or uptake rate of dyes by isolated organelles, and their usually increased lability. However, FCM analysis of isolated organelles provides insight of subcellular functions and structures in experimental models where a higher degree of metabolic control can be achieved (Table 1 and Table 2).

Table 4
Biochemical assays by flow cytometry: difficulties

Critical points
• Preparation of single-cell suspensions from adherent cell populations
• Maintenance of cell viability along the experimental period
• Isolation of subcellular elements from cells and tissues
• Readjusting conditions for subcellular analysis
• Identification of small cells and particles from background noise
• Adequate access of probes to intracellular sites or processes
• Adequate retention of substrates and probes
• Noninterference of probes with cell functions
• Adequate selection of time-windows for kinetic assays
• Assay calibration for data expression in absolute units

Rhodamine 123 and other membrane-potential (MP)-sensitive dyes (*15*, *31*, *38*) have been used for functional analysis of isolated mitochondria, whereas other MP-independent mitochondrial dyes can be applied to determine the mitochondrial content in whole cells (*39*). Manipulation of membrane potential in isolated mitochondria induced predictable changes in Rh123 fluorescence and revealed mitochondrial heterogeneity in liver cells and heterogeneous responses to physiological and nutritional conditions (*40*, *41*). Isolated mitochondria have been used also for toxicological and pharmacological studies, which yielded data complementary to those obtained using whole cells (*42*).

FCM has been applied also to analyze the binding of fluorescent lectins to isolated chloroplasts (*43*) and Golgi vesicles (*44*) for the study of their oligosaccharide content. The data thus obtained may be of relevance to the study of the normal and altered mechanisms of glycoprotein maturation and sorting.

Cell-Free Cytometry: Quantifying Soluble Analytes

FCM is not limited to the analysis of biochemical components in suspensions of cellular or subcellular particles. On the contrary, a recently developed strategy known as multiplexed

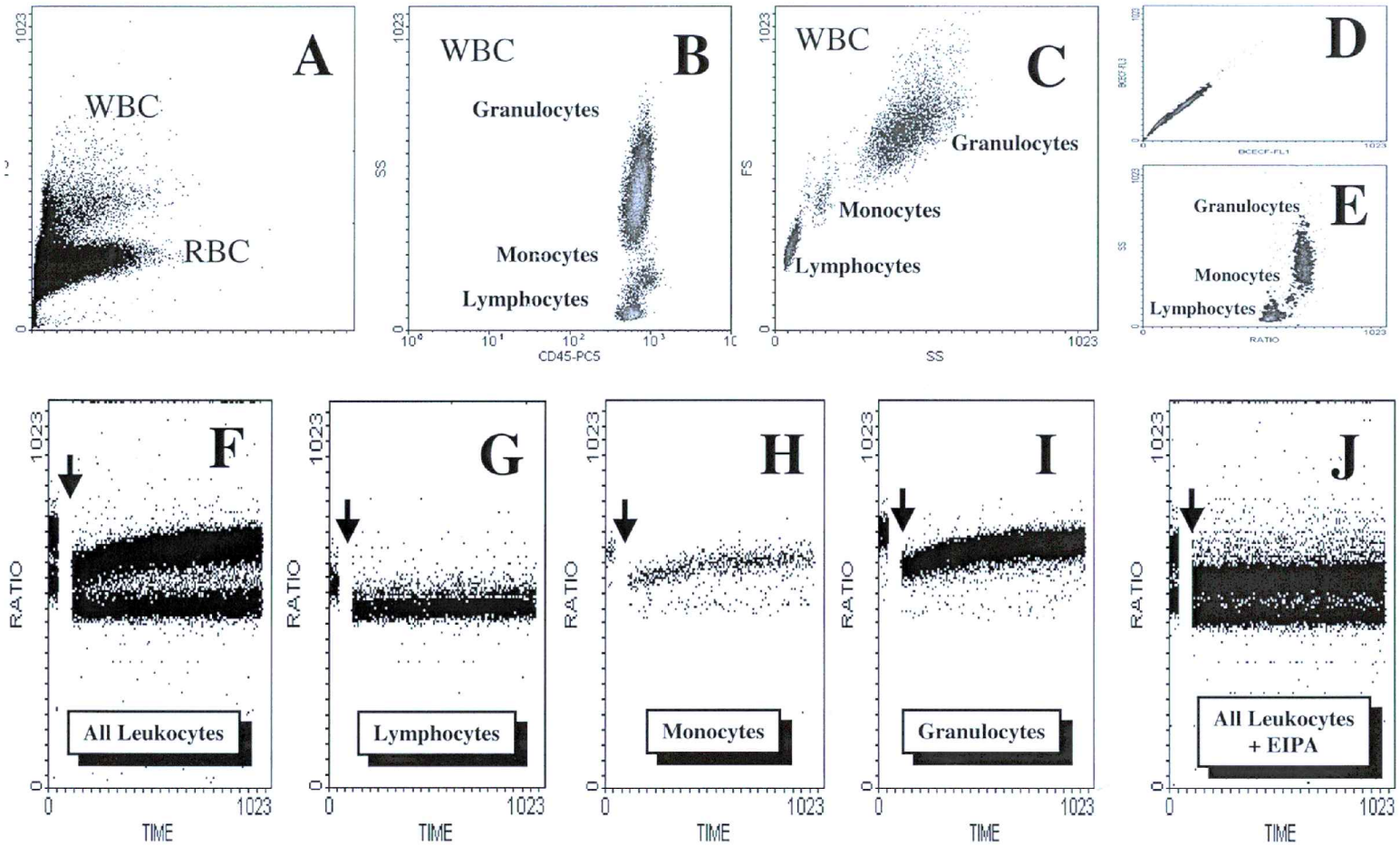

Figure 2. Example of the multiparametric data acquisition and multivariate data analysis in a single-tube FCM biochemical assay. First, 25 μL-samples of human whole blood were incubated with 5 μL of pan-leukocyte antibody CD45-PCy5 for 15 min and diluted to 1 mL with RPMI medium. Then, 5 μL of pH-indicator, 1 mM BCECF-AM, were added and the sample incubated for 15 min at 37°C in the dark in the presence or absence of 4 μM ethyl-isopropyl-amyloride (EIPA), a Na^+/H^+ antiport inhibitor. Samples were run on an EPICS XL-MCL flow cytometer and the following parameters acquired: Forward scatter (FS, an estimation of cell size), side scatter (SS, an estimation of cell granularity), Log FL1 (BCECF-AM green fluorescence), Log-FL2 (BCECF-AM yellow fluorescence), Log FL4 (CD45-PCy5 red fluorescence), Ratio FL2/FL1 (the ratio between BCECF-AM yellow and green fluorescences), an estimation of intracellular pH (pHi), and Time (the x-axis scale represents 300 s). Sample was run for 10 s, then paused, and 50 μL of 100 mM propionic acid (ProH) added (arrow). Data acquisition was re-started to show induced acidification as a decrease in the ratio FL2/FL1. Analysis of whole blood does not distinguish leukocytes (WBC) from erythrocytes (RBC) based in scatter parameters (A). However, gating on CD45 shows clearly leukocyte subpopulations (B) and their typical morphology (C). Analysis of BCECF FL1 and FL2 separately (D) does not provide information. However, bivariate plot of FL2/FL1 ratio vs SS (E) shows that leukocyte subpopulations differ in resting pHi, granulocytes being more alkaline. Kinetic analysis of pHi following acidification with ProH shows heterogeneity in WBC (F). Selection of specific subpopulations on dotplot (C) shows that lymphocytes do not recover pHi (G), whereas monocytes (H) and granulocyte (I) return rapidly to resting pHi. The participation of Na^+/H^+ antiport is confirmed by the inhibitory effect of EIPA in a second tube run in the same conditions (J).

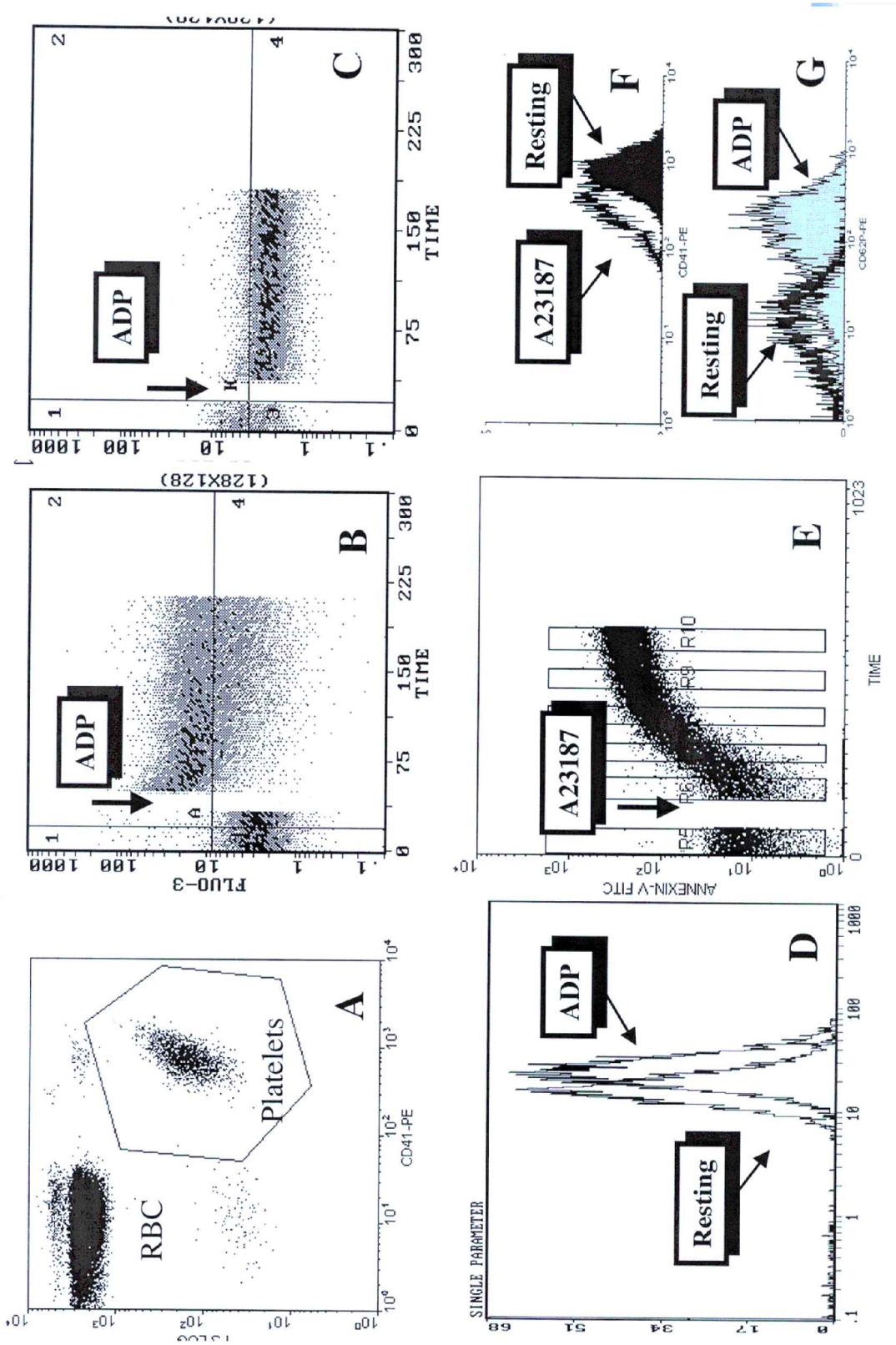

Figure 3. Example of an integrated (multiple tubes) flow cytometric assay of biochemical parameters: Study of platelet activation in whole blood. (A) Platelets are identified in whole blood with an antibody specific for glycoprotein complex gpIIb-IIIa (CD41-PE). (B) Rise in cytoplasmic Ca^{++} detected by the green fluorescent chelator Fluo-3 AM following platelet activation with ADP. (C) Rapid degranulation response upon platelet activation followed by kinetic analysis of loss of the complexity-dependent signal SS. (D) Rearrangement of cytoskeleton (change of actin G to actin F) using FITC-labelled phalloidin in platelets fixed following addition of ADP. The biochemical events depicted in panels B–D are considered very early events in the functional changes induced in platelet by activating agonists. The surface expression of phosphatidylserine can be analyzed kinetically with FITC-labelled annexin V following addition of calcium ionophore A23187, an experimental model of induction of platelet pro-coagulant surface (E). Under these experimental conditions, platelets release membrane microparticles, as evidenced by the loss of constitutive membrane glycoprotein CD41 (F). Data in this graph are obtained by displaying CD41 fluorescence in annexin V-positive cells at the last of the time slices defined in the plot of panel D. The overexpression on platelet surface of the α-granule protein gmp140 (CD62-P) is one of the latest sequential biochemical changes following pseudophysiological platelet activation with ADP (G).

analysis allows simultaneous quantification of multiple analytes in solution. The basis for each measurement consists of a set of microspheres identifiable by embedded fluorophores. Individual sets of microspheres are modified with reactive components such as antigens, antibodies, or oligonucleotides, and then mixed to allow multiple independent reactions to be analyzed simultaneously. The use of microspheres with different ratios of red and orange fluorescence provides the multiplexed format, and FCM analysis simultaneously identifies both the microsphere type and the fluorescent green signal, revealing the capture of the particular analyte (*45*). This measurement system can analyze up to 64 analytes in a single sample.

With the microsphere-associated technology, the applications for basic and clinical flow cytometry in the future are enormous. For instance, the system has been used to perform simultaneous detection of multiplex-amplified human immunodeficiency virus type 1 RNA, hepatitis C virus RNA, and hepatitis B virus DNA (*46*). This approach has been found to be more accurate, sensitive, and reproducible than the conventional microtitre ELISA for qualitative and quantitative immunoassays for several proteins. For instance, this assay can accurately quantitate 15 cytokines in 100 μL-samples, whereas the same analysis by ELISA requires 1.5 mL (100 μL for each cytokine assay) (*47*). Also, multiplexed flow cytometric analyses have been developed to measure simultaneously cytokine receptor expression, internal cytokine expression, and cytokine secretion by activated T-cells in vitro (*48*), thus opening an interesting approach to the study of cell activation responses.

A series of novel applications illustrates the potential of genomic analysis with microsphere arrays and FCM using subnanomolar concentrations of sample in small volumes at rates of one sample per minute or faster, without a wash step. Thus, the system has been used to perform DNA sequence analysis by multiplexed competitive hybridization of sequence-specific oligonucleotide probes (*49*) and for multiplexed analysis of dozens of single nucleotide polimorfisms (*50*). These results demonstrate the sensitivity and accuracy of flow cytometry-based minisequencing, a powerful new tool for genome- and global-scale SNP analysis.

There is a significant number of websites dedicated to basic FCM that should be visited to obtain further information as well as related links and FCM freeware. For the sake of brevity, readers are encouraged to bookmark http://www.biochem.mpg.de/valet/cytorel.html (Cytorelay, Max-Planck Institut for Biochemistry); http://flowcyt.cyto.purdue.edu/ (Purdue University Cytometry Laboratories), and http://www.bio.umass.edu/mcbfacs/flowhome.html (Flow Cytometry Facility, University of Massachussets at Amherst).

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