1
Perspectives in Cytometry

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1.1
Background

Cytometry is the general term for quantitative single cell analyses. Without cytometric analyses, work in modern life sciences would be unthinkable. Since its introduction, cytometry has been influencing and promoting development in biology and medicine. A high number of molecular parameters are analyzable within heterogeneous cell systems by cytometry. If the normality of a heterogeneous cell system is known, changes can be identified. Hence, biological alterations induced by malignancies, infections, and so on, are diagnosable. Such phenotypic changes allow for understanding disease-related (or induced) alterations of molecule expression patterns and hence, the functionality of the whole biological system. This interest to unravel molecular properties of single cells of healthy and diseased organisms (and to compare them) led to the development of the first cytophotometric instruments in the middle of the last century [1].

Analyses in those days were usually based on different light absorption capabilities of cell constituents of cells fixed on microscopic slides, with or without staining (e.g., Feulgen). Since these analyses were very time consumptive (5–10 min per nucleus or cytoplasm region), measurements of high cell numbers were simply not possible [2]. This technology was followed by instruments for blood cell counting with a higher throughput where cell concentrations were enumerated by counting electrical voltage pulse during cell transit [3].

Application of fluorescence dyes opened the way for obtaining more information per cell. In 1961, the first use of fluorescence for quantitation was reported [4]. Since then, development of new instruments was focused toward fluorescence analysis. In 1969, the first impulse cytophotometer (ICP-11) (Phywe GmbH, Göttingen [5]) was commercially launched where the fluorescence (resulting from mercury arc lamp excitation) of several thousand cells per second was measured by photomultiplier tubes (PMTs). Later, lasers were employed as stable light sources for excitation of fluorescence dyes. The first flow cytometer equipped with two lasers was available in 1976 [6]. Several fluorescence dyes could now be measured simultaneously. The basic principle of this technology is still applied in modern flow cytometers: cells

Advanced Optical Flow Cytometry: Methods and Disease Diagnoses, First Edition. Edited by Valery V. Tuchin.

are separated by sheath fluid, (hydrodynamically) focused, and excited by (laser) light in flow. The scattered and emitted or absorbed light is measured.

The demand for comprehensive analyses and with it the simultaneous detection of several parameters on many (thousands to millions of) individual cells in one sample led to further developments in the field of cytometric analyses. More lasers as well as detectors were included to be able to perform three- or four-color measurements (plus information of scattered light) routinely.

This was sufficient for many applications, or at least, it had to be. Detailed cellular subtyping, coexpression of specific markers, cytokine analyses of certain cell types, cell–cell interaction, and so on, are, in the majority of cases, not possible by using only four fluorescence parameters. The list of applications is long where multiparametric analyses are essential.

1.2 Basics of Cytometry

The beauty of labeling specific markers or cellular functions with fluorescence dyes lies in its multicolor approach and therewith the feasibility of simultaneous analysis of many parameters. If cells are stained with different ''colors,'' each single color can be distinguished from each other and multiple information can be obtained for single cells. Admittedly, differentiation of more than three colors by the eye is almost impossible, but light detectors in cytometers (PMTs or camera), in combination with appropriate bandpass filters, are able to detect wavelength ranges (i.e., specific ''colors'') of interest. Discrimination of fluorescence dyes is hereby possible by defining certain wavelength ranges, suitable for specific fluorescence dyes. However, the usually very broad emission spectra of fluorescence dyes make it sometimes hard to differentiate between dyes in one detection filter owing to spectral overlap. This problem is known for fluorescence dyes such as fluorescein isothiocyanate (FITC) and phycoerythrin (PE) but can be mathematically solved by compensation, that is, ''purification'' of specific fluorescence from unwanted signals. Another novel possibility to overcome the problem of spectral overlap is multispectral analyses (known from microscopy) although it is rarely used in flow cytometry (FCM) [7].

In general, there are two different types of cytometric analyses named by the analytical technique: FCM and slide-based cytometry (SBC). As previously mentioned, cytometry has its roots in the analysis of cells on a slide. Owing to the higher throughput, development moved to the FCM, although now, with higher computing and storage capacity of workstations, cytometry by microscopy has been revitalized. Nevertheless, both methods are quite similar and identical in many details.

1.2.1 Flow Cytometry

It is apparent from the name cytometry that cells are analyzed in flow. Generally, cells in suspension are sucked or pressed into the cytometer by overpressure or

mechanical pumps. Covered with sheath fluid, cells are separated (like pearls on a string) and move actively to the place of analysis. Lasers (or also light emitting diodes nowadays) excite the cell (i.e., the fluorescence dye on it) and the emitted fluorescence is detected by PMTs. On the basis of specific characteristics (mainly fluorescence of a certain label but also light scattering properties), separation of wanted cell types and its concentration and purification can be accomplished. However, fluorescence-activated cell sorting (FACS) is necessary for that, which can be time consumptive. In ''normal'' FCM, the sample is usually lost after analysis. Up to 50 000 cells can be measured per second, although the normal throughput is usually around 1000–10 000 cells per second.

Fluorescence information of the cells can be displayed as histograms and dotplots. Clever experimental setup (marker selection, fluorescence combination) and smart gating strategies allow for extraction of multiple information out of a three- or four-color staining. Nevertheless, detailed subtyping or functional information (e.g., activation) of specific cellular subtypes can hardly be obtained by such low-color analyses [8].

Although the main principle in FCM has not dramatically changed since its beginning, there are of course some new developments besides increasing number of detectors and lasers. There is not only hydrodynamic focusing of cells (with the need for utilization of sheath fluid) but also focusing derived from acoustic radiation pressure forces [9] or the utilization of photodetectors for sensing the position of particles in the sample stream without sheath fluid [10]. Without sheath fluid (but with a unique flow cell design), even usage of FCM in space is conceivable [11]. Another development is the implementation of imaging in FCM. There are flow cytometers available that are able to capture images of analyzed cells in flow for morphological analysis [12].

Cellular analyses in FCM, however, are restricted to cell suspensions. Solid tissue or adherent cells cannot be analyzed, that is, not without prior trypsinization or disintegration of tissue. For these types of specimens, SBC was developed.

1.2.2 Slide-Based Cytometry

There are two major types of SBC systems: camera-based detection in combination with lamp illumination (e.g., [13]) and laser excitation and fluorescence detection via PMTs (e.g., [14]). However, mixed systems, for example, lasers and camera, are available, too. No matter which modality is used for excitation and detection, the core of these instruments is a fluorescence microscope. But that does not mean that every fluorescence microscope is capable of cytometric analysis. Cytometric analysis means quantitative analysis of the whole cell, that is, it requires optics with a relatively low numerical aperture. Analysis of single slices of a cell, as, for example, in confocal microscopy, is not cytometric. Moreover, analyses using microscopes with lamps or diodes as excitation source and no corrections (optical or software solutions) for light excitation intensity, that is, stability of the excitation light, are also not cytometric. Owing to unstable excitation intensity, one cannot be

certain that the resulting fluorescence intensity of cells in different fields of view (or different samples, irrespective of the same acquisition setups) will provide the same fluorescence intensity. Qualitative statements about existing fluorescences are possible but no quantitative conclusion can be made about cell activation or other marker expression of cells. Prerequisites for cytometric analyses with microscopes are stable excitation power, even illumination of the sample, and a steady and sensitive detection of the emitted fluorescence.

Even though cytometric analyses were slide based at the beginning and the modern concept of SBC was presented in the 1980s, the first type of such instruments, the laser scanning cytometer (LSC), became commercially available a decade later [15]. The reason for this was probably the time needed for image analyses in the past. However, higher processing power and storage capacities of modern computers promoted development in this field.

Unlike FCM, samples in SBC analyses are fixed on a slide or plate. Although mainly developed for tissue analysis, LSC was used for many different applications, for example, cell cycle studies [16–18], apoptosis [19, 20], immunophenotyping [21–24], tumor analysis in solid tissue [25], fine-needle aspirate biopsies [26], circulating tumor cell analysis [27], stem cell analysis [28, 29], or study of the effects of drugs [30, 31].

The principle of LSC analysis is comparable to FCM. Fluorescence dyes on (or in) cells are excited by laser light and the emitted fluorescence is split into certain wavelength bands by optical filters and detected by PMTs. The deviation from FCM is that cells remain on the slide and can be further analyzed or even cultured.

LSC allows for studying growth and the variety of expression of specific markers during development of cells in their ''natural'' environment [31]. Possible effects of cell preparation, for example, stress or activation caused by detachment of cells from the surface (like for FCM analyses), can be avoided. Moreover, interactions between single cells can hardly be observed on detached cells. This applies for cell cultures as well as tissue sections. Another advantage is that cells can be traced and analyzed during culturing [29].

1.3 Cytomics

Since the complexity of biological systems is very high, a multiplicity of different information from cells, their interaction, and triggered reactions (e.g., by external stimuli or diseases) is necessary to understand such systems. For this purpose, different concepts were and still are under development to get a better insight into biological processes in organisms. Cytomics is one of these concepts. Its aim is to characterize single cells in cell systems and to unravel the interactions of cells within these systems [32]. Another concept is systems biology. The aim of systems biology is similar to that of cytomics, but it focuses more on understanding intracellular behavior, that is, the interaction of single cellular constituents such as genes, proteins, metabolites, and organelles and *in silico* modeling [33]. Interconnection

of different analyses is very important for this purpose, that is, to obtain all needed information and combine them appropriately. In contrast to other concepts like genomics (characterization of genome [34]), proteomics (analysis of proteome [35]), lipidomics (cellular lipid constituents [36]), or other -omics, where only certain components of cells are in the focus of interest, cytomics and systems biology focus on interaction of cells and cellular constituents.

Always, biological conditions are the result of the interaction of all components of a complex system. Therefore, such a system must be analyzed as a unit to unravel its secrets. For example, different developmental stages of an organism have the same genome but are different (also phenotypically) in their protein composition [37]. Cytomics and systems biology start there and go even further. Not only single components are under investigation but also the relations and interactions between different components. Therewith, changes in cell systems can be understood – from work flows within the cell (systems biology) to interactions of the whole system (cytomics). If these actions are known, alterations (even before clinical manifestation) can be classified and can lead to predictive and preventive individualized medicine [38, 39]. Cells are the elementary building units of an organism and hence, their analysis is the easiest way to identify diseases or reasons for diseases. Alterations to healthy conditions can be found by differential screening, that is, examining a multiplicity of cell types for phenotype, activation, or cytokine production (multiparameter analyses), and extracting important and relevant cell types and marker combinations for a further diagnostic panel. However, it is clear that the mass of information obtained from multiparametric cytometry must be analyzed appropriately to find causal connections. Bioinformatics tools, that is, algorithms for cluster analyses, can be applied [40–42].

1.4 Cytometry – State of the Art

Routine are still fluorescence analyses with a relatively low amount of measured parameters (sample stained with three to four colors). However, developments of new fluorescence dyes with promising spectral characteristics, for example, UV excitable dyes for protein labeling (e.g., Quantum dots [43]) and, of course, instruments capable for multiparametric analyses, led to the increased usage of multiparametric, that is, multicolor, analyses in laboratories worldwide. Cellular analyses with 6–12 colors (polychromatic cytometry) simultaneously are no longer a rarity (e.g., [8, 22, 44]).

Multicolor analyses allow for detailed understanding of complex cellular structures, cell subtypes, and cell–cell interactions. All fluorescence information, that is, the tagged biological components, generate a network from where information of interest can be extracted. Admittedly, there are attempts to perform it with three- or four-parameter analyses. To this end, as an example, in FCM analyses many aliquots of the same sample (e.g., blood) are stained for different antibodies. All these single tubes are measured and afterward, information obtained from

each tube is combined, for example, for cellular subtyping of the sample (but not on a single cell level). The presence of main cellular subtypes can certainly be detected but it is not possible to get details. For example, a staining for CD3, CD4, and CD8 in one tube yields information on the amount and distribution of T helper and cytotoxic T cells as well as double positive and double negative T cells. Conclusions on further subtypes, for example, existence of $\alpha\beta$-TCR (T cell receptor) or $\gamma\delta$-TCR or expression of CD16 or CD56 in NKT cells, are not possible. If cells are stained for CD3, CD19, and CD16/CD56 one can get an overview of lymphocyte subtypes (i.e., T cells, B cells, NK cells, and NKT cells) but merging this information with one of the other tubes and drawing a conclusion on, for example, the presence of $\alpha\beta$-TCR$^+$CD4$^+$CD8$^+$ NKT cells is impossible. Therefore, one needs this information on a single cell basis, for example, a sample with CD3, CD4, CD8, CD16/CD56 (or even better, CD16 and CD56), and $\alpha\beta$-TCR. Only then, the required information can be extracted from the analysis.

Admittedly, some recent developments promise to combine several (three to four color) FCM measurements of a sample into one metadata file (i.e., a virtual multiparameter analysis by data merging) on the basis of a leading antibody [45] but this might not be feasible with every desired cellular subtype and, of course, needs verification with a true polychromatic analysis. For answering complex questions, multiparametric analyses are indispensable [8]. Another advantage of multiparametric analyses is the fact that information density increases with each parameter added to the existing setting. The resulting network provides the opportunity to find interactions of cells, coexpression of markers, and so on, that were never be thought of before. Moreover, polychromatic cytometry reduces costs of analysis. Although a complex pattern of fluorescence dyes means also the use of ''unusual'' dyes or fluorescence marker combinations (mostly more expensive than the commonly used ones), the combination of markers in one analysis prevents the repetitive use of markers in several tubes for identifying main populations.

1.4.1 Multiparametric Analyses

As an example for the complexity of multicolor analyses, an eight-color measurement on LSC [22] is described here.

For precise analysis and correct interpretation of data, a careful selection of antibodies and their corresponding fluorescence dyes is essential [46]. Only optimal experiment settings allow for unequivocal identification of cell types, clear discrimination between positive and negative cells, and doubt-free analysis of data. This means that in most of the cases different antibody fluorescence combinations need to be tested before selecting the final experiment settings. For the eight-color experiment on LSC, the following panel was selected for a simultaneous staining of several surface markers in a sample of human peripheral blood leukocytes: CD14-FITC, CD4-PE, CD56-PECy5, CD16-PECy7, CD45-APC (Allophycocyanin), CD8-APCCy5.5, and CD3-APCCy7. This mixture allowed for identification of the required lymphocyte cell subtypes (focus on NK and NKT cells) by an appropriate

gating strategy. In Figure 1.1, analysis including gating strategy is shown. Since LSC lacks side scatter, which is usually used in FCM to discriminate between leukocyte subsets (neutrophils, monocytes, lymphocytes), fluorescence signals were used for this purpose. Nevertheless, leukocytes could be subdivided because of their different expression of CD45 (pan-leukocyte marker) and CD14 (LPS-Receptor) into the subsets: monocytes, lymphocytes, granulocytes (neutrophilic and eosinophilic), and basophilic granulocytes after excluding cell debris, artifacts, and aggregates based on their forward scatter maximum pixel and area values (for details, see [22]). The lack of a CD14 signal on basophils enables their differentiation from neutrophils and eosinophils (both $CD14^{weak}$) and monocytes ($CD14^{bright}$). Within the monocytes there is a small but prominent population of $CD16^{+}$ cells with a slightly reduced CD14 expression: proinflammatory monocytes. $CD16^{weak}$ cells within the granulocyte population are eosinophils. Thereby, the main leukocyte populations were discriminated.

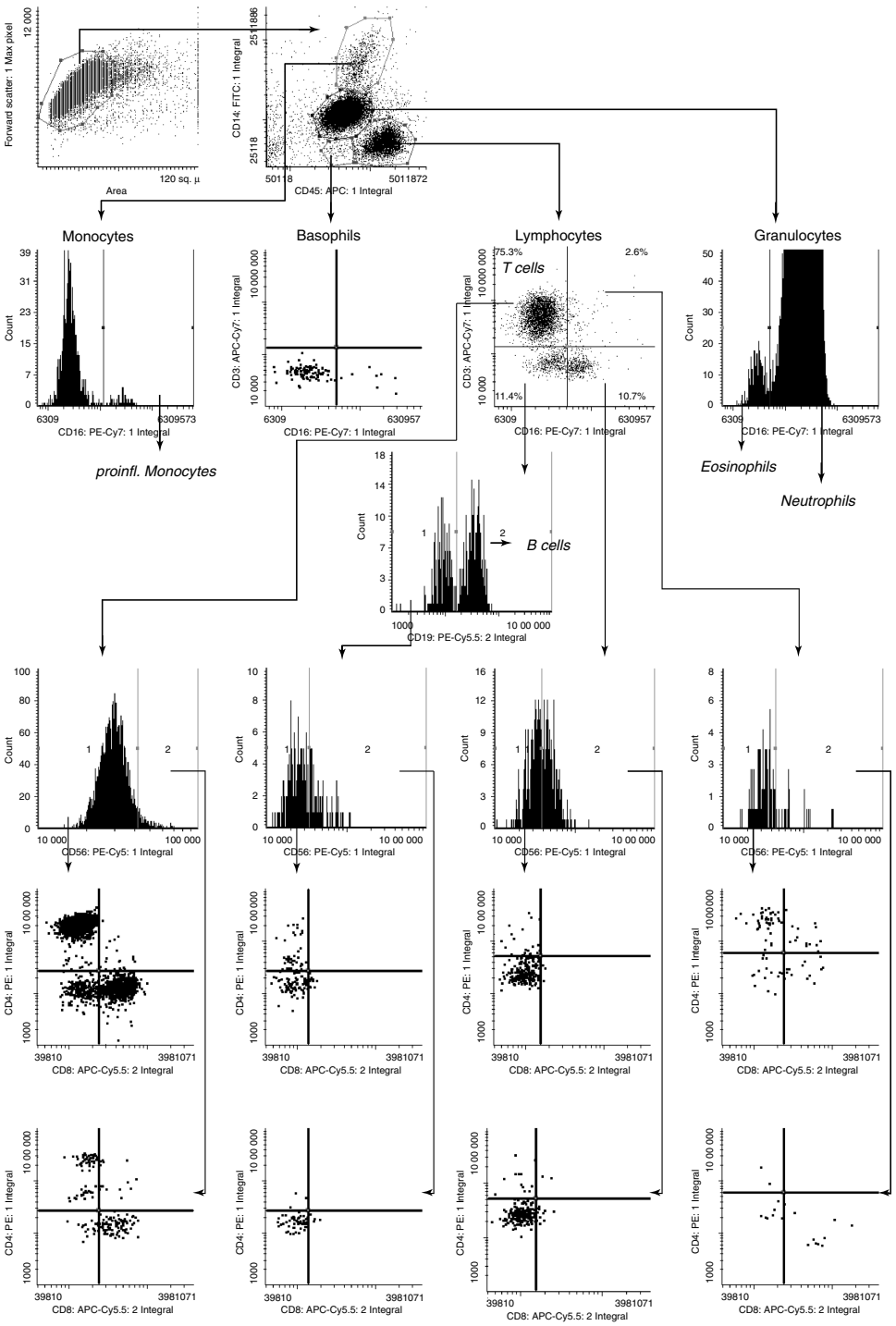
The focus of this LSC analysis though was lymphocytes. Lymphocytes were subtyped by their CD3 and CD16 expression: $CD3^{+}CD16^{-}$, $CD3^{+}CD16^{+}$, $CD3^{-}CD16^{-}$, and $CD3^{-}CD16^{+}$. CD19 expression allowed for unequivocal identification of B cells in the $CD3^{-}CD16^{-}$ population.

Since cytomics and multiparametric analyses provide complex networks of data (fluorescence, scatter, size, etc.) on a single cell level, expression of every marker can be verified for each cell, or more practically, for each identified cell population.

Without further investigation, one would expect all the $CD3^{+}CD16^{-}$ population to be T cells. But that is not the truth. CD56 expression revealed a small population ($\sim$7%) of $CD3^{+}CD16^{-}$ NKT cells. The presence or absence of CD56 (in combination with the previous step: CD16) allowed for discrimination of several NK and NKT cell subphenotypes also within the other lymphocyte subsets ($CD3^{+}CD16^{+}$, $CD3^{-}CD16^{+}$, $CD3^{-}CD16^{-}$) with varying combinations of CD16 and/or CD56. All of these newly identified populations of T cells, NK cells, and NKT cells were further investigated for their expression of CD4 and CD8. As can be seen in Figures 1.1 and 1.2, the diversification of all these populations goes deeper and deeper. One can imagine that with additional antibodies further subsets can be discriminated, although it must be clearly said that only at a certain concentration, that is, number of cells, a cell population can be identified as a ''population'' [47]. If only a very low number of cells can be expected at the end of such a gating cascade, a very high number of cells must be analyzed in the beginning.

1.5 Perspectives

Biological systems never were simple – but their analyses were, in the past. The reason for these ''simple'' analyses may be limitations in understanding such systems or limitations of techniques for their analyses. However, understanding is based on observations that are only possible with suitable instruments. Most important for unraveling complex systems is the ability to look at many parameters at a time


Forward scatter: 1 Max pixel
12 000
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CD14: FITC: 1 Integral
25118
5011872
CD45: APC: 1 Integral
Monocytes
Basophils
Lymphocytes
Granulocytes
Count
CD16: PE-Cy7: 1 Integral
6309
6309573
630957
CD3: APC-Cy7: 1 Integral
10 000
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75.3%
2.6%
11.4%
10.7%
T cells
proinfl. Monocytes
Eosinophils
Neutrophils
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CD19: PE-Cy5.5: 2 Integral
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CD56: PE-Cy5: 1 Integral
CD4: PE: 1 Integral
CD8: APC-Cy5.5: 2 Integral
39810
3981071

Figure 1.1 Eight-color analysis by LSC. EDTA anticoagulated blood was stained for CD3, CD4, CD8, CD14, CD16, CD19, CD45, and CD56. Artifacts were excluded by FSC (forward light scatter) MaxPixel versus area for further analysis (top left) and events of nonleukocyte origin by the lack of CD45 expression (top center). Leukocytes were further subdivided by their different antigen expression. Figure was published earlier in: Mittag *et al*. [22].

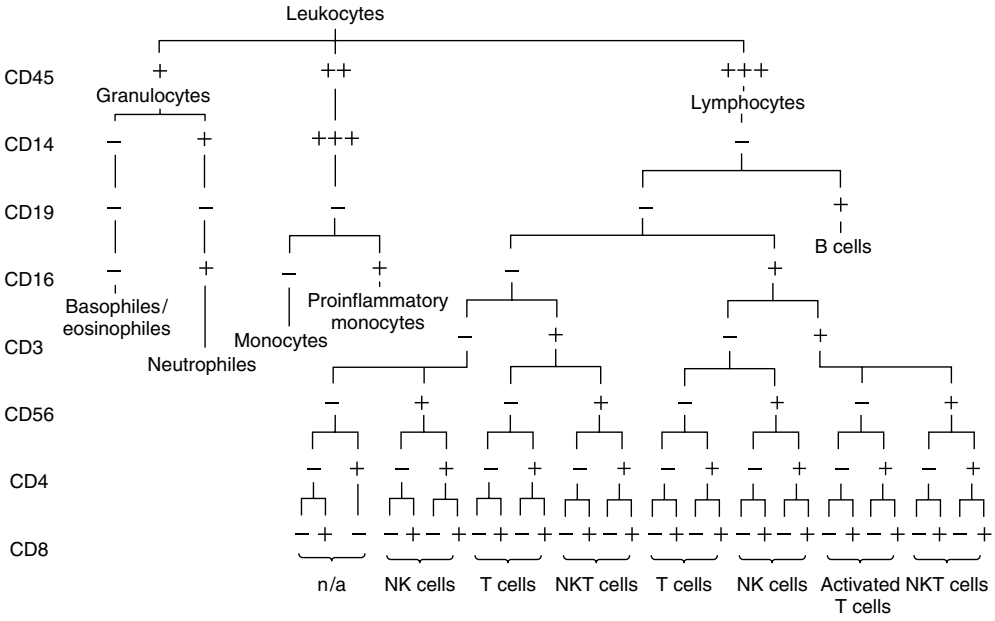

Figure 1.2 Polychromatic characterization of leukocytes. Detailed subtyping of cells, leukocytes in this example, is only possible with polychromatic cytometry, that is, the simultaneous use of several markers. Figure was published earlier in: Mittag [48].

to find interactions and causal connections. Thus, there is an increasing demand for multiparametric analyses in the field of biological and clinical investigations.

Perfetto *et al.* [8] point out that markers for identification of different developmental stages of T cells are expressed on several T cell types (e.g., CD45RA, CD27, CD28, CD62L, CCR7) and that in the case of four-color experiments one cannot make a statement about expression of (presumably analog) markers such as CD62L or CD27 on $CD45^{-}CCR7^{+}$ cells. Only if more markers are added to the commonly used "simple" protocols for immunophenotyping, questions about coexpression of antigens can be answered unequivocally without theoretical assumptions. This applies even more to the investigation of antigen specificities or cell functions [8]. The authors, therefore, emphasize the need for technologies for polychromatic cytometry with more than six colors. Only then can investigations of cellular interrelations be accomplished as well as a more precise analysis of rare cell

populations feasible. It may also help explain immunopathogenesis of infectious or autoimmune diseases and will substantially improve currently available diagnostic tools. Hence, the discovery of sensitive and specific panels of biomarkers might be essential for future diagnostic and therapeutic monitoring of complex diseases [49].

One of the perspectives of cytometry therefore definitely is cytomics and multiparametric analyses, that is, their further development, whereby the information density of the analyzed material is exponentially increased with each additional analyzed parameter. Technical possibilities are exhausted and new methods for multiparametric approaches are under development.

1.5.1 New Technologies and Methods

Perfetto *et al.* [8] reported an FCM analysis of 17 fluorescence dyes simultaneously. This high number of fluorescence-labeled markers is necessary to get to the bottom of the immunological reasons for disease patterns. For example, even if the overall number of T cells is not significantly altered, alterations in the composition of the immune system can be detected by a closer look on T cell subsets of patients with diseases caused by HIV [8]. Alterations in T cell subsets, such as depletion of naïve or newly produced T cells or viral infection of memory and HIV-specific $CD4^+$ T cells, provide information on the progression of disease or are valuable for diagnosis or prognosis [50–54]. For studying functions of these cellular subtypes (e.g., cytokine pattern analysis) or other disease-related characteristics, multiparametric analysis is mandatory.

To the best of our knowledge, there is no cytometer available yet on the market which is capable of measuring such a high amount of parameters simultaneously as described by Perfetto *et al.* [8]. The authors built, that is, modified their own instrument in a way that it fulfilled the requested tasks. Nevertheless, manufacturers of cytometers recognized the signs of the need for multicolor analyses in laboratories. Several instruments capable of 10- to 12-color measurements were launched recently. This might be enough for cytometric investigations at the moment, at least for routine analyses, but researchers tend to look a little bit deeper. This means, to make it short, there can never be enough parameters in cytometric analyses. The only problem is that, at a certain point, hardware has its limitations. Software solutions for increasing measurable parameters might therefore be a good addition or even an alternative. Implementation of additional lasers for excitation and PMTs for fluorescence detection changed standard 3-4 colour FCM analysis to >8 colour polychromatic cytometry. However, for many technical reasons this development will not continue for ever. SBC seems to be a very interesting alternative in this field. Besides its ability to analyze not only cell suspensions but also cells in culture or tissue, the main advantage is that the sample is not lost after analysis. This opens the way for reanalysis and merging of information for one sample from different analyses – on a single cell level. Cellular reactions of added compounds can be tested [31] or a detailed phenotyping of cells by sequential analysis [22], for example, restaining [55], is possible, among other exciting possibilities.

1.5.1.1 Sequential Analyses

In the eight-color leukocyte analysis by LSC mentioned before, merging of two separate measurements was part of the analysis [22]. Although the sample was stained for all eight markers simultaneously, the cytometric analysis consisted of two parts, that is, two different measurements. This was necessary because of the limit of four PMTs in LSC. In combination with a 488 and a 633 nm laser for excitation, there is a limit for fluorescence detection of six parameters. The exchange of filters between the measurements enables an analysis of the fluorescence signals, the detection of which was excluded by the settings for the first measurement. This second measurement, that is, the obtained data, was merged with the first data set. In the resulting data file, all information, obtained from measurement 1 or 2, is available for each individual cell. In this example, only two scans were performed and only eight markers were analyzed. However, this method of sequential analysis can be extended; there is no limit to merging data files.

Another application of this approach is the sequential staining of samples. This means staining of samples with one set of antibodies (or only one marker) with a subsequent analysis, followed by staining with a second set (same fluorescence dyes can be applied). Between the two measurements a bleaching step can be applied, although it is not mandatory as Laffers *et al.* [55] could demonstrate. Bleaching, of course, allows ''resetting'' the sample and starting with a ''blank one'' but with the information from the previous analysis [56, 57]. The combination of staining, analysis, bleaching, and restaining could be extended and brought to perfection by Schubert *et al.* [56] for an analysis of more than 90 molecules in one tissue section. The multiepitope-ligand-cartography (MELC) technology, developed by this group, allows for mapping the topological position of many proteins simultaneously in a cell and can therewith unravel hierarchies of proteins. This, in turn, indicates cell function or dysfunction and allows identification of specific proteins in protein networks of cells and tissues [58]. Mapping of toponome, that is, identification of protein colocalization, could be demonstrated on peripheral blood leukocytes, on a rhabdomyosarcoma cell line as well as on skin biopsies and rat spinal cords by repetitive fluorescence labeling of proteins [56]. This technology might be relevant for pharmacology to find new target proteins for drugs.

1.5.1.2 Spectral Analyses

If bandpass filters are used for analysis, always a part of the fluorescence will not be detected since most of the fluorescence dyes have a very broad emission spectrum. The narrower the bandpass is in front of the detector the more specific is the detection of fluorescence signals (i.e., without substantial interferences of spillover signals from other dyes) of the dyes in multicolor experiments. However, a lesser amount of light is transmitted, resulting in lower fluorescence signals (i.e., signal-to-noise ratios). This can be of hindrance in analysis of weakly expressed antigens.

An alternative to splitting fluorescence emission of samples into several broad bands by filters is spectral analysis. Image stacks of 1 nm distance (up to broader image acquisition steps) can cover the whole range of visible and invisible light

although main applications (based on fluorescence characteristics) are in the visible range. Wavelength changes in spectral imaging are usually done by acousto-optic tunable filters (AOTFs) or liquid crystal tunable filters (LCTFs) as used in some *in vivo* imaging systems (e.g., [59]). AOTFs, implemented in microscopic systems (e.g., spectrofluorometer, confocal laser scanning microscopy), allow for multiparametric analyses [60]. In such analyses, the spectrum or at least the spectral range of interest is analyzed and recorded (''spectral fingerprinting''). All used fluorescence dyes (or cell type specific spectra – without staining) can be extracted (spectral deconvolution) and distinguished by reference values (e.g., measurement of ''pure'' fluorescence signals) or virtual filters [44].

However, spectral analysis is not applicable only for SBC. Spectral measurements of fluorescence and Raman labels are also feasible with a commercial (although modified) large particle analyzer using a spectrograph and charge coupled device (CCD) array detector [7].

1.5.1.3 Fluorescence Modifications for Analyses

When using SBC, one is confronted with photobleaching of fluorescence signals. Most fluorescence dyes are sensitive to prolonged light exposure. In SBC, the microscope-based cytometry technology, excitation times are by at least one order of magnitude longer than in FCM. Therefore, photobleaching will affect analytical results of SBC. On the one hand, bleaching effects are very annoying, but on the other hand, it is possible to use bleaching as a tool for analysis. The combination of stable and photosensitive fluorescence dyes for staining biological samples can be used to extend the number of measurable parameters per analysis [61, 62]. Bleaching of fluorescence dyes was also used as a tool for single-cell fluorescence spectroscopy. The electronic interaction of DNA base pairs leads to oxidation of bound fluorescence dye and this in turn to fluorescence bleaching, which enables discrimination of DNA information at the single-molecule level [63]. Moreover, fluorescence recovery after photobleaching (FRAP) can be used for biological investigations of molecular dynamics in living cells. Examples for FRAP analyses are studies on integrin dynamics [64] or RNA motion [65].

Fluorescence resonance energy transfer (FRET) is probably the main application of directed bleaching in cytometry. With FRET analyses, interactions of surface molecules on two interacting cells can be studied [66] or the intracellular trafficking and state of toxin subunits can be determined [67].

Alternatively, studies of protein interactions can also be performed with a new technology, called *in situ proximity ligation assay* [68]. This method utilizes antibodies coupled to oligonucleotides. If the target proteins are in close proximity, they can form a circular DNA molecule that can be amplified and detected by adding complementary oligonucleotides labeled with a fluorescence dye. This fluorescence can then be analyzed by FCM [68].

Another interesting technique for manipulation of fluorescence to gain information is photoconversion [69]. Fluorescent proteins (e.g., KAEDE) or fluorescence dyes (e.g., Lucifer Yellow) are transferred into stable reaction products by light excitation. These reaction products can be detected and analyzed by microscopic techniques [70, 71]. Similar to FRET analyses, there is a change in

the ''color'' of emitted fluorescence. This method is usually used for the study of fluorescence-labeled cellular structures [70].

All these methods can provide additional information from already stained samples. Mechanical manipulation of cells is not necessary. However, for some applications no manipulation of the cells at all (i.e., also no staining) is desirable. A prominent example is the isolation of stem cells for therapeutic use.

1.5.1.4 **Label-Free Analyses**

Fluorescence analyses enable specific labeling and characterization of biomarkers; even analyses using combination of multiple markers are possible (polychromatic cytometry). Nevertheless, tagging cells with antibodies or the use of DNA dyes influences cells' behavior and can be toxic as well. For live cell analyses and the intention to further use these cells (e.g., for therapeutic use or cell differentiation studies), characterization of cells by a label-free approach would be preferable.

Some promising efforts were made for label-free analysis of cells. Impedance analysis is one of them. With this technology it is possible to analyze adherent cells, for example, for detection of ischemic effects on cardiomyocytes [72] or detection of tau hyperphosphorylation in a neuroblastoma cell line [73]. Impedance analysis is also feasible with flow cytometric techniques, that is, analysis of cells in flow [74]. Cells can be analyzed at different frequencies. The analysis over a wide frequency range provides information of cellular characteristics depending on the frequency, that is, cell size (0.1–20 MHz, but mainly determined in the range of 0.1–1 MHz), membrane capacitance (1–5 MHz), and cytoplasm conductivity (3–20 MHz). The amplitude, opacity, and phase information can be used for discrimination between different cell populations without the use of cell markers [75], viability and apoptosis analyses, or the visualization of microbiological life cycle phases [76].

Conventional FCM can also be used for label-free analysis. Scatter as a parameter provides opportunity for label-free detection since all particles scatter light when illuminated. Commonly used in FCM are forward light scatter (which is dependent on cell size and refraction index) and orthogonal side scatter (provides information of granularity). These two parameters are sufficient for rough leukocyte discrimination [77, 78] but cannot be used for separation of cells with similar size, shape, or structure. Therefore, an analysis of the complex scatter pattern of particles (e.g., cells) is necessary, that is, information about size, shape, refraction index, density, and morphology obtained by different light scattering properties. Rajwa *et al.* [79] demonstrated the classification of particles on the basis of their discrete scatter patterns with a modified flow cytometer capable of measuring five different angles of scattered light and axial light loss. Label-free detection of bacterial pathogens (colonies growing on agar plates) can be accomplished by their forward-scatter signatures at 635 nm and image analysis. Multiple bacterial pathogens can be identified although their visual morphology is similar [80].

Another approach is based on image analysis. Label-free analysis intends to replace evaluated markers by parameters formerly not used for identification of specific cells or characteristics. Newberg and Murphy [81] demonstrated an automated image analysis of subcellular patterns of proteins by immunohistochemistry

for studying the functions of proteins. For this purpose, proteins were stained and analyzed using morphological parameters for further prediction. Ishikawa *et al*. [82] succeeded in identifying alga cells without using any labels. Fujita and Smith [83] outline the importance of label-free analysis for the examination of living cells. The authors describe the chemical and biochemical impact of markers on living cells and suggest using Raman scattering and coherent anti-Stokes Raman scattering (CARS) to image molecules in cells. Moreover, biomolecular composition of tissue can be determined by Raman spectroscopy, for example, applied for discrimination of normal liver, viable tumor, and fibrotic hepatoblastoma [84]. For Raman scattering analysis, a relatively high concentration of molecules (or high laser intensity) is necessary. Spectrum of single molecules, therefore, cannot be detected. Surface enhanced raman scattering (SERS) can solve this problem and make this method sensitive enough for FCM [85].

Another vibrational spectroscopic technique, near infrared spectroscopy (NIRS), allows for noninvasive monitoring of oxygenation and perfusion [86–89], functional brain imaging [90], or examination of normal and malignant tissue, for example, applied for breast cancer imaging [91]. Although this method is mainly used for imaging in clinical applications, it might also be interesting for tissomics and cytometry, for example, for distinguishing intact cartilage from the enzymatically digested one [92] or the characterization, discrimination, and identification of microorganisms [93].

Autofluorescence can also be used as a relevant discriminatory parameter in analysis. The combination of second-harmonic generation (SHG) and multiphoton excited autofluorescence (MAF) signals can be used for a label-free discrimination of cancerous from noncancerous lung tissue [94].

1.5.2 Automation

Particularly in cytometric analyses for diagnostics, high-throughput and standardized reliable data are mandatory. Therefore, instruments capable of automated measurement and analysis are necessary. This applies, of course, also for multiparametric analysis. Automated measurement might not be the problem; robot units are applied in many routine laboratories, but an automated analysis, that is, precise setting of gates for analysis, is still at its beginning.

Hence, modern instruments will be equipped with a high-precision hardware and sophisticated, reliable software suitable for high-speed data acquisition and computer-assisted fully automated data analysis. Currently available cytometers allow for detection of increasing numbers of cell surface markers. However, this development is challenging for ''traditional'' methods of identifying cell populations, that is, manual gating for positive and negative cells. Easy-to-use software tools are needed, allowing for fast and effective analysis of multiparametric datasets based on supervised or unsupervised data mining algorithms [95]. The routine generation of vast amounts of data makes cytometric analyses a logical target for the application of data mining techniques. Clustering algorithms help

arrange the multidimensional datasets containing large numbers of parameters describing molecular features of individual cells in a small number of relatively homogeneous clusters on the basis of differences and similarities between analyzed objects [40–42]. The rapid advance in automatic cytometric analysis will facilitate the development of computer-assisted clinical decision-making systems and will provide additional assistance to medical professionals [96].

Automation in cellular analyses implies not only automatic gating of flow cytometric data but also automated image analysis. Image analysis can be simple if only the size of objects or fluorescence intensity is of interest. But image analysis provides more opportunities. A long list of numerical parameters can be extracted from images or more precisely from identified objects, describing shape, texture, pixel-intensity statistics, and so on. Classification algorithms can then be trained to discriminate between cell phenotypes [97]. Such software applications can be of high accuracy in classifying cell types but reach their limits in recognizing new phenotypes. Hence, the trend points to ''intelligent'' classification systems, which automatically learn and define new classes with similar characteristics [97]. However, such an automatic identification and classification system can also be used for protein analyses (proteomics). It is a valuable tool in location proteomics, that is, specifying the location of proteins within cells [98].

The main obstacle in live cell image analysis is the monitoring of single cells over time. Cell motility hinders direct retrieval of cells in single images. Cell tracking algorithms, however, allow for connecting objects in time. Even tracking of object splitting (cell division) or merging (cell fusion) is possible. Analysis of time-lapsed data sets can provide information of, for example, individual cell cycle progression [99], cell migration [100], or cell motility behavior [101].

1.5.3
Cytometry – the Other Side

Multiparametric cytometry has been the major trend for many years. However, more and more complex analyses cause more and more costs, of reagents, instrumentation, and so on. But not everywhere can money be spent for such purposes although cellular analyses are badly needed – simple analyses, easy to accomplish, but too expensive to be done in some areas of this world. CD4 counting in HIV patients in Africa is such an example. The procedure of counting cells of a single cell population is far from being tricky but the technology is too expensive as are the costs for each analysis. Howard Shapiro's appeal in 2000 on a Purdue Cytometry Discussion Forum for low-cost and effective CD4 testing tools was a starting point for many researchers to take a serious look at the situation in Africa. Since then, the development of low-cost tests for CD4 counting or for malaria for resource-limited countries is another trend in cytometry.

FCM, as well as SBC, is of interest for reducing costs and developing low-cost instruments. Since FCM is expensive because of the sophisticated technology and costs for maintenance, the cheaper alternative will be SBC. However, SBC instruments available today with sophisticated microscopes and complex optics

(e.g., [14, 44, 102, 103]) are expensive. If they could be reduced to inexpensive light sources (e.g., diodes), elementary optics, and simple image analysis software, such instruments will become a valuable tool for low-cost cell analyses and adequate diagnostics in third-world countries [104]. An example of such an approach is the device developed by Moon *et al.* [105], which utilizes immobilized anti-CD4 antibodies, a CCD sensor, and an automatic cell-counting software.

Nevertheless, an FCM is also in the focus of low-cost analyzers. Zaragosa [106] developed a portable, low-cost flow cytometer that uses acoustic micromanipulating techniques to focus particles in a flow stream instead of the commonly used hydrodynamic focusing. This technique reduces maintenance costs because sheath fluid, a big cost factor in consumables, is no longer required. Additionally, inexpensive light sources and cheaper detector systems (less sensitive) can be used, which further reduce costs [106]. However, costs for preparation of blood samples should not be neglected. Greve *et al.* [107] presented a method for less expensive determination of CD4 T cells. With a no-lyse, no-wash flow-cytometric method in combination with a simplified gating strategy, it is possible to reduce the costs per sample from €30 to below €2 [107, 108].

It should be kept in mind that not only HIV patients need adequate diagnostics but also patients suffering from other global level diseases such as malaria and tuberculosis. In this context, Wolfgang Göhde and his great effort in developing and spreading affordable diagnostic tests cannot be forgotten. Göhde and the Partec Essential Healthcare program aim to bring innovative techniques directly to where they are needed most, including to those patients far from large cities, living in remote areas where the existing health services have not reached until now [109].

1.6 Conclusion

Cytometry offers many opportunities for cellular investigations. It varies from flow- to slide-based cytometry and from analysis of cell suspension to solid tissue. As broad the methodological spectrum is so broad is the range of applications. Nevertheless, two major trends can be identified: (i) very complex, multiparametric (mainly multicolor) analyses and (ii) ''back to basics'' – low-cost and simple analyses for diagnostics in resource-limited countries. Multiparametric cytometry is mainly for gaining deeper understanding of biological processes or identifying rare cell types (for diagnoses or drug target screening) in the western world, while simple (partially only monocolor or even label-free) analyses are definitely cheaper and can be utilized in a wider area, where they are desperately needed.

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