Cellular Diagnostics
Basic Principles, Methods and Clinical Applications of Flow Cytometry
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 (Oberkochern, 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 cell 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], ‘Impulsmicrophotometrie’ [28], ‘pulse cytophotometry’ [30], ‘micro-flow fluorometry’ [31], and ‘micro-flow 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

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 CytoFluograph (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 piezocrystal-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.
Experimental and Clinical DNA Cytometry

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-bisbenzimidazole derivatives) [78] for DNA measurements or DANS (1-dimethylaminonaphthalene-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 remained limited, 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 ratio dyes 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-phthalaldehyde [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 polylysine 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-trifluoromethylcoumarinyl-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 (DIAGNOSI [213], CLASSIFI [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 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.

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