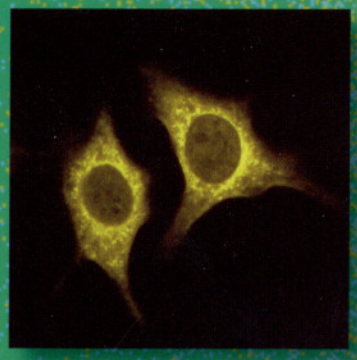
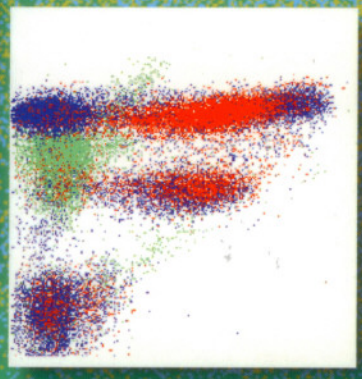
Ulrich Sack Attila Tárnok Gregor Rothe (Eds.)

# Cellular Diagnostics

## Basic Principles, Methods and Clinical Applications of Flow Cytometry





KARGER

IFCC International Federation of Clinical Chemistry and Laboratory Medicine

Sack U, Tárnok A, Rothe G (eds): Cellular Diagnostics. Basics, Methods and Clinical Applications of Flow Cytometry. Basel, Karger, 2009, pp 29–52

# Concept Developments in Flow Cytometry

Günter Valet

Max Planck Institute for Biochemistry, Martinsried, Germany

## Introduction

Cytometry fundamentally influences the development of modern biosciences because even our highly detailed knowledge on the sequenced genomes does not currently explain how the coded biomolecules assemble in order to ultimately develop the architecture and function of living cells. The multitude of theoretical possibilities for the formation of biostructures and the highly compartmentalized functionality of complex metabolic pathways limit the precise understanding of the molecular networks in living cells by deductive hypothesis and mathematical modeling. In this context, cytometry opens the possibility of performing high-speed simultaneous measurements of several molecular parameters of single cells in heterogeneous cell mixtures consisting of many different cell types. This can be done under fairly physiological conditions. Comparing cell measurements from healthy and diseased individuals permits detection of disease-associated changes in molecular expression or functionality in the form of differential molecular cell phenotypes. Measurement parameters can be selected according to system analysis concepts rather than to prove or disprove a particular molecular pathway hypothesis. For example, data analysis can be focused on the capacity of cell parameters to predict the therapy-dependent outcome of disease. Such parameters and parameter patterns can be used for inductive hypothesis development in an effort to unify the observations in particular molecular pathways and to understand the molecular causes of disease.

Given the multitude of published articles in the field, this review predominantly centers on work that initiated essential further developments in flow cytometry.

## Cytophotometry

The stimulus for the development of cytophotometric instrumentation originated from endeavors to distinguish cells from healthy and diseased organisms based on

their molecular properties [1]. Cells in such studies were typically attached to microscopic glass slides. Light absorption at a given wavelength was measured by stepwise overlap-free motion of the microscope stage in front of the opening of a small diaphragm in the focus plane of the microscope. The integrated optical density of a given area of interest such as the cell nucleus could be determined in this way. The wavelength was between 250 and 260 nm to determine the cellular DNA content from the photometric absorption of the DNA bases. The use of the stoichiometric Feulgen stain [2] with a maximum light absorption between 550 and 570 nm [3] permitted measurements without ultraviolet (UV) optics as required otherwise. The cell protein content was determined at 280 nm by the light absorption of aromatic amino acids like tyrosine, tryptophan and phenylalanine. The Zeiss-UMSP1 universal microspectrophotometer (Oberkochem, Germany) was frequently used for such investigations. The comparatively slow speed of measurement of between 5 and 10 min per cell nucleus or cytoplasm area, however, precluded the investigation of larger cells numbers.

## Flow Technology

Blood cell counters worked much faster, counting between 500 and 5,000 cells/s. Light scatter [4, 5] or electrical voltage pulse counting during the transit of electrically nonconductive blood cells in physiological saline suspension through a cylindrical capillary orifice of typically between 70 and 100 μm as in the Coulter [6, 7] were used to enumerate erythrocyte, leukocyte and thrombocyte concentrations in blood. As the electrical pulse height is proportional to cell volume, cell volume distribution curves can be recorded as well. Furthermore, the absolute cell volume can be calculated from capillary geometry, conductivity of the suspension medium and cell form during capillary transit. The first cell sorter, for example, used electrical cell sizing to sort erythrocytes according to cell volume [8].

## Impulse Cytophotometry

A significant improvement compared with slide-oriented cytophotometry was achieved by aspirating cells into a laminar and hydrodynamically focused [9] narrow beam through the capillary orifice of a measurement chamber at a speed of around 1 m/s. Following specific staining with a fluorescent dye, the cell beam can be axially Koehler epi-illuminated through a 40× or 63× high numerical aperture objective by UV light from a high-pressure mercury arc lamp (HBO-100, Osram, Munich, Germany) [10]. The emerging fluorescence is observed in the focus plane of the microscope through the same objective as for epi-illumination through a confocal pinhole

to lower the amount of captured fluorescence noise around the cell beam. The fluorescence pulses from the stained cells are amplified by a photomultiplier tube, followed by analogue-to-digital conversion and classified according to their maximum amplitude or pulse area in a multichannel analyzer.

The first commercial flow cytometer, the ICP-11 impulse cytophotometer (Phywe Company, 1969, Göttingen, Germany) [10], typically measured the fluorescence of several thousand cells per second. Two photomultipliers permitted simultaneous collection of fluorescence from two types of molecules of similar fluorescence excitation but different emission characteristics. The separation of the emitted fluorescence light was achieved by a wavelength-sensitive dichroic mirror and long- and short-pass filters that collected the light in defined wavelength windows in each of the two light paths. Cell doublets and cell aggregates were eliminated by setting thresholds for the pulse height over pulse area ratio [11, 12], a procedure that is widely used today in flow cytometers or cell sorters.

The ICP-11 instrument [10, 13] was available at a time when doubts still existed as to whether fluorescence cytometry would be able to reach the same sensitivity as absorption cytometry using specific cytochemical cell staining [14–16]. Progress in absorption cytometry led to the development of the Hemalog-D [17, 18] blood cell analyzers (Technicon, Tarrytown, NY, USA). Although its technology is still used in today's instruments, most researchers considered fluorescence the more promising approach.

As a consequence, the Bio/physics CytoFluorograph (Bio/Physics Systems Inc, Mahopac, NY, USA) with a 488-nm argon ion laser for fluorescence excitation was developed (1970) from the initial Cytograph absorption cytometer with its 633-nm HeNe laser light source for light absorption measurements in cells.

The Phywe ICP-11 and ICP-22 as well as the Biophysics developments were purchased by Ortho Diagnostics (Johnson and Johnson Unternehmensbereich, Westwood, MA, USA) in 1976. The CytoFluorograph development was continued while the Phywe development disappeared from the market. The Coulter Company (Miami, FL, USA) followed with the EPICS cell sorter in 1977, and Becton Dickinson (Mountain View, CA, USA), with the FACSIV cell sorter [19] in 1978.

The Partec Company (Münster, Germany) was at the origin of the early Phywe ICP-11/ICP-22 development and continued the development of flow cytometers on its own after the takeover of Phywe by Ortho Diagnostics. A closed piezocrystal cell sorter [20–22] became available around 1986, proving particularly useful for sorting large particles (pancreatic islands [23]) and infectious material. Partec also developed various new types of instruments like the PASI, PASII, PASIII flow cytometers with different possibilities for illumination, measurement and piezo cell-sorting chambers as well as a number of analysis parameters.

## Nomenclature

The variety of initial nomenclature and publication languages for the new technology explains the difficulty of gathering an adequate impression of the early developments, especially in Europe. Terms like 'Impulsfluorimetrie' [13, 24], 'Impulsfluorometrie' [25], 'Impulszytophotometrie' [26, 27, 29, 30], 'Impulsmikrophotometrie' [28], 'pulse cytophotometry' [30], 'micro-flow fluorometry' [31], and 'microflow fluorometry' [32] were used in Europe whereas 'flow cytometry' [33], 'flow microfluorimetry' [34] or 'flow microfluorometry' [35] were preferred in the USA. The term 'flow cytofluorometry' [36] was used both in Europe and the USA. A further term, 'flow DNA analysis' [37], was also coined.

The term 'flow cytometry' was accepted in 1976 by an international consensus at the 5th American Engineering Foundation Conference in Pensacola, FL, USA. Due to the nomenclature change and the publication habits, especially of the German proponents of flow cytometry, a significant part of the groundbreaking work in the technical, and especially in the clinical areas is not adequately appreciated. In view of the various terms, the ISI Web of Knowledge during the first decade of flow cytometry (1969–1978) mentioned 60 articles for the various European terms versus 44 articles for the terms flow cytometry, flow microfluorimetry, flow cytoflorimetry and flow cytofluorometry, predominantly used by US authors. This comparison does not take into account the significant number of European articles in catalogued books and periodicals that were not listed by the Institute for Scientific Information (ISI).

## Clinical Impulse Cytophotometry

A first meeting of the growing group of clinical scientists interested in 'impulse cytophotometry' was organized by Michael Andreeff in 1972 in Heidelberg, Germany, with follow-up meetings in Nijmegen, The Netherlands (1973), Münster, Germany (1975), Vienna, Austria (1977), Voss, Norway (1979) and Rome, Italy (1980). The organizers were Clemens Haanen, Wolfgang Göhde, Dieter Lutz, Ole Laerum and Francesco Mauro. The scientific contributions were edited and published by C.A.M. Haanen, H.F.P. Hillen, J.M.C. Wessels (*Pulse Cytophotometry I*), W. Göhde, J. Schumann, T. Büchner (*Pulse Cytophotometry II*) and D. Lutz (*Pulse Cytophotometry III*) and printed by European Press, Ghent, Belgium in 1975, 1976 and 1978, respectively. *Flow Cytometry IV* was edited by O.D. Laerum, T. Lindmo, E. Thorud and printed by Universitetsforlaget, Bergen, Norway in 1980. A reprint of this book appeared in *Acta Pathologica et Microbiologica Immunologica Scandinavica A, Supplement* (1981;274:1–535). The abstracts of the Rome Congress were edited by F. Mauro and G. Mazzini for *Basic and Applied Histochemistry* (1980;24:229–398). A separate congress proceedings monograph with the publications of these presentations is not available.

The significance of the early flow cytometry congresses held between 1972 and 1980 is shown by the fact that 20 of the cited publications in this review mark conceptual focus points for later developments. Seven additional articles in *The* Journal of *Histochemistry and Cytochemistry* represent European contributions at the American Engineering Foundation Congresses, which were organized during the same period and alternated between the USA and Europe (*http://www.isac-net.org/content/category/4/132/42*).

The Society for Analytical Cytology (SAC) was founded in 1978 during the 6th American Engineering Foundation Conference on Automated Cytology in Schloss Elmau near Mittenwald (Germany). This resulted in the initially controversial tendency to abandon the organization of cytometry meetings in Europe and to work within SAC. SAC congresses were subsequently organized in the USA and Europe, and the journal *Cytometry* was founded in 1980.

The European scene was reorganized between 1985 and 2000 in the form of national cytometric societies in Italy (GIC, SICICS), France (AFC), Portugal/Spain (SIC), Germany (DGfZ), Denmark, UK (section of the Royal Microscopic Society, RMS), Belgium (BVC/ABC), Switzerland (SCS), Sweden, Poland (PCS) and Austria (OEGfZ) totaling more than 2,000 members to date. In addition to SAC, the significance of national and regional organizations is evident, as shown by the foundation of the European Society for Analytical Cellular Pathology (ESACP) in 1986 with its journal *Analytical Cellular Pathology* (ACP) published since 1989, and the foundation of the European Working Group for Clinical Cell Analysis (EWGCCA) in 1996, which cooperates with the *Journal of Biological Regulators and Homeostatic Agents* (JBRHA) since 2002. Quality assurance for clinical immunophenotyping and other clinical cytometry applications at the European level started with the collaboration between EWGCCA and Eurostandards/UK-NEQAS (Sheffield, UK) and resulted in the foundation of the European Society for Clinical Cell Analysis (ESCCA) in 2007 and participation in the journal *Cytometry Part B* (*Clinical Cytometry*). ESACP and the Society for Diagnostic Quantitative Pathology (ISDQP) merged in 2003 as the International Society for Cellular Oncology (ISCO) with the journal ACP reappearing as *Journal of Cellular Oncology* (JCO). SAC emphasized their international ambition by changing their name to International Society for Analytical Cytology (ISAC) in 1990 and, under the same acronym, to International Society for Advancement of Cytometry in 2008.

The changes in organizational frameworks mirror the remarkable developments in flow and image cytometry.

### Further Instrument Developments

During the initial expansion phase of the ICP-11 flow cytometers, several research groups developed their own instrumentation because some of the measurement

tasks were only partially possible with the ICP-11 and its successor ICP-22. The HBO-100 mercury arc lamp was suitable for measurements in the UV fluorescence excitation range, but no light scatter or electrical cell volume measurements were possible. It was also difficult to measure the low-intensity immunofluorescences of fluorescein isothiocyanate (FITC)-labeled antibodies on cells with the limited excitation intensity of the mercury arc lamp between 470 and 490 nm. The CytoFluorograph (Bio/Physics 1970, Ortho Diagnostics 1976) as well as the Coulter EPICS and the Becton Dickinson FACS instruments were typically equipped with argon ion laser fluorescence excitation at 488 nm and captured small-angle (1–3 degrees) forward-scatter and orthogonal (90 degrees) side scatter light.

The necessity to determine more than two fluorescence parameters led to the development of the first double-laser [38] flow cytometer and cell sorter at the Deutsches Krebsforschungszentrum (DKFZ, Heidelberg, Germany). An instrument with a particularly tightly bundled laser beam [39, 40] for fast and precise length measurement of cells and cell aggregates was developed by the Gesellschaft für Strahlenforschung (GSF), Hanover, Germany, and later commercialized by Kratel Instrumente (Stuttgart-Leonberg, Germany).

The Metricell [41] and Fluvo-Metricell instruments [42] were developed at the Max Planck Institute for Biochemistry to determine relative analyte concentrations in cells and average surface densities of molecules on the cell surface. A measurement chamber with a hydrodynamically focused electrical sizing orifice for the fast determination of cell volumes was initially combined with the optical bench of an ICP-11 instrument. A multichannel analyzer and a computer served for histogram display and storage of listmode data [43]. The Fluvo-Metricell was subsequently redesigned with a new optical setup, a Z-80 microprocessor computer, and manufactured by HEKA Elektronik (Forst/Weinstraße, Germany) between 1985 and 1990.

The generally observed right skew of erythrocyte volume distribution curves by electrical sizing with Coulter measurement capillaries initially presented a significant obstacle for the precise characterization of normal or abnormal erythrocyte populations, in particular the analysis of mixed, discrete erythrocyte populations of various size as sometimes found in newborn or x-irradiated mammalian organisms, after a hemorrhage or induced erythropoiesis in the post-partum phase [44]. Several cell volume peaks were observed under these conditions for certain time periods.

Significant work on the right skew issue led to the development of the piezo-crystal-driven [45] droplet cell sorter [8]. The exact cause of the artifact was finally elucidated by high-speed photography of native and fixed erythrocytes during their passage through the orifice using microsecond laser pulses [46] or an argon arc flashlight [47]. These investigations revealed that the right skew is caused by cells flowing close to the edge of the capillary entrance through zones of increased electrical field strength [9, 46, 48], leading to M-shaped electronic

pulses. The electronic rejection of M-shaped pulses [49], the use of short measurement capillaries, or the widening of the orifice outlet side [50] of the capillary only diminishes the right skew, while hydrodynamic focusing of the cells as a narrow cell beam through the orifice reliably avoids it altogether [9, 51–53]. AEG Telefunken (Ulm, Germany) used hydrodynamically focused electrical sizing [51] in its AEG-Telefunken particle analyzer (1972). The AEG development was acquired by the Coulter Company (Miami, FL, USA) and subsequently taken off the market.

The AEG engineers observed a transcellular ion flux through erythrocytes or nucleated cells during elevated electrical-field strength in the measurement capillary. The ion flux is caused by a temporary dielectric breakdown of the cell membrane [54–56]. These observations are used for controlled molecule transport through cell membranes by electroporation [57], a method frequently applied for transfection in molecular biology.

High-speed photography enables fast imaging in cell flow [58], an idea taken up recently as high-throughput technology by the ImageStream100 instrument (Amnis, Seattle, WA, USA) [59], taking advantage of far more sensitive imaging technologies. The narrow focusing of the fluorescence exciting laser beam to a focus of around 0.5 μm permits the determination of single-cell [60] or chromosome [61] slit-scan profiles in flow.

The requirement for fast signal processing, histogram display and signal ratios led to the use of software-driven microprocessors [62–64] instead of a hardware circuit or computer control. The early modular instrumentation was partly developed by Dr. O. Ahrens Meßtechnik (Bargteheide, Germany), a company which continues to develop DNA image analysis systems.

The interest in time- and temperature-controlled flow-cytometric cell function experiments led to the development of a particularly adapted laser flow cytometer and cell sorter in Cambridge (UK) [65]. Besides several lasers, particular measurement chambers for the sensitive fluorescence light collection from large spatial angles were developed [66, 67].

The measurement of microorganisms like bacteria or yeast cells [68] initiated the development of a particularly sensitive epi-illumination system [69] with an HBO-100 mercury arc lamp for fluorescence excitation in Oslo (Norway). The instrument was successively produced under the names MPV flow cytometer by Leitz (Wetzlar, Germany), Argus100 by Skatron (Tranby, Norway) and Bryte HS by BioRad-Laboratories (Hercules, CA, USA).

Bruker-Odam (Wissembourg, France) produced the ATC3000 flow cytometer and cell sorter between 1990 and 1993. The instrument was developed under the patronage of the French Commissariat à l'Energie Atomique (CEA) with a hydrodynamically focused electrical cell-sizing chamber. Fast graphics with the possibility to evaluate a high number of simultaneous histogram windows of polygonal or elliptic shape represented the particular feature of this instrument.

Ethidium bromide (2,7-diamino-10-ethyl-9-phenylphenantridiniumbromide) [13, 24], acriflavin-auramine [70], propidium iodide [71], mithramycin [72], ethidium bromide-mithramycin [73], chromomycin A3 [74], acridine orange [75, 76], DAPI (4′,6-diamidino-2-phenyindol) [77], Hoechst 33342 and Hoechst 33258 (2,6-bis-benzimidazole derivatives) [78] for DNA measurements or DANS (1-dimethylamino-naphthalene-5-sulfonic acid) [24] and FITC [79] as fluorescent protein stains were introduced for flow-cytometric measurements. DNA against protein was the first two-parameter fluorescence combination in flow cytometry [24], including mathematical histogram analysis [80, 81].

Cell nuclei were prepared from biological tissues by digestion with 0.5% acid pepsin solution at pH 1.8 [29, 82] or with pronase [83]. Alternatively, cellular RNA was removed by tissue treatment with 0.1–1% RNase [13, 29, 30, 84]. High and low salt concentrations at pH 10 and pH 5.8 in the presence of RNase and detergents [85], or trypsin in combination with detergent [86] proved useful for the measurement of narrow DNA distribution curves of cell nuclei. The successful enzymatic preparation of cell nuclei from paraffin block [83] material permitted to access archive material from pathological institutes, a methodology that became quite popular later on [87].

The low coefficients of variation (CV = 100 × standard deviation/mean) (see also 'Quality Control and Standardization', pp. 159) of the DNA distribution of cell nuclei as obtained by measurements with Phywe or Partec instruments, and the use of the intensive ethidium bromide-mithramycin fluorescence staining [73, 88] finally opened the way for the analytical and preparative x- and y-sperm cell separation [89, 90] and flow-cytometric chromosome analysis [91, 92] with mercury arc lamp flow cytometers.

The clinical interest in cellular DNA measurements was often related to the detection of DNA aneuploidy as an indicator of malignancy. The degree of aneuploidy was expressed by the DNA index. Furthermore, the fraction of cells in the S-phase of the cell cycle was determined when possible [29, 30, 82, 93–95]. The characterization of precancerous lesions [96], stomach cancers [27, 97], leukemias and lymphomas [93, 98–100] or abnormal granulopoiesis or erythropoiesis [101] and the measurement of synovial [102], skin [32] or bladder cancer [103, 104] cells account for the immediate and widespread clinical interest in the new technology.

The use of DNA aneuploidy and S-phase determinations in daily clinical routine remainedlimited, however [105–109] despite the concerted efforts of many scientists, reflected in more than 1,000 clinically oriented scientific publications since 1969.

## Chromosome Analysis, FISH

The use of intensive and narrowly focused lasers allowed the analytical and preparative separation of chromosomes [35, 110–113] to establish specific DNA libraries as

one of the preconditions for the subsequent human genome project. Fluorescence in situ hybridization (FISH) was important for the visualization of specific DNA strands in chromosomes [114–117].

### DNA Cell Cycle Analysis, Micronuclei, Hematopoietic Stem Cells

An essential part of the early experimental work was devoted to DNA cell cycle analysis under different growth conditions of cells, especially during and after the action of cytostatic drugs like Velbe [24], daunomycin [26], bleomycin [118], combinations of Adriamycin and bleomycin [79] or after ionizing irradiation [79, 119–121]. Cell cycle duration [122], synchronization within the cell cycle by x-irradiation and daunomycin [123], mechanisms of contact inhibition [124, 125] and lectin (concanavalin A)-induced cell agglutination [126] concerned other areas of the initial interest.

The flow-cytometric bromodeoxyuridine (BrdU)/Hoechst 33258 fluorescence quenching technique [127] offered a fast and excellent nonradioactive alternative for the study of cell regulation, similar to the use of fluorescence-labeled anti-BrdU antibodies [128–130]. The monoclonal Ki-67 antibody for the analysis of cell proliferation in healthy and diseased tissues was frequently used [131].

The flow-cytometric determination of micronuclei in peripheral blood or in tissue culture opened new possibilities for the evaluation of the mutagenic potential of substances, cytostatic drugs or ionizing radiation [132].

Hematopoietic stem cells were characterized and enriched based on their light scatter properties [133] and antibody-binding characteristics [134] following centrifugal elutriation and cell sorting.

### Predictive Cytology and Cytopathology by DNA Image Cytometry

While flow-cytometric DNA analysis is typically faster and superior in precision to DNA image cytometry on Feulgen stained-cell nuclei, the combination of the morphological analysis of normal, dysplastic or tumor cells with DNA measurement for aneuploidy provides sensitive information. Several investigations show that DNA aneuploidy in dysplastic lesions of the lung [135], larynx [136] and cervix uteri [137] predisposes a high percentage of cases to future malignant tumors. DNA image cytometry also permits to reliably detect few DNA aneuploid cells as an early sign of tumor relapse [138].

DNA image cytometry is therefore increasingly used as a reference method for the detection of malignant cells in cytological slides [139, 140], with a better discrimination rate for malignant cells by DNA measurement as compared to the exclusive use of morphological criteria. DNA image cytometry achieves >95% correct

single-case predictions for the development of subsequent malignancies in oral leukoplakia [141, 142].

Quantitative DNA image cytometry paves the way for cytology and cytopathology in predicting malignant disease, with the apparent exception of DNA euploid malignant tumors, which cannot be detected this way. This suggests that the early recognition of DNA aneuploid cells by DNA image techniques may finally be of more clinical value to the individual patient than the preferred flow-cytometric investigations.

### Immunophenotyping

The discrimination of lymphocytes, monocytes and granulocytes by their different forward and sideward light scatter characteristics [143, 144] is an essential prerequisite for the generalized use of flow-cytometric immunophenotyping in clinical medicine and research. The technique combines the differential binding of fluorescently labeled antibodies [145] and fluorescence compensation to remove overlap in the individual light collection paths of the flow cytometer [146], and may include cell sorting [145, 147].

### Fluorescence Anisotropy, FRET, Polarized Light and Raman Scatter

Fluorescence anisotropy [148] and fluorescence resonance energy transfer (FRET) [38, 88, 149–152] in single cells can be determined by flow cytometry to investigate membrane fluidity and the spatial proximity of biomolecules, including their degree of interaction. The degree of polarization of scattered light [153] from leukocytes permits stain-free discrimination of lymphocytes, monocytes, granulocytes, and basophil and eosinophil granulocytes [154] in diluted blood. Confocal Raman microscopy permits label-free visualization of the functionality of proteins, lipids or other molecules in viable cells [155].

### Measurement of Cell Functions

The interest in cell functions as fast-reacting parameters for cell biochemical, cell physiological or clinical alterations of cells led to the development of fluorescence indicator molecules for various specific cell functions. The indicators frequently pervade the cell membrane in a diffusion-controlled way as electrically uncharged and nonfluorescent precursor molecules. The fluorescent indicator molecules of positive or negative electric charge are released following intracellular enzymatic cleavage or

activation by other mechanisms. Positively charged molecules tend to auto-accumulate inside of cells or organelles due to the electrically negative transmembrane and mitochondrial membrane potentials while negatively charged molecules are easier to expel by the cells through active excretion or diffusion. The tendency of positively charged molecules to auto-accumulate significantly increases their detection sensitivity.

The flow-cytometric determination of esterases with fluorescein diacetate [17, 156, 158, 159], of phosphatases and β-d-glucuronidase [160], the simultaneous determination of esterases, phosphatases (umbelliferone phosphate) [161, 162], and peptidases and transpeptidases [163, 164] in flow, represented initial challenges for enzyme activity assessment in single cells. Simultaneous DNA staining of viable cells with Hoechst 33342 and in dead cells with propidium iodide discriminated both types of cells in the same assay [165] while phagocytosis of fixed and FITC-labeled bacteria [166] or of monodisperse fluorescence particles [167] or viable bacteria [168] provided insight into cell function changes during phagocytosis.

Interest in the intracellular pH value as an indicator of the metabolic state of viable cells in experimental and clinical settings led to the use of fluorescent pH indicator substances like fluorescein, which maintains a pH-dependent fluorescence excitation spectrum at a constant emission range. This required the sequential excitation of cells in the flow cytometer with a single laser in two runs of a cell batch at two different wavelengths. The observed fluorescence excitation ratio from the double wavelength excitation represents a measure of the average intracellular pH of all cells [169], but not that of a single cell. The technically more demanding setup of two sequential laser beams in the same instrument, with 4-methylumbelliferone [170]) as pH indicator, provided single-cell pH values. The earlier alternative idea of using substances with pH dependent fluorescence emission spectra such as 1,4-dicyano-hydroquinone [171]) required only a single light source and two fluorescence emission channels to determine intracellular pH values from fluorescence emission ratios. The use of fluorescence emission ratio dyes made intracellular pH measurements accessible to most standard flow cytometers without hardware modification. The use of fluorescence emission ratios determined from various emission channels has become common practice [172] for many flow- and image-cytometric applications.

Further challenges concerned the determination of cell stimulation from changes of intracellular $Ca^{2+}$ levels (Indo 1 [173]), stopped-flow calcium kinetics [174, 175], the assessment of oxidative burst activities with dihydrorhodamine 123 [176] or hydroethidine [177]), of intracellular free glutathione with *o*-phthaldialdehyde [178] or monobromobimane with N-ethylmaleimide protein thiol group blocking [179] as indicators of the reductive cell potential, as well as of the negative surface charge density with FITC-fluoresceinated polycations like polylysin or polyornithine as a measure of the electrophoretic mobility of cells [180] (see 'Determination of Cell Physiological Parameters: pH, $Ca^{2+}$, Glutathione, Transmembrane Potential', pp. 325).

The accurate determination of protease activities in viable cells was of interest for granulocyte function studies in intensive-care patients as early indicators of imminent sepsis or polytraumatic shock [181]. Efforts to increase sensitivity and specificity of the initially used fluorogenic endopeptidase substrate (Z-$Arg_2$-4-trifluoromethyl-coumarinyl-7-amide [182] led to the development of the significantly more sensitive and specific rhodamine110 proteinase substrates for serin-, cystein- [183–185] and aminopeptidases [186].

Of further interest were not only cell function assays for the assessment of the effect of cytostatic drugs on patient cells [187] but also the simultaneous determination of cell concentration and function of blood lymphocytes, monocytes, granulocytes, erythrocytes and thrombocytes [188, 189] in peripheral blood samples. The long-lasting lack of an absolute cell counter device in commercial flow cytometers led to the addition of a known amount of fluorescent monodisperse microparticles as internal counting and fluorescence standard for flow-cytometric measurements [189], a method that is widely used in the clinical environment under the name of 'single-platform absolute cell counting'. In flow cytometers with an integrated absolute counting feature [190], monodisperse fluorescent particles remain useful to monitor the long-term performance of fluorescence and light scatter measurements.

### Apoptosis

In situ nick translation with DNA polymerase I in the presence of either fluorescein-12-dUTP (uridine triphosphate) or digoxigenin-labeled 11-dUTP (deoxyUTP) opened the way for the flow-cytometric assessment of DNA fragmentation during apoptosis [191].

### Microbiology and Biotechnology

The interest in flow-cytometric determination of microorganisms led to the detection of DNA, RNA and protein in yeast cells [192, 193] and bacteria [68] as an early effort toward biotechnology as well as food quality testing and antibiotics efficiency assessment.

### Data Analysis

Data analysis in flow cytometry is of major importance for the evaluation of the histograms or of multiparametric listmode data. The initial interest concerned the exact determination of cell fractions in various phases of the cell cycle (cell cycle analysis)

[80, 194] from one-parameter DNA histograms and the mathematical analysis of two-parameter flow-cytometric DNA/protein measurements [81]. Furthermore, one- [195, 196], two- [180] or three-parameter [197] linear or logarithmic Gaussian distributions were adapted to single or multiparametric flow cytometer measurements in order to simplify the results as well as the development of scientific hypotheses concerning the biological regulation of various cell populations by the organism.

Besides the above primary data evaluation, significant efforts have been invested in extracting further information or knowledge from the measurements, usually by parametric mathematical or statistical methods like cluster [198, 199] or principal-component analysis [200], multivariate statistics [201–203], knowledge-based [204, 205] or hierarchical classification [206–208], fuzzy logic [201, 209], neuronal networks [201, 210–212] or self-organizing matrices [212].

These methods depend in part on mathematical assumptions for observed value distributions, for example, that they are Gaussian. Frequently, multipoint clusters in multidimensional space are evaluated in which the coherence of the data points of the individual experiment or patient is lost. Data complexity in this instance is tentatively reduced by making *models* to evaluate the conceptually most promising parameters or those being most significantly different between experimental series or patient groups by trial and error. The consequence for medicine is that therapy-dependent extrapolations for predicting the course of a disease are expressed as *prognoses,* indicating the *statistical* future of patient groups (Kaplan-Meier statistics) but not, as would be desirable, the *individualized future* of a given patient before the start of a calculated therapy.

Individualized patient prognosis is obtained by the linked evaluation of the multiparameter values (data pattern) of each patient by algorithmic (nonparametric) data pattern analysis (DIAGNOS1 [213], CLASSIF1 [214–216]). In case of large numbers of available parameters per patient, they are split into portions of, for example, 50 parameters. Then, at the first level, the five most discriminatory parameters between the investigated patient groups are determined for each parameter portion. At the second evaluation level, the most discriminatory parameters of the first level are subsequently merged with 50 new parameter portions. The five most discriminatory parameters of each portion of the second level are merged with the third level, and so on until the most discriminatory data pattern of all parameters is obtained at the last level.

This hierarchical information concentration of data pattern analysis can be imagined as a multilevel sieve cascade with increasingly coarser mesh size (data sieving). The calculations can be automated and parallel computed in an unattended way with an essentially unlimited number of parameters. Data sieving does not depend upon data models. The resulting knowledge extraction is of importance for standardized flow-cytometric diagnostics as well as for therapy-dependent predictions of disease course in individual patients, i.e. predictive medicine, personalized or individualized medicine.

## Predictive Medicine by Cytomics

The concept of predictive medicine by cytomics is a consequence of the initial observation that the results of cell function measurements provided a >80% correct extrapolation for the later occurrence of sepsis, posttraumatic shock, an intermediate state or normal recovery in surgical intensive-care patients three days in advance [181].

The multimolecular cytometric analysis of the heterogeneity of cells and cell systems (*cytomes, system cytometry*) [217], in conjunction with exhaustive bioinformatics knowledge extraction (*cytomics*), constitutes the basis of this concept [218–223]. Likewise, the analysis of data from flow-cytometric multiplex particle arrays, multiparameter cell-oriented DNA or proteomics arrays, and of clinical and clinical chemistry or image analysis data is equally possible. This permits generalized access to therapy-dependent predictions for the further course of disease in individual patients, often with >95% accuracy. These predictions may, among others, reduce tissue damage or tissue loss, thus supporting the efforts of preventive medicine. Improved adaptation of therapy to the individual patient also permits the pretherapeutic exclusion of therapy-resistant patients. Additionally, there is the potential to reduce adverse drug effects.

Reverse engineering of the predictive data patterns by cell systems biology [224] has the potential to analytically explore molecular disease courses in patients instead of exploring them in disease models that are not necessarily representative of the patient's situation. This may lead to the discovery of new drug targets for the pharmaceutical industry [225]. Such goals can furthermore be realized within the framework of the proposed human cytome project [226].

## Outlook

Rapid technological progress is currently leading to a merger between flow and image cytometry, integrating them as cell-oriented bioinformatics into cytomics. Examples of this development are the fast acquisition of fluorescence images in a flow cytometer [59], the laser scanning cytometer [227] (see 'Technical and Methodological Basics of Slide-Based Cytometry', pp. 89), and the 4Pi-microscope [228]. Chip and particle arrays [229], instrument miniaturization by nanotechnologies and progress in bioinformatics generate an increasing interest in the molecular analysis of single cells and their molecular environment.

The interaction with systems biology [230, 231] opens new potentials as biomedical cell systems biology [232, 233] for modeling disease pathways and optimizing therapy.

The fascination with the biocomplexity of intact cells as the organism's elementary function units and their intriguing molecular analysis will remain a primary

driving force behind this cross-disciplinary approach. Concerning the medical field, implementing the acquired knowledge for the patients' benefit will be a major goal.

## Summary

The development of flow cytometry has provided an important driving force for the advancement of molecular single-cell research in biomedical and clinical domains. Continually evolving research concepts for instrument development combined with the analysis of multiparameter results from immunological data, cell function tests, DNA measurements, and microbiological and biotechnological applications have profoundly shaped this area of research. They remain essential prerequisites for the solution of future scientific challenges.

Flow cytometry addresses the high complexity of the assembled molecular architecture of single cells and cell systems (cytomes). Molecular cell phenotypes develop during the life of an organism and represent the cumulative result of genotype and exposure influences. This makes them particularly interesting for biomedical research. The merging of flow and image cytometry with multiparametric bioinformatics into cytomics initiates a new approach to predictive medicine. Some of its potentials include therapy-dependent prediction of further disease course for individual patients (i.e. personalized, individualized medicine, improved detection of molecular disease pathways, and the identification of new targets in pharmaceutical research and drug discovery. These developments are accompanied by rapid progress in molecular fluorescence technologies and microscopy instrumentation. New insights into the molecular expression of the genomic information derived from the observed heterogeneity of cells and cell systems may contribute to the organization of large scale international research projects such as the human cytome project.

## Acknowledgments

The support of W Göhde, M Stöhr, A Böcking, and A Tárnok in the supply of information concerning the widely distributed and often electronically uncatalogued publications of the initial development phase as well as valuable discussions and comments are gratefully acknowledged.

## References

1 Caspersson TO: Cell Growth and Cell Function: A Cytochemical Study. New York, Norton, 1950, pp 1–185.
2 Chieco P, Derenzini M: The Feulgen reaction 75 years on. Histochem Cell Biol 1999;111:345–358.
3 Kasten FH: The chemistry of Schiff's reagent. Int Rev Cytol 1960;10:1–100.
4 Moldavan A: Photoelectric technique for the counting of microscopic cells. Science 1934;80:188–189.

5 Mullaney PF, Van Dilla MA, Coulter JR, Dean PN: Cell sizing – a light scattering photometer for rapid volume determination. Rev Sci Instrum 1969;40:1029–1032.
6 Coulter WH: Means for counting particles suspended in a fluid. US patent 2656508 priority Aug 27, 1949.
7 Coulter WH: High speed automatic blood cell counter and cell size analyzer. Proc Natl Electronics Conf 1956; 12:1034–1040.
8 Fulwyler MJ: Electronic separation of biological cells by volume. Science 1965;150:910–911.
9 Crosland-Taylor PJ: A device for counting small particles suspended in a fluid through a tube. Nature 1953;171:37–38.
10 Göhde W: Automatisches Meß- und Zählgerät für die Teilchen einer Dispersion. Patent DE1815352, priority date Dec 18, 1968.
11 Göhde W: Process for automatic counting and measurement of particles. US patent 4021117, priority date Jun 23, 1973(DE).
12 Göhde W, Schumann J, Fruh J: Coincidence eliminating device for pulse-cytophotometry; in Göhde W, Schumann J, Büchner Th (eds): Pulse Cytophotometry II. Ghent, European Press, 1976, pp 71–78.
13 Dittrich W, Göhde W: Impulsfluorimetrie bei Einzelzellen in Suspensionen. Z Naturforsch 1969;24b:221–228.
14 Kamentsky LA, Derman H, Melamed MR: Spectrophotometer: New instrument for ultrarapid cell analysis. Science 1965;150:630–631.
15 Kamentsky LA: Discussion remarks; in Evans DMD (ed): Cytology Automation. Proc 2nd Tenovus Symp, Cardiff Oct 24–25, 1968. Edinburgh, Livingstone, 1970, pp 55, 141, 196.
16 Kamentsky LA: Rapid cell spectrophotometry for cell identification and sorting; in Evans DMD (ed): Cytology Automation. Proc 2nd Tenovus Symposium, Cardiff, 1968. Edinburgh, Livingstone, 1970, pp 177–185.
17 Ornstein L, Ansley HR: Spectral matching of classical cytochemistry to automated cytology. J Histochem Cytochem 1974;22:453–469.
18 Mansberg HP, Saunders AM, Groner W: The Hemalog D white cell differential system. J Histochem Cytochem 1974;22:711–724.
19 Fulwyler MJ, McDonald CW, Haynes JL: The Becton Dickinson FACS IV; in Melamed MR, Mullaney PF, Mendelsohn ML (eds): Flow Cytometry and Sorting. New York, Wiley, 1979, pp 653–667.
20 Dühnen J, Stegemann C, Wiezorek C, Mertens H: A new fluid switching flow sorter. Histochemistry 1983;77:117–121.
21 Göhde H, Schumann J: Method and apparatus for sorting particles. US patent 4756427, priority date Sep 11, 1984 (CH).
22 Göhde W: Verfahren und Vorrichtung zur Sortierung von mikroskopischen Partikeln. Patent EP0177718, priority date Aug 08, 1985.
23 Derek WR, Gray W, Göhde W, Carter N, Heiden T, Morris PJ: Separation of pancreatic islets by fluorescence-activated sorting. Diabetes 1989;38:133–135.
24 Göhde W, Dittrich W: Simultane Impulsfluorimetrie des DNS- und Proteingehaltes von Tumorzellen. Z Anal Chem 1970;252:328–330.
25 Göhde W, Dittrich W: Impulsfluorometrie – ein neuartiges Durchflußverfahren zur ultraschnellen Mengenbestimmung von Zellinhaltsstoffen. Acta Histochem 1971; 41(suppl 10):429–437.
26 Göhde W, Dittrich W: Cytostatische Wirkung von Daunomycin im Impulscytophotometrie Test. Arzneimittelforschung1971;21:1656–1658.
27 Schwabe M, Wiendl HJ: Die Bedeutung der Impulszytophotometrie für die Früherkennung benigner und maligner Erkrankungen der Magenschleimhaut. Münch Med Wochenschr 1974;116:1005–1008.
28 Göhde W: Automation of cytofluorometry by use of the Impulsmicrophotometer; in Thaer AA, Sernetz M (eds): Fluorescence Techniques in Cell Biology. Berlin, Springer, 1973, pp 79–88.
29 Reiffenstuhl G, Severin E, Dittrich W, Göhde W: Die Impulscytophotometrie des Vaginal- und Cervicalsmears. Arch Gynäkol 1971;211:595–616.
30 Schumann JF, Ehring W, Göhde W, Dittrich W: Impulscytophotometrie der DNS in Hauttumoren. Arch Klin Exp Dermatol 1971;239:377–389.
31 Farsund T: Preparation of bladder mucosa cells for micro-flowfluorometry. Virchows Arch B 1974;16:35–42.
32 Clausen OPF, Lindmo T, Sandnes K, Thorud E: Separation of mouse epidermal basal and differentiating cells for microflow fluorometric measurements – Methodological study. Virchows Arch B 1976;20:261–275.
33 Barrett DL, King EB, Jensen RH, Merrill JT: Cytomorphology of gynecologic specimens analyzed and sorted by 2-parameter flow cytometry. Acta Cytol 1976;20:585–586.
34 Yataganas X, Mitomo Y, Traganos F, Strife A, Clarkson B: Evaluation of a Feulgen-type reaction in suspension using flow microfluorimetry and a cell separation technique. Acta Cytol 1975;19:71–78.

35 Gray JW, Carrano AV, Moore DH, Steinmetz LL, Minkler J, Mayall BH, Mendelsohn ML: High speed quantitative karyotyping by flow microfluorometry. Clin Chem 1975;21:1258–1262.
36 Stephens SO: Analysis of effect of fluorescence intensity on distributions obtained by flow cytofluorometry. Exp Cell Res 1974;89:228–230.
37 Auer G, Tribukait B: Comparative single cell and flow DNA analysis in aspiration biopsies from breast carcinomas. Acta Pathol Microbiol Scand A 1980;88:355–358.
38 Stöhr M: Double beam application in flow techniques and recent results; in Göhde W, Schumann J, Büchner T (eds): Pulse Cytophotometry II. Ghent, European Press, 1976, pp 39–45.
39 Eisert WG, Ostertag R, Niemann EG: Simple flow microphotometer for rapid cell-population analysis. Rev Sci Instrum 1975;46:1021–1024.
40 Eisert WG, Nezel M: Internal calibration to absolute values in flowthrough particle-size analysis. Rev Sci Instrum 1978;49:1617–1621.
41 Kachel V: Basic principles of electrical sizing of cells and particles and their realization in the new instrument Metricell. J Histochem Cytochem 1976;24:211–230.
42 Kachel V, Glossner E, Kordwig E, Ruhenstroth-Bauer G: Fluvo-Metricell, a combined cell volume and cell fluorescence analyzer. J Histochem Cytochem 1977;25:804–812.
43 Benker G, Kachel V, Valet G: A computer-controlled data management system for multiparameter flow cytometric analysis; in Laerum OD, Lindmo T, Thorud E (eds): Flow Cytometry IV. Bergen, Universitetsforlaget, 1980, pp 116–119.
44 Valet G, Hofmann H, Ruhenstroth-Bauer G: The computer analysis of volume distribution curves: demonstration of two erythrocyte populations of different size in the young guinea pig, and analysis of the mechanism of immune lysis of cells by antibody and complement. J Histochem Cytochem 1976;24:231–246.
45 Sweet RG: High frequency recording with electrostatically deflected ink jets. Rev Sci Instrum 1965;36:131–136.
46 Thom R, Hampe A, Sauerbrey G: Die elektronische Volumen-Bestimmung von Blutkörperchen und ihre Fehlerquellen. Z Gesamte Exp Med 1969;151:331–349.
47 Kachel V, Metzger H, Ruhenstroth-Bauer G: Der Einfluß der Partikeldurchtrittsbahn auf die Volumenverteilungskurven nach dem Coulter-Verfahren. Z Gesamte Exp Med 1970;153:331–347.
48 Thom R, Kachel V: Fortschritte für die elektronische Größenbestimmung von Blutkörperchen. Blut 1970;21:48–50.
49 Kachel V: Eine elektronische Methode zur Verbesserung der Volumenauflösung des Coulter-Partikelvolumenverfahrens. Blut 1973;27:270–274.
50 Göhde W: Kapillare zur Zählung und Messung kleiner Teilchen. Patent DE1973692, priority date: Aug 25, 1967.
51 Thom R: Anordnung zur Gewinnung von Größen, die den Mengen von in der Untersuchungsflüssigkeit enthaltenen Teilchen verschiedenen Volumens entsprechen. Patent DE1806512, priority date Nov 02, 1968.
52 Thom R: Vergleichende Untersuchung zur elektronischen Zellvolumen-Analyse. Telefunken Hochfrequenztechnik N1/EP/V 1698, Ulm, 1972, pp 1–59.
53 Spielman L, Goren SL: Improving resolution in Coulter counting by hydrodynamic focusing. J Colloid Interface Sci 1968;26:175–182.
54 Schulz J, Nitsche HJ: Nachweis des transzellulären Ionenflusses bei der Volumenbestimmung von nativen Humanerythrozyten; in Thom R (Hrsg): Vergleichende Untersuchung zur elektronischen Zellvolumen-Analyse. Telefunken Hochfrequenztechnik N1/EP/V 1698, Ulm, 1972, pp 60–61.
55 Zimmermann U, Schulz J, Pilwat G: Transcellular ion flow in *Escherichia-coli* B and electrical sizing of bacteria. Biophys J 1973;13:1005–1013.
56 Zimmermann U, Pilwat G, Riemann F: Dielectric breakdown of cell membranes. Biophys J 1974;14:881–899.
57 Riemann F, Zimmermann U, Pilwat G: Release and uptake of hemoglobin and ions in red blood-cells induced by dielectric breakdown. Biochim Biophys Acta 1975;394:449–462.
58 Kachel V, Benker G, Lichtnau K, Valet G, Glossner E: Fast imaging in flow: a means of combining flow-cytometry and image analysis. J Histochem Cytochem 1979;27:335–341.
59 Basiji DA, Ortyn WE: Imaging and analyzing parameters of small moving objects such as cells. US patent 6249341 priority date Jan. 24, 2000.
60 Wheeless LL, Patten SF: Slit scan cytofluorometry. Acta Cytol 1973;17:333–339.
61 Gray JW, Peters D, Merrill JT, Martin R, Van Dilla MA: Slit-scan flow cytometry of mammalian chromosomes. Cytochemistry 1979;27:441–444.
62 Ahrens O, Albrecht U, Rajewsky MF: Microprocessor-based data acquisition-system for flow cytometers; in Laerum OD, Lindmo T, Thorud E (eds): Flow Cytometry IV. Bergen, Universitetsforlaget, 1980, pp 112–115.
63 Kachel V, Meier H, Stuhlmüller P, Ahrens O: Ultra fast digital calculation of ratios of flow cytometric values; in Laerum OD, Lindmo T, Thorud E (eds): Flow Cytometry IV. Bergen, Universitetsforlaget, 1980, pp 109–111.

64 Kachel V Benker G, Weiss W, Glossner E, Valet G, Ahrens O: Problems of fast imaging in flow; in Laerum OD, Lindmo T, Thorud E (eds): Flow Cytometry IV: Bergen, Universitetsforlaget, 1980, pp 49–55.
65 Watson JV: A twin laser multi-parameter analyzing flow cytometer (abstract 42). Br J Cancer 1980;42:184.
66 Watson JV: A method for improving light collection by 600% from square cross-section flow-cytometry chambers. Br J Cancer 1985;51:433–435.
67 Watson JV: Flow-cytometry chamber with 4-Pi light collection suitable for epifluorescence microscopes. Cytometry 1989;10:681–688.
68 Steen HB, Boye E: Bacterial-growth studied by flow-cytometry. Cytometry 1980;1:32–36.
69 Steen HB, Lindmo T: Flow cytometry – high resolution instrument for everyone. Science 1979;204:403–404.
70 Van Dilla MA, Trujillo TT, Mullaney PF, Coulter JR: Cell microfluorometry: a method for rapid fluorescence measurements. Science 1969;163:1213–1213.
71 Crissman HA, Steinkamp JA: Rapid simultaneous measurement of DNA, protein and cell volume in single cells from large mammalian cell populations. J Cell Biol 1973;59:766–771.
72 Crissman HA, Tobey RA: Cell-cycle analysis in 20 minutes. Science 1974;184:1297–1297.
73 Zante J, Schumann J, Barlogie B, Göhde W, Büchner T: New preparation and staining procedures for specific and rapid analysis of DNA distributions; in Göhde W, Schumann J, Büchner T (eds): Pulse-Cytophotometry II. Ghent, European Press, 1976, pp 97–106.
74 Jensen RH: Chromomycin A3 as a fluorescent probe for flow cytometry of human gynecologic samples. J Histochem Cytochem 1977;25:573–579.
75 Darzynkiewicz Z, Traganos F, Sharpless T, Melamed MR: Thermal denaturation of DNA in situ as studied by acridine orange staining and automated cytofluorometry. Exp Cell Res 1975;90:411–428.
76 Darzynkiewicz Z, Traganos F, Sharpless T, Melamed MR: Conformation of RNA in situ as studied by acridine-orange staining and automated cytofluorometry. Exp Cell Res 1975;95:143–153.
77 Göhde W, Schumann J, Zante J: The use of DAPI in pulse cytophotometry; in Lutz D (ed): Pulse Cytophotometry III. Ghent, European Press, 1978, pp 229–232.
78 Lämmler G, Schütze HR: Vital-Fluorochromierung tierischer Zellkerne mit einem neuen Fluorochrom. Naturwissenschaften 1969;56:286–286.
79 Göhde W, Schumann J, Büchner T, Barlogie B: Influence of irradiation and cytostatic drugs on proliferation patterns of tumor cells; in Haanen CAM, Hillen HFP, Wessels JMC (eds): Pulse Cytophotometry I. Ghent, European Press, 1975, pp 138–152.
80 Baisch H, Göhde W, Linden WA: Analysis of PCP-data to determine the fraction of cells in the various phases of cell cycle. Radiat Environ Biophys 1975;12:31–39.
81 Baisch H, Göhde W: Mathematical analysis of DNA-protein two-parameter pulse-cytophotometric data; in Göhde W, Schumann J, Büchner Th (eds): Pulse Cytophotometry II. Ghent, European Press, 1976, pp 71–78.
82 Berkhan E: DNS Messung von Zellen aus Vaginalabstrichen. Ärztl Lab 1972;18:77–79.
83 Schonefeld J: DNS Verteilungsmuster in Hauttumoren – Impulszytophotometrische Messungen an fixierten Geweben; Dissertation Münster, 1974.
84 Göhde W, Dittrich W: Die cytostatische Wirkung von Daunomycin im Impulscytophotometrie-Test. Arzneimittelforschung 1971;21:1656–1658.
85 Vindelov LL: Flow microfluorometric analysis of nuclear-DNA in cells from solid tumors and cell-suspensions – New method for the rapid isolation and staining of nuclei. Virchows Arch B 1977;24:227–242.
86 Vindelow LL, Christensen IJ, Nissen NI: A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry 1983;3:323–327.
87 Hedley DW, Friedlander ML, Taylor IW: Application of DNA flow-cytometry to paraffin-embedded archival material for the study of aneuploidy and its clinical significance. Cytometry 1985;6:327–333.
88 Zante J, Schumann J, Göhde W, Hacker U: DNA-fluorometry of mammalian sperm. Histochemistry 1977;54:1–7.
89 Meistrich ML, Göhde W, White RA: Resolution of x and y spermatids by pulse cytophotometry. Nature 1978;274:821–823.
90 Meistrich ML, Göhde W, White RA, Longtin JL: Cytogenetic studies of spermatids of mice carrying Cattanach's translocation by flow cytometry. Chromosoma 1979;74:141–151.
91 Otto FJ, Oldiges H: Flow cytogenetic studies in chromosomes and whole cells for the detection of clastogenic effects. Cytometry 1980;1:13–17.
92 Otto F, Oldiges H, Göhde W, Dertinger H: Flow cytometric analysis of mutagene induced chromosomal damage; in Laerum OD, Lindmo T, Thorud E( eds): Flow Cytometry IV. Bergen, Universitetsforlaget, 1980, pp 284–286.
93 Büchner T, Dittrich W, Göhde W: Die Impulscytophotometrie in der hämatologischen Cytologie. Klin Wochenschr 1971;40:1090–1092.

94 Büchner T, Göhde W, Schneider R, Hiddemann W, Kamanabroo D: Zellsynchronisation und cytocide Effekte durch Chemotherapie der Leukämie in der Klinik anhand der Impulscytophotometrie; in Andreeff M (ed): Impulscytophotometrie. Berlin, Springer-Verlag, 1975, pp 77–86.
95 Barlogie B, Göhde W, Johnston DA, Smallwood L, Schumann J, Drewinko B, Freireich EJ: Determination of ploidy and proliferative characteristics of human solid tumors by pulse cytophotometry. Cancer Res 1978;38:3333–3339.
96 Fey F, Gibel W, Schramm T, Teichmann B, Ziebarth D: Untersuchung über den Wert der Impulscytophotometrie bei der Erkennung präkanzeröser Veränderungen. Arch Geschwulstforsch 1972;39:1–7.
97 Gibel W, Weiss H, Schramm T, Gütz HJ, Wolff G: Untersuchungen über die Möglichkeiten einer Magenkrebsfrüh- und Differentialdiagnostik mittels Impulscytophotometrie. Arch Geschwulstforsch 1972;40:263–267.
98 Büchner T, Dittrich W, Göhde W: Impulscytophotometrie von Blut- und Knochenmarkszellen. Verh Dtsch Ges Inn Med 1971;77:416–418.
99 Müller D, Reichert E, Lang HD, Simon A, Benöhr HC: Die Möglichkeiten der Impulszytophotometrie für die Bestimmung der Zellproliferation bei Hämoblastosen; in Groß R, van de Loo J (eds): Leukämie. Berlin, Springer, 1972, pp 221–228.
100 Büchner Th: Impulszytophotometrie in der Hämatologie. Blut 1974;28:1–7.
101 Müller D, Reichert E, Lang HD, Orywall D: Zellcyclusänderungen und ineffektive Zellneubildung bei gestörter Granulo- und Erythropoese. Klin Wochenschr 1974;52:384–393.
102 Klein G, Altmann H: Impulscytophotometrische Untersuchungen zur Proliferationskinetik von Synovialzellen. Wien Klin Wochenschr 1973;85:774–778.
103 Pedersen T, Larsen JK, Krarup T: Characteriztaion of bladder tumors by flow cytometry on bladder washings. Eur Urol 1978;4:351–355.
104 Tribukait B, Esposti PL: Quantitative flow-microfluorometric analysis of DNA in cells from neoplasms of urinary-bladder – Correlation of aneuploidy with histological grading and cytological findings. Urol Res 1978;6:201–205.
105 Wheeless LL, Badalamant RA, deVere White RW, Fradet Y, Tribukait B: Consensus review of the clinical utility of DNA cytometry in bladder cancer. Cytometry 1993;14:478–481.
106 Hedley DW, Clark GM, Cornelisse CJ, Killander D, Kute T, Merkel D: Consensus review of the clinical utility of DNA cytometry in carcinoma of the breast. Cytometry 1993;14:482–485.
107 Bauer KD, Bagwell BC, Giaretti W, Melamed M, Zarbo RJ, Witzig TE, Rabinovitch PS: Consensus review of the clinical utility of DNA flow cytometry in colorectal cancer. Cytometry 1993;14:486–491.
108 Duque RE, Andreeff M, Braylan RC, Diamond LW, Peiper SC: Consensus review of the clinical utility of DNA flow cytometry in neoplastic hematopathology. Cytometry 1993;14:492–496.
109 Shankey TV, Kallionemi OP, Koslowski JM, Lieber ML, Mayall BH, Miller G, Smith GJ: Consensus review of the clinical utility of DNA content cytometry in prostate cancer. Cytometry 1993;14:497–500.
110 Gray JW, Carrano AV, Steinmetz LL, Van Dilla MA, Moore DH, Mayall BH, Mendelsohn ML: Chromosome measurement and sorting by flow systems. Proc Natl Acad Sci U S A 1975;72:1231–1234.
111 Stubblefield E, Cram S, Deaven L: Flow microfluorometric analysis of isolated Chinese hamster chromosomes. Exp Cell Res 1975;94:464–468.
112 Gray JW, Langlois RG, Carrano AV, Burkhart-Schultz K, Van Dilla MA: High resolution chromosome analysis: One and two parameter flow cytometry. Chromosoma 1979;73:9–27.
113 Carrano AV, Gray JW, Langlois RG, Burkhart-Schultz KJ, Van Dilla MA: Measurement and purification of human chromosomes by flow cytometry and sorting. Proc Natl Acad Sci U S A 1979;76:1382–1384.
114 Bauman JGJ, Wiegant T, Borst P, Van Duijn P: A new method for fluorescent microscopical localization of specific DNA-sequences by in-situ hybridization of fluorochrome-labeled DNA. Exp Cell Res 1980;128:485–490.
115 Landegent JE, Dewal NJI, Baan RA, Hoeijmakers HJ, Van Der Ploeg: 2-Acetylaminofluorene-modified probes for the indirect hybridocytochemical detection of specific nucleic-acid sequences. Exp Cell Res 1984, 153:61–72.
116 Hopman AHN, Wiegant J, Van Duijn P: Mercurated nucleic-acid probes, a new principle for non-radioactive in-situ hybridization. Exp Cell Res 1987;169:357–368.
117 Pinkel D, Landegent J, Collins C, Fuscoe J, Seagraves R, Lucas J, Gray J: Fluorescence in situ hybridization with human chromosome-specific libraries: Detection of trisomy 21 and translocations of chromosome 4. Proc Natl Acad Sci U S A 1988;85:9138–9142.
118 Schumann J, Göhde W: Die zellkinetische Wirkung von Bleomycin auf das Ehrlich-Karzinom der Maus in vivo. Strahlentherapie 1974;147:298–307.
119 Dittrich W, Göhde W: Phase progression in two dose response of Ehrlich ascites tumour cells. Atomkernenergie 1970;15–36:174–176.

120 Kal HB: Distribution of cell volume and DNA content of rhabdomyosarcoma cells growing in vitro and in vivo after irradiation. Eur J Cancer 1973;9:77–79.
121 Otto F, Göhde W: Effects of fast neutrons and x-ray irradiation on cell kinetics; in Göhde W, Schumann J, Büchner Th (eds): Pulse Cytophotometry II. Ghent, European Press, 1976, pp 244–249.
122 Reddy SB, Erbe W, Linden WA, Landen H, Baigent C: Die Dauer der Phasen im Zellzyklus von L-929 Zellen. Vergleich von Impulscytophotometrischen und Autoradiographischen Messungen. Biophysik 1973;10:45–50.
123 Linden WA, Zywietz F, Landen H, Wendt C: Synchronisation des Teilungszyklus von L-Zellen in der G2-Phase durch fraktionierte Röntgenbestrahlung und Daunomycin. Strahlentherapie 1973;146:216–225.
124 Smets LA: Contact inhibition of transformed cells incompletely restored by dibutyryl cyclic AMP. Nat New Biol 1972;239:123–124.
125 Smets LA: Activation of nuclear chromatin and the release from contact-inhibition of 3T3 cells. Exp Cell Res 1973;79:239–343.
126 Smets LA: Agglutination with ConA dependent on cell cycle. Nat New Biol 1973;245:113–115.
127 Böhmer RM: Flow cytometric cell-cycle analysis using the quenching of 33258 Hoechst fluorescence by bromodeoxyuridine incorporation. Cell Tissue Kinet 1979;12:101–110.
128 Gratzner HG, Leif RC, Ingram DJ, Castro A: The use of antibody specific for bromodeoxyuridine for the immunofluorescent determination of DNA replication in single cells. Exp Cell Res 1975;95:88–94.
129 Gratzner HG, Pollack A, Lingram DJ, Leif RC: Deoxyribonucleic acid replication in single cells and chromosomes by immunologic techniques. J Histochem Cytochem 1976;24:34–39.
130 Gratzner HG, Leif RC: An immunofluorescent method for monitoring DNA synthesis by flow cytometry. Cytometry 1981;1:385–389.
131 Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H: Cell-cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal-antibody KI-67. J Immunol 1984;133:1710–1715.
132 Nüsse M, Kramer J: Flow cytometric analysis of micronuclei found in cells after irradiation. Cytometry 1984;5:20–25.
133 Van den Engh G, Visser J: Light scattering properties of pluripotent and committed haematopoietic stem cells. Acta Haematol 1979;62:289–298.
134 Van den Engh G, Visser J, Bol S, Trask B: Concentration of hematopoietic stem-cells using a light-activated cell sorter. Blood Cells 1980;6:609–623.
135 Auffermann W, Böcking A: Early detection of precancerous lesions in dysplasias of the lung by rapid DNA image cytometry. Anal Quant Cytol Histol 1985;7:218–226.
136 Böcking A, Auffermann W, Vogel H, Schlöndorff G, Goebbels R: Diagnosis and grading of malignancy in squamous epithelial lesions of the larynx with DNA cytophotometry. Cancer 1985;56:1600–1604.
137 Böcking A, Hilgarth M, Auffermann W, Hack-Werdier C, Fischer-Becker D, von Kalkreuth G: DNA-cytometric diagnosis of prospective malignancy in borderline lesions of the uterine cervix. Acta Cytol 1986;30:608–615.
138 Sun D, Biesterfeld S, Adler CP, Böcking A: Prediction of recurrence in giant cell bone tumors by DNA cytometry. Anal Quant Cytol Histol 1992;14:341–346.
139 Böcking A, Motherby H: Abklärung zervikaler Dysplasien mittels DNA-Bild-Zytometrie. Pathologe 1999;20:25–33.
140 Grote HJ, Nguyen HVQ, Leick AG, Böcking A: Identification of progressive cervical epitelial cell abnormalities using DNA-image cytometry. Cancer Cytopathol 2004;102:373–379.
141 Remmerbach TW, Weidenbach H, Pomjanski N, Knops K, Mathes S, Hemprich A, Böcking A: Cytologic and DNA-cytometric early diagnosis of oral cancer. Anal Cell Pathol 2001;22:211–221.
142 Remmerbach TW, Weidenbach H, Hemprich A, Böcking A: Earliest detection of oral cancer using non-invasive brush biopsy including DNA-image-cytometry: Report on four cases. Anal Cell Pathol 2003;25:159–166.
143 Salzman G, Crowell JM, Martin JC, Trujillo TT, Romero A, Mullaney PF, Labauve OM: Cell classification by laser light-scattering – Identification and separartion of unstained leukocytes. Acta Cytol 1975;19:374–377.
144 Loken MR, Sweet RG, Herzenberg LA: Cell discirmination by multiangle light scattering. J Histochem Cytochem 1976;24:284–291.
145 Bonner WA, Sweet RG, Hulett HR, Herzenberg LA: Fluorescence-activated cell sorting. Rev Sci Instrum 1972;43:404–409.
146 Loken MR, Parks DR, Herzenberg LA: Two-color immunofluorescence using a fluorescence-activated cell sorter. J. Histochem Cytochem 1977;25:899–907.
147 Julius MH, Masuda T, Herzenberg LA: Demonstration that antigen binding cells are the precursors of antibody producing cells after purification using a fluorescence activated cell sorter. Proc Natl Acad Sci U S A 1972;69:1934–1938.

148 Jovin TM, Arndt-Jovin DJ: The measurement of structural changes in cells using fluorescence emission anisotropy in flow systems; in Göhde W, Schumann J, Büchner T (eds): Pulse Cytophotometry II. Ghent, European Press, 1976, pp 33–38.
149 Jovin TM: Fluorescence polarization and energy transfer: theory and application; in Melamed MR, Mullaney PF, Mendelsohn ML (eds): Flow cytometry and sorting. New York, Wiley, 1979, pp 137–165.
150 Chan SS, Arndt-Jovin DJ, Jovin TM: Proximity of lectin receptors on the cell-surface measured by fluorescence energy-transfer in a flow system. J Histochem Cytochem 1979;27:56–64.
151 Szollosi J, Tron L, Damjanovich S, Helliwell SH, Arndt-Jovin D, Jovin TM: Fluorescence energy-transfer measurements on cell-surfaces – A critical comparison of steady-state fluorimetric and flow cytometric methods. Cytometry 1984;5:210–216.
152 Tron L, Szollosi J, Damjanovich S, Helliwell SH, Arndt-Jovin DJ, Jovin TM: Flow cytometric measurement of fluorescence resonance energy-transfer on cell surfaces – quantitative evaluation of the transfer efficiency on a cell-by-cell basis. Biophys J 1984;45:939–946.
153 De Grooth BG, Terstappen LWMM, Puppels GJ, Greve J: Light scattering polarization measurements as a new parameter in flow-cytometry. Cytometry 1987;8:539–544.
154 Terstappen LWMM, De Grooth BG, Visscher K, Van Kouterik FA, Greve J: 4-Parameter white blood-cell differential counting based on light-scattering measurements. Cytometry 1988;9:39–43.
155 Puppels GJ, Demul FFM, Otto C, Greve J, Robert-Nicoud M, Arndt-Jovin DJ, Jovin TM: Studying single living cells and chromosomes by confocal Raman microsprectroscopy. Nature 1990;347:301–303.
156 Hulett HR, Bonner WA, Barrett J, Herzenberg LA: Cell sorting – automated separation of mammalian cells as a function of intracellular fluorescence. Science 1969;166:747–749.
157 Sengbusch GV, Couwenbergs C, Kühner J, Müller U: Fluorogenic substrates in single living cells. Histochem J 1976;8:341–350.
158 Wilder ME, Cram LS: Differential fluorochromasia of human lymphocytes as measured by flow cytometry. J Histochem Cytochem 1977;25:888–891.
159 Watson JV: Enzyme kinetic-studies in cell populations using fluorogenic substrates and flow cytometric techniques. Cytometry 1980;1:143–151.
160 Dolbeare FA, Phares WF: Napthol AS-BI(7-bromo-3-hydoxy-2-naphtho-*o*-anisidine) phosphatase and naphtol AS-BI-beta-d-glucoronidase in chinese-hamster ovary cells – biochemical and flow cytometric studies. J Histochem Cytochem 1979;27:120–124.
161 Watson JV: Enzyme kinetic-studies in cell-populations using fluorogenic substrates and flow cytometric techniques. Cytometry 1980;1:143–151.
162 Malin-Berdel J, Valet G: Flow cytometric determination of esterase and phosphatas activities and kinetics in hematopoietic cells with fluorogenic substrates. Cytometry 1980;1:222–228.
163 Dolbeare FA, Smith RE: Flow cytometric measurement of peptidases with use of 5-nitrosalicylaldehyde and 4-methoxy-beta-napthylamine derivatives. Clin Chem 1977;23:1485–1491.
164 Vanderlaan M, Cutter C, Dolbeare F: Flow microfluorometric identification of liver cells with elevated gamma-glutamyltranspeptidase activity after carcinogen exposure. J Histochem Cytochem 1979;27:114–119.
165 Stöhr M, Vogt-Schaden M: A dual staining technique for simultaneous flow cytometric DNA analysis of living and dead cells; in Laerum OD, Lindmo T, Thorud E (eds): Flow Cytometry IV. Bergen, Universitetsforlaget, 1980, pp 96–99.
166 Bassoe CF, Solsvik J, Laerum OD: Quantitation of single cell phagocytic capacity by flow cytometry; in Laerum OD, Lindmo T, Thorud E (eds): Flow Cytometry IV. Bergen, Universitetsforlaget, 1980, pp 170–174.
167 Raffael A, Valet G: Distinction of macrophage subpopulations: measurement of functional cell parameters by flow-cytometry; in Norman SJ, Sorkin E (eds): Macrophages and Natural Killer Cells. New York, Plenum Press, 1982, pp 453–459.
168 Rothe G, Valet G: Phagocytosis, intracellular pH and cell volume in the multifunctional analysis of granulocytes by flow-cytometry. Cytometry 1988;9:316–324.
169 Visser JWM, Jongeling AAM, Tanke HJ: Intracellular pH determination by fluorescence measurements. J Histochem Cytochem 1979;27:32–35.
170 Gerson DF, Kiefer H, Eufe W: Intracellular pH of mitogen-stimulated lymphocytes. Science 1982;1009–1010.
171 Valet G, Raffael A, Moroder L, Wünsch E, Ruhenstroth-Bauer G: Fast intracellular pH determination in single cells by flow-cytometry. Naturwissenschaften 1981;68:265–266.
172 Valet G, Ruhenstroth-Bauer G, Wünsch E, Moroder L: Process for determining the pH value in the interior of a cell. US patent 4677060 priority date Feb 6, 1981 (DE).

173 Valet G, Raffael A: Determination of intracellular calcium in vital cell by flow cytometry. Naturwissenschaften 1985;72:600–602.
174 Dubben H, Meyerhoff W, Tarnok A: Selection strategy for neuropeptide receptor expressing cells. Cell Prolif 1991;1:81.
175 Tarnok A: Improved kinetic analysis of cytosolic free calcium in pressure sensitive cells by fixed time flow-cytometry. Cytometry 1996;23:82–89.
176 Rothe G, Oser A, Valet G: Dihydrorhodamine 123: a new flow cytometric indicator for respiratory burst activity. Naturwissenschaften 1988;75:354–355.
177 Rothe G, Valet G: Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2′7′-dichlorofluorescein. J Leukoc Biol 1990;47:440–448.
178 Treumer J, Valet G: Flow-cytometric determination of glutathione alterations in vital cells by *o*-phthaldialdehyde (OPT) staining. Exp Cell Res 1986:163:518–524.
179 Poot M, Verkerk A, Koster JF, Jongkind JF: De novo synthesis of glutathione in human-fibroblasts during in vitro aging and some metabolic diseases as measured by a flow cytometric method. Biochim Biophys Acta 1986;883:580–584.
180 Valet G, Bamberger S, Ruhenstroth-Bauer G: Flow cytometric determination of surface charge density of the erythrocyte membrane using fluorescinated polycations. J Histochem Cytochem 1979;27:342–349.
181 Rothe G, Kellermann W, Valet G: Flow cytometric parameters of neutrophil function as early indicators of sepsis- or trauma-related pulmonary or cardiovascular organ failure. J Lab Clin Med 1990;115:52–61.
182 Rothe G, Assfalg-Machleidt I, Machleidt W, Valet G: Independent regulation of endopeptidase activity and respiratory burst activity of neutrophils analyzed by flow cytometry; in Burger G, Oberholzer H, Voijs GP (eds): Advances in Analytical Cellular Pathology. Amsterdam, Excerpta Medica, 1990, pp 119–120.
183 Assfalg-Machleidt I, Rothe G, Klingel S, Banati R, Mangel WF, Valet G, Machleidt W: Membrane permeable fluorogenic rhodamine substrates for selective determination of cathepsin L. Biol Chem Hoppe Seyler 1992;373:433–440.
184 Rothe G, Klingel S, Assfalg-Machleidt I, Machleidt W, Zirkelbach C, Banati RB, Mangel WF, Valet G: Flow cytometric analysis of protease activities in vital cells. Biol Chem Hoppe Seyler 1992;373:547–554.
185 Klingel S, Rothe G, Kellermann W, Valet G: Flow cytometric determination of cysteine and serine proteinase activities in living cells with rhodamine110 substrates. Methods Cell Biol 1994;41:449–459.
186 Ganesh S, Klingel S, Kahle H, Valet G: Flow cytometric determination of aminopeptidase activities in viable cells using fluorogenic rhodamine110 substrates. Cytometry 1995;20:334–340.
187 Valet G, Warnecke HH, Kahle H: New possibilities of cytostatic drug testing on patient tumor cells by flow cytometry. Blut 1984;49:37–43.
188 Valet G: A new method for fast blood cell counting and partial differentiation by flow cytometry. Blut 1984;49:83–90.
189 Valet G: Method for the simultaneous quantitative determination of cells and reagent therefor. US patent 4751188, priority date: Oct 15, 1982 (DE).
190 Cassens U, Greve B, Tapernon K, Nave B, Severin E, Sibrowski W, Göhde W: A novel true volumetric method for the determination of residual leucocytes in blood components. Vox Sang 2002;82:198–206.
191 Gold R, Schmied M, Rothe G, Zischler H, Breitschopf H, Wekerle H, Lassmann H: Detection of DNA fragmentation in apoptosis – application of in-situ nick translation to cell-culture systems and tissue-sections. J Histochem Cytochem 1993;41:1023–1030.
192 Hutter KJ, Göhde W, Emeis CC: Untersuchungen über die DNS-, RNS- und Proteinsynthese ausgewählter Mikroorganismenpopulationen mit Hilfe der Zytophophotometrie und der Impulscytophotometrie. Food Chem Mikrobiol Techn 1975;4:29–32.
193 Hutter KJ, Stöhr M, Eipel H: Simultaneous DNA and protein measurements of microorganisms; in Laerum OD, Lindmo T, Thorud E (eds): Flow cytometry IV: Bergen, Universitetsforlaget, 1980, pp 100–102.
194 Baisch H, Beck HP, Christensen IJ, Hartmann NR, Fried J, Dean PN, Gray JW, Jett JH, Johnston DA, White RA, Nicolini C, Zietz S, Watson JV: Comparison of evaluation methods for DNA histograms measured by flow-cytometry; in Laerum OD, Lindmo T, Thorud E (eds): Flow Cytometry IV. Bergen, Universitetsforlaget, 1980, pp 152–155.
195 Ruhenstroth-Bauer G, Valet G, Kachel V, Boss N: Die elektrische Volumenmessung von Blutzellen bei der Erythropoese, bei Rauchern, Herzinfarkt- und Leukämiepatienten, sowie von Leberzellkernen. Naturwissenschaften 1974;61:260–266.
196 Valet G, Hofmann H, Ruhenstroth-Bauer G: The computer analysis of volume distribution curves: demonstration of two erythrocyte populations of different size in the young guinea pig and analysis of the mechanism of immune lysis of cells by antibody and complement. J Histochem Cytochem 1976;24:231–246.

197 Valet G: Graphical representation of three-parameter flow cytometer histograms by a newly developed FORTRAN IV computer program; in Laerum OD, Lindmo T, Thorud E (eds): Flow Cytometry IV. Bergen, Universitetsforlaget, 1980, pp 125–129.
198 Eisen MB, Spellman PT, Brown PO, Botstein D: Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 1998;95:1486–1488.
199 Wilkins MF, Hardy SA, Boddy L, Morris CW: Comparison of five clustering algorithms to classify phytoplankton from flow cytometric data. Cytometry 2001;44:210–217.
200 Leary JF: Strategies for rare cell detection and isolation; in Z Darzynkiewicz, JP Robinson, HA Crissman (eds): Methods in Cell Biology: Flow Cytometry. San Diego, Academic Press, 1994, ed 2, pt B, vol 42, pp 331–358,
201 Molnar B, Szentirmay Z, Bodo M, Sugar J, Feher J: Application of multivariate, fuzzy set and neural network analysis in quantitative cytological examination. Anal Cell Pathol 1993;5:161–175.
202 Davey HM, Jones A, Shaw AD, Kell DB: Variable selection and multivariate methods for the identification of microorganisms by flow cytometry. Cytometry 1999;35:162–168.
203 Hokanson JA, Rosenblatt JI, Leary JF: Some theoretical and practical considerations for multivariate statistical cell classification useful in autologous stem cell transplantation and tumor cell purging. Cytometry 1999;36:60–70.
204 Thews O, Thews A, Huber C, Vaupel P: Computer-assisted interpretation of flow cytometry data in hematology. Cytometry 1996;23:140–149.
205 Diamond LW, Nguyen DT, Andreeff M, Maiese RL, Braylan RC: A knowledge based system for the interpretation of flow cytometric data in leukemia and lymphoma. Cytometry 1994;17:266–273.
206 Beckman RJ, Salzman GC, Stewart CC: Classification and regression trees for bone marrow immunophenotyping. Cytometry 1995;20:210–217.
207 Dybowski R, Grant VA, Riley PA, Phillips I: I. Rapid compound pattern classification by recursive partitioning of feature space. An application in flow cytometry. Pattern Recog Lett 1995;16:703–709.
208 Decaestecker C, Remmelink M, Salmon I, Camby I, Goldschmidt D, Petein M, Van Ham P, Pasteels JL, Kiss R: Methodological aspects of using decision trees to characterise leiomyomatous tumors. Cytometry 1996;24:83–92
209 Peltri G, Bitterlich N: Increased predictive value of parameters by fuzzy logic-based multiparameter analysis. Cytometry B Clinical Cytometry 2003;53B:75–77.
210 Zheng Q, Milthorpe BK, Jones AS: Direct neural network application for automated cell recognition. Cytometry A 2003;57A:1–9.
211 Frankel DS, Frankel SL, Binder BJ, Vogt RF: Application of neural networks to flow cytometry data analysis and real-time cell classification. Cytometry 1996;23:290–302.
212 Boddy L, Wilkins MF, Morris CW: Pattern recognition in flow cytometry. Cytometry 2001;44:195–209.
213 Valet G: Automated diagnosis of malignant and other abnormal cells by flow cytometry using the newly developed DIAGNOS1 program system; in Burger G, Ploem B, Goerttler K (eds): International Symposium Histometry. London, Academic Press, 1987, pp 58–67.
214 Valet G, Valet M, Tschöpe D, Gabriel H, Rothe G, Kellermann W, Kahle H: White cell and thrombocyte disorders: Standardized, self-learning flow cytometric list mode classification with the CLASSIF1 program system. Ann NY Acad Sci 1993;677:183–191.
215 Valet G: Human cytome project: A new potential for drug discovery; in Las omicas genomica, proteomica, citomica y metabolomica: modernas tecnologias para desarrollo de farmacos. Madrid, Real Academia Nacional de Farmacia, 2005, pp 207–228.
216 Valet G, Höffkes HG: Automated classification of patients with chronic lymphatic leukemia and immunocytoma from flow cytometric three colour immunophenotypes. Cytometry Comm Clin Cytometry 1997;30:275–288.
217 Valet G, Cytometry, a biomedical key discipline; in Robinson JP (ed): Purdue Cytometry CD-ROM, vol 4. Purdue University, West Lafayette, 1997. *www.cyto.purdue.edu/cdroms/flow/vol4/8_websit/valet/keyvirt1.htm.*
218 Valet G: Human disease; in Robinson JP (ed): Purdue Cytometry CD-ROM, vol 2. West Lafayette, Purdue University, 1996. *www.cyto.purdue.edu/cdroms/flow/vol2/14/valet/disease.htm.*
219 Valet G. Cytometry and human disease; in Robinson JP (ed): Purdue Cytometry CD-ROM, vol 5. West Lafayette, Purdue University, 2000. *www.cyto.purdue.edu/cdroms/flow/vol5/websites/cytrelay/disease.htm.*
220 Valet G: Predictive medicine by cytomics: potential and challenges. J Biol Regul Homeost Agents 2002;16:164–167.
221 Valet G, Tarnok A: Cytomics in predictive medicine. Cytometry B Clin Cytometry 2003;53B:1–3.
222 Valet G, Repp R, Link H, Ehninger G, Gramatzki M, SHG-AML study group. Pretherapeutic identification of high-risk acute myeloid leukemia (AML) patients from immunophenotype, cytogenetic and clinical parameters. Cytometry 2003;53B:4–10.

223 Valet G: Concepts and history of flow cytometry and cytomics at Max-Planck-Institut für Biochemie, Martinsried (1960–2006); in ed JP Robinson, 60 Years Innovation in Cytometry, Purdue University Cytometry Laboratories, ISBN 978–1–890473–10–5 (2007)
224 Valet G, Murphy RF, Robinson JP, Tárnok A, Kriete A: Cytomics – from cell states to predictive medicine; in Kriete A, Eils R (eds): Computational Systems Biology. Elsevier, Amsterdam 2006, p 363–381.
225 Valet G: Cytomics as a new potential for drug discovery. DDT 11:785–791(2006).
226 Valet G, Leary JF, Tarnok A: Cytomics – new technologies: towards a human cytome project. Cytometry A 2004;59A:167–171.
227 Martinreay DG, Kamentsky LA, Weinberg DS, Hollister KA, Cibas ES: Evaluation of a new slide-based laser-scanning cytometer for DNA analysis of tumors – comparison with flow-cytometry and image-analysis. Am J Clin Pathol 1994;102:432–438.
228 Hell S, Stelzer EHK: Properties of a 4PI confocal fluorescence microscope. J Opt Soc Am A – Opt Imag Sci Vis 1992;9:2159–2166.
229 Khan SS, Smith MS, Reda D, Suffredini AF, McCoy JP: Multiplex bead array assays for detection of soluble cytokines: comparison of sensitivity and quantitative values among kits from multiple manufacturers. Cytometry 2004;61B:35–39.
230 Hood L, Heath JR, Phelps ME, Lin B: Systems biology and new technologies enable predictive and preventive medicine. Science 2004;306:640–643.
231 Liebman M: Systems biology: top-down or bottom-up. Bio-IT World 2004: *www.bioitworld.com/archive/031704/horizons_horizons_comm.html.*
232 Valet G: Cytomics: an entry to biomedical cell systems biology. Cytometry A 2005;63A:67–68.
233 Valet G: Human cytome project, cytomics and systems biology: the incentive for new horizons in cytometry. Cytometry A 2005;64A:1–2.