Chapter 10
Cytomics and Predictive Medicine for Oncology

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Abstract Cytomics combines the multimolecular cytometric analysis of cell and cell system (cytome, cytomes) heterogeneity on a single cell level with the exhaustive bioinformatic knowledge extraction from all analysis results (cytomics = system cytometry + bioinformatics). It therefore yields a maximum of information about the apparent molecular cell phenotype.

At present, in the typical *hypothesis driven* way the high amount of information collected by multiparameter single cell flow- or slide-based cytometry measurements is preferentially used to investigate the molecular behaviour of specific cell populations in the perspective of the hypothesis. The information outside the scope of the hypothesis remains frequently unused.

In contrast, under the *predictive medicine by cytomics* concept, the entire available information is processed ("sieved") in a *data driven* way under the general data mining hypothesis that such data may contain useful information for clinical diagnosis and especially for therapy related predictions about disease progress in individual patients.

The present experience from clinical data sets of various malignant and other diseases suggests that this is a promising concept for cancer patients since it has amongst others the potential to identify high risk patients prior to an anticipated therapy as being unsusceptible with accuracies of greater 95% or 99%. This opens the way for early decision on alternative therapies by objective and molecularly standardised criteria. This has been traditionally difficult by current prognosis evaluation according to the widely used Kaplan-Meier statistics for patient groups.

The cytomics concept is also useful for cancer research in general because it favours the enrichment of informative parameters concerning disease outcome in individual organisms or cell cultures from an essentially unlimited number of parameters.

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The selected parameters are useful as a starting point for mathematical modelling in systems biology without requirement for detailed pre-existing knowledge about potential disease inducing mechanisms. It has therefore the potential for the discovery of new molecular cell pathways and for their subsequent molecular reverse engineering.

10.1 Background

Single cell measurements by flow or slide-based cytometry in cancer medicine and cancer research are typically performed in a multiparameter setup using hypothesis driven parameter selection in order to simultaneously gather a maximum of diagnostic or prognostic information by the accurate assessment of the molecular phenotype of patient or experimental cells (Table 10.1).

Table 10.1 Comparison of flow- *vs.* slide-based cytometry**.** Some principle advantages and specific features of flow- (FCM) and slide-based (SBC) cytometry are listed. Depending on the specific setting one approach alone or both in combination might be applied. Whereas FCM allows to measure large sets of parameters in unsurpassed speed, SBC offers the unique feature to keep cells in their natural environment, i.e. tissue context and to follow up "individual" cells at different time points of experimental settings

	FCM	SBC
Technical specifications	Rapid speed	Slow
	Low CVs	Broader CVs
		Higher background, bleaching, during measurement
	Standard 6 colours	Standard 6 colours
	Optional 17 colours	Optional 8-colour, theoretical n-colour
Logical features	Single-cell	Multi-cellular complexes: tissue sections, cell/tissue cultures
	In suspension: cell-network destroyed	On slide: topology kept intact
	High-content analysis	High-content analysis
	Limited structural resolution	Morphological re-evaluation
Clinical/practical aspects	Large specimens	Hypocellular specimens
	Consumptive unless cell sorting	Non-consumptive, no cell loss
	Detection of ultra-rare events	Analysis of cell interaction
	Bulk-sorting of specific cell-subtypes	Re-analysis on a single-cell basis
		Combination of data at different functional states (pre-/post-fixation, pre-/post-stimulation) at single-cell level

With cells as the elementary function units of organisms at the one hand and diseases being caused by molecular alterations in cells and cellular systems (cytomes) at the other hand (Valet 2002), the disease associated molecular cell phenotype is a correlate of the disease process and a result of genotype realisation and the lifetime history of cell exposure to external and internal influences. The molecular cell phenotype (Fig. 10.1) is of interest for disease diagnosis but also for predictions about the future disease progress in individual patients.

These considerations lead to the development of the *data driven* system cytometry (Valet 1997) and cytomics (Valet 2002) approaches with the aim to extract knowledge from the entire available information of *hypothesis driven* cytometry and other investigations. Both, system cytometry and cytomics, regard a single cell as being a single biochemical cuvette. The analysis of the utmost cellular complexity instead of cellular monosystems constitutes the central feature of system cytometry. This requires to combine both: (a) to collect as much biochemical information as possible in a maximum of potentially related but nevertheless different cell populations of complex cellular systems (blood, bone marrow, transplant biopsies etc); and (b) extract this enormous amount of information

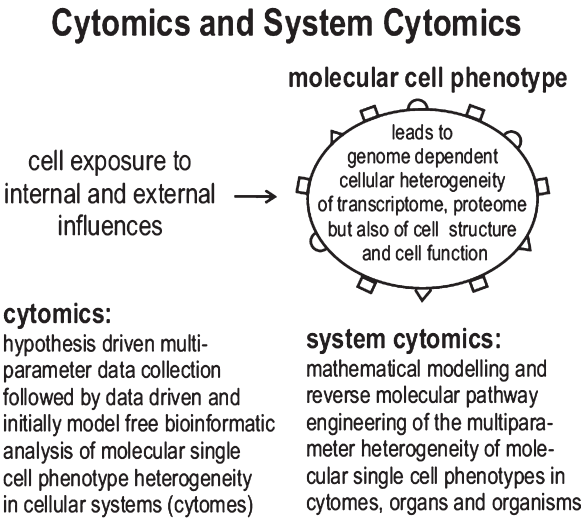

Fig. 10.1 Cytomics. Cytomics and system cytomics address the molecular heterogeneity of single cells in cell systems (cytomes). Genome expression in cytomes adapts to environmental influences and may lead to altered disease susceptibility in genetically identical organisms (Wirdefeldt et al. 2005). The resulting molecular cell phenotype represents a useful indicator of the actual balance between genetic setup and exposure in healthy and diseased individuals. It provides information for therapy dependent predictions on future disease course in individual patients (predictive medicine by cytomics) and is of interest for the molecular reverse engineering of disease pathways (Valet 2005b, 2002, 1997) by system cytomics as well as for purposes of drug discovery (Valet 2006)

efficiently by standardised multiparameter data classification (SMDC). This concept dates back to 1982 when a periodic system of cells was drafted for the first time (Schwemmler 1982).

Nevertheless, still multiparameter cytometry measurements are used in many instances to discriminate particular cell populations of interest while the information of the other cell populations remains unconsidered. This constitutes a significant waste of information since it is by no means certain that non-evaluated cells lack relevant diagnostic, prognostic, or predictive information.

Antigen as well as forward (FSC) and sideward (SSC) light scatter distributions of cells are typically broad with coefficients of variation in the 20–50% range. Cellular antigens are frequently expressed with little correlation to each other as evidenced by the presence of nearly round or spherical clusters in cytometric two or three parameter histograms displays (Fig. 10.2). Antigen expression, the correlations between different antigen expressions, and the spreads of value distributions

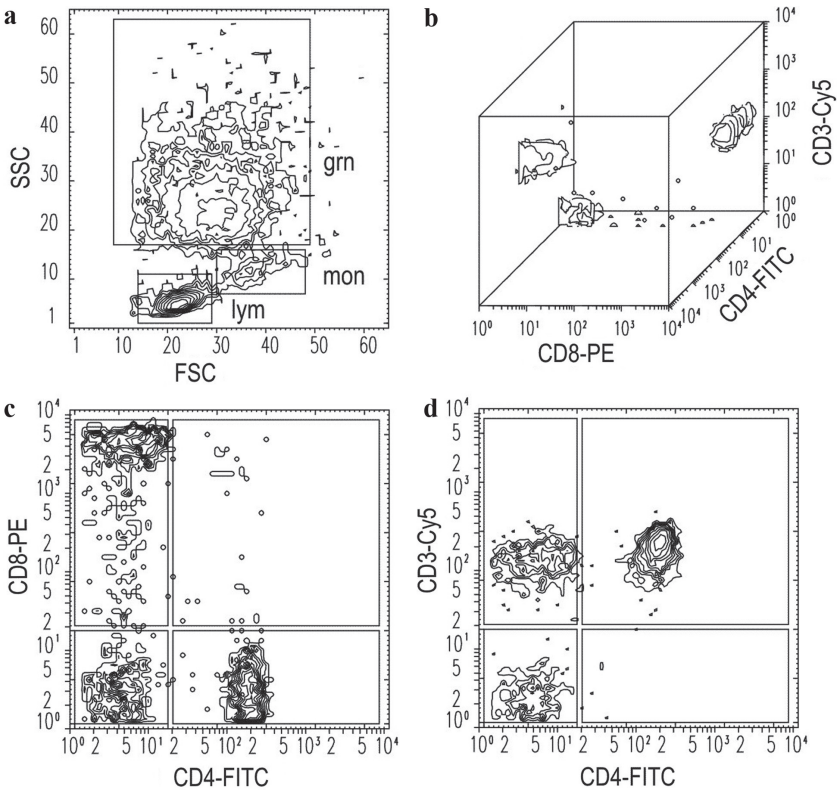

Fig. 10.2 CD4, CD8 and CD3 antigen expression on peripheral blood leukocytes of a healthy adult person by flow cytometry as determined by fluorescence labelled antibodies (data file from Valet et al. 2002). The spheroid and oblongated cell clusters show significant spread and little parameter correlation (**a**–**d**). The coefficients of variation (CVs) of the cell clusters are typically between one and two orders of magnitude higher than CVs from DNA cell cycle analysis where

may change during disease or experimental conditions. The observed cellular heterogeneity may furthermore contain information about the high adaptivity of the hemato- and immunopoietic systems. It seems worthwhile to study these phenomena by systematic, automated, and exhaustive information and knowledge extraction from all cells or biological particles in slide-based or flow cytometric measurements by bioinformatic data mining (Valet et al. 1986) under the general hypothesis that the thus obtained information may prove useful for disease diagnosis or especially for therapy related predictions on disease progress in individual patients. Data mining can be performed by statistical or algorithmic methods. Algorithmic data pattern classification (data sieving) (Valet et al. 1993) was found particularly efficient for this purpose.

10.2 Flow Cytometry

10.2.1 Clinically Oriented Studies

Initial efforts concerned cell functions with intracellular pH, esterase activity, and viable *E. coli* K12 phagocytosis as potential outcome predictors for intensive care patients with regard to recovery, development of sepsis, or posttraumatic shock (Rothe et al. 1990). Although at this stage not predictive, granulocyte function parameters showed a potential for individualised outcome predictions when granulocyte serine protease activity was flow cytometrically determined (Valet et al.

Fig. 10.2 (continued) G0/G1 phase CV values around and below 1% lead to the resolution of x- and y-spermatides by flow cytometry (Meistrich et al. 1978). The form and spread of the cell clusters indicates molecular heterogeneity of cell populations and not uncertainty of measurement. The uncorrelated heterogeneity of cell parameters is informative in view of knowledge extraction by data mining (Valet et al. 1993, 2003). Self-adjusting gates (Valet et al. 1993) separate peripheral blood leukocytes into lympho-, mono- and granulocytes (A). The CVs (CV = 100*standard deviation/mean) of the FSC/SSC clusters of lympho- (lym), mono- (mon) and granulocytes (grn) are 10.2/21.6%, 10.7/16.6%, 23.2/25.1% with correlation coefficients between FSC and SSC of r = 0.441, 0.269, 0.099. The selective display of cells within the lymphocyte gate (**a**) separate CD4/CD3 positive and CD8/CD3 positive T-lymphocyte clusters as well as a CD3/CD4/CD8 negative cell cluster together with some CD8 positive but CD3 negative cells (**b**). The CD4 and CD8 positive T-cell clusters (**c**) show coefficients of variation of 24.6% and 46.9% with CD4 to CD8 correlations of r = 0.183 and -0.053. The CVs for CD3 expression on CD3 positive/CD4 negative and CD3/CD4 positive lymphocytes (**d**) are 37.2% and 33.3% with 25.0% for the CD4 positive cells. The CD4 to CD3 correlations are r = 0.056 and 0.273 for the two lymphocyte clusters. The automated evaluation of the list mode file with the parameters forward (FSC) and sideward (SSC) light scatter, fluorescein isothiocyanate (FITC), phycoerythrin (PE) and phycoerythrin-cyanine 5 (Cy5) antibody labels, provides histograms containing 9789 (**a**), 2514 (**b**), 1872 (**c**) and 2384 (**d**) cells. Contour lines were drawn on a 3-decade logarithmic amplitude scale in 10% linear steps downwards from the respective maximum logarithmic channel content of each histogram (max = 253, 22, 70 cells) (**a**, **c**, **d**). The 10% contour line delimits the 3D clusters (max = 118 cells) (**b**). The lowest contour lines contour histogram channels containing minimally a single cell thus assuring the display of all cells in each histogram

1998). It was equally possible to identify risk patients for myocardial infarction from peripheral thrombocyte surface antigens CD62, CD63, and thrombospondin by algorithmic data pattern classification (Valet et al. 1986).

With this background, predictions in malignant diseases were addressed using clinical data collected outside of the own institution. It was possible from an initial data set of six parameters to prognosticate by meta-analysis individualised 10 year survival in melanoma patients at diagnosis (Valet et al. 2001) from the parameter triplet tumor diameter, tumor infiltration depth, and percentage of S-phase cells with negative and positive predictive values of around 80.3% for survivors and 79.8% for non-survivors (Valet et al. 1986).

Acute myeloid leukemia (AML) was investigated in the context of a multicenter study of the South German Hemoblastosis Group (SHG). Data on the expression of 23 of the most common cytogenetic abnormalities, of the frequency of positive cells for 36 antigen specificities, and of 9 clinical parameters at diagnosis were available (Valet et al. 2003). The predictive data pattern consisted of the following seven selected parameters: patient age, sample cellularity, percent CD2, CD4, CD13, CD36, and CD45 positive cells. This pattern identified with positive predictive values of 100% a subset of 49.8% and 91.5% of high risk non-survivors in the learning and unknown test sets. The result indicates an identifiable patient subgroup that will only have a survival chance upon immediate bone marrow stem cell transplantation. This clinically important conclusion cannot be derived from the concomitant prognostic analysis of the same data by Kaplan-Meier statistics (Repp et al. 2003).

Survivor AML patients, in contrast, are not well identified with 15.1% negative predictive value by the above data pattern (Valet et al. 2003). A more discriminatory data pattern may help. It may be important to analyse the “normal” cell populations by flow cytometry appearing during the first remission and to classify these results against final outcome to better understand the influence of non-malignant cells on final AML outcome.

Individualised outcome prediction by data pattern classification is not restricted to flow cytometry. The expression of mRNA in diffuse large B-cell lymphoma (DLBCL) was assessed on a Lymphochip cDNA array with 7,399 evaluable spots (Rosenwald et al. 2002). In the original study, this high amount of information produced a Kaplan-Meier prognosis graph, similar to the one for the international prognostic index (IPI) (The International Non-Hodgkin’s Lymphoma Prognostic Factors Project 1993). The IPI is constituted of only five clinical parameters, namely age over 60 years, late-stage disease (stages 3 and 4), more than one extra-nodal site, high LDH, and poor general health. Since the Lymphochip evaluation heat maps did not allow individualised predictions it was of interest whether data pattern classification could do so.

High risk non-survivor patients were well identified at diagnosis by data pattern classification with 98.1% and 78.3% positive predictive values in the learning as well as in the unknown test set of patients (Valet and Höffkes 2004). Survivors, similarly as in the AML study, were less well recognised with negative predictive values of 67.3% and 45.3%.

In conclusion, data pattern classification has been shown to be of significant immediate clinical interest for the identification of high risk patients at diagnosis. The selected data patterns may be useful for research purposes and it may be promising to additionally classify "normal" remission cells against AML- or DLBCL-outcome.

10.2.2 Multiparameter Data Mining

Individualised predictions of therapy related disease progress in medicine are frequently considered impossible because in the majority of patients their future under therapy is evaluated by Kaplan–Meier statistics for patient groups (Bland and Douglas 1998). This approach is directed towards overall therapy optimisation but does not address the individual patient.

Parameter values of patients are typically introduced into value clusters in multiparameter data mining. At this point the concatenation, i.e. the coherence of the parameter values of a given patient, is lost. The value clusters for the various parameters are then correlated between the various classification categories and the parameters with the most discriminatory clusters are selected for classification. The lost coherence of patient parameters values leads typically to probabilistic conclusions with the inherent difficulty to generate individualised disease course predictions at accuracy levels greater than 95% or 99%.

Accurate individual predictions of therapy related disease progress are, however, important for patients and doctors but also for the public and private health care systems since potential adverse drug reactions in non-responders as well as higher health care costs due to inefficient therapies.

Data pattern classification according to the CLASSIF1 algorithm (Valet et al. 1993, 2001; Valet 2005a) performs always a concatenated parameter classification of the originally measured multiparameter data patterns of each patient to achieve individualised predictions. The most discriminatory parameters of the measured data patterns are selected during the iterative learning phase and kept whereas lesser discriminating parameters are eliminated from consideration after the end of the iteration process.

All parameter values of a data pattern are classified at the beginning of the learning phase, then one parameter is temporarily removed, followed by reclassification of the remaining learning set. If the removed parameter contained information, an effect of this removal on the classification result will be observed (deterioration or improvement); this result is retained. The temporarily removed parameter is then reinserted and the next parameter is temporarily removed for the next iteration, and so on until the last parameter. Only parameters that have improved the classification result are kept at the end.

The ensuing predictive classification patterns consist typically of between 5 and 20 parameters. The statistical probability that such patterns occur randomly decreases with 3^{-n} and is lower than 1% from five-parameter classification patterns onward. Statistical probabilities do therefore not significantly influence the classification result.

The robustness of the CLASSIF1 data patterns against random statistical fluctuations permits to further analyse them in order to discriminate subgroups of patients for example of particular genotype or exposure background as an additional data mining aspect. CLASSIF1 classifiers are typically standardised on a reference group of patients or on standard particles. The similarity of reference groups is verified by classifying them against each other. If they are indistinguishable classifiers can be compared between institutions and databases can be merged to build up standardised relational classification system of cells at the molecular level suitable for a human cytome project or for a periodical system of cells where normal and diseased cells can be compared in a standardised classification system for normal and abnormal cells (Valet 2005a, b).

10.3 Slide-Based Cytometry

Slide-based cytometry offers a different technological platform for the multiparametric analysis of cells. First instruments actually date back as early as 1955 (Caspersson et al. 1955; Göhde and Dittrich 1970): instead of passing the sample as a single-cell solution flowing through an analysis chamber as in flow cytometry, for slide-based cytometry the sample is prepared on an objective slide as for conventional microscopy or on some other kind of adequate solid support. The different commercially available instruments have in common that this slide with the fluorescent cells on it is moved by a motorised stage under the objective. Cells are illuminated by lasers or lamps (Xenon lamp or mercury arc lamp) and the fluorescence is detected by CCDs or photomultiplier tubes. This chapter does not aim to describe the different technical features in detail that have been realised in order to obtain stable illumination, spectral separation of different fluorochromes, detection of the fluorescence, or calculation of fluorescence intensity (for detailed comparison, see Mittag et al. 2009). We would rather concentrate on the potential of this technology in general in the context of clinical oncology.

The slide-based design opens several opportunities that have so far not been accessible by flow cytometry or image cytometry based on conventional chromatic cytological staining. First, fluorescence can be stoichiometrically detected so that the number of antigens bound by an antibody can be calculated (Rimm 2006), and modern fluorochromes allow combinations of up to 17 dyes that can be detected in parallel (Perfetto et al. 2004). This is a major advantage as compared to image cytometry. So far, for a slide-based system up to eight colours have been combined at a single run using two excition wavelengths (488 nm by an Argon- and 633 nm by a HeNe-laser) (Gerstner et al. 2002; Mittag et al. 2006). This can easily be increased by adding another wavelength (405 nm by a violet-dye laser) and adapting the filter systems. In image analysis there is in general only one channel available; taking the most popular application, which is the determination of DNA-ploidy by Feulgen-staining (Feulgen and Rossenbeck 1924), the staining protocol includes procedures that destroy most antigens keeping only cellular morphology intact.

Second, for image analysis in general the investigator selects just 150–300 single "typical" cells for analysis whereas the other cells on the slide are not analysed and therefore the information contained in these cells is lost. In slide-based cytometry, all cells on the slides can easily be included into the analysis.

The third advantage is that samples not necessarily have to be prepared as a single-cell solution. Whereas this separation yields to no loss of information in a tissue that constitutes of single cells physiologically, as it is the case in blood, in any other tissue crucial information is contained within the architecture of the tissue itself. A good example is the case of metastatic disease to a lymph node. Disrupting the architecture of the node for preparation of a single-cell solution only allows to detect the presence of metastatic cells among the lymphocytes; however, clinical even more relevant information such as extracapsular spread is lost. Detection of this topological information demands an intact anatomical specimen. This, however, would be inappropriate for flow cytometric assays.

In principle, any specimen that can be placed on a slide can be analysed in some way by slide-based cytometry. Modern instruments allow the analysis of chromatic dyes, too.

Since objects are immobilised on the slide at a fixed position they can be identified according to their x–y-co-ordinates. This puts the investigator into the position to rule out any artefact and to obtain proof that a given fluorescence signal is in fact generated by a cell. Also, cells are not lost; this has the advantage that sample too small for flow cytometry can be readily analysed by slide-based systems, such as hypocellular fine-needle aspirate biopsies or exfoliative swabs. For better visualisation of cell morphology cells can be re-stained by conventional chromatic dyes and inspected afterwards. Instead using chromatic dyes, specimens can be bleached and stained with another set of fluorochromes for another analyses. The data of the first analysis can be merged with those of the subsequent analyses to yield a data stack per cell. In principle, bleaching can even be omitted since the fluorescence of the prior staining can be subtracted from the subsequent staining (Mittag et al. 2006). Slides also can be stored as a conventional pathological specimen which is of utmost importance keeping medicolegal issues in mind.

The sum of these specific characteristics makes slide-based cytometry the superior technology for clinical applications in the oncology of solid tumors. Although the concept of predictive medicine by cytomics opens up n-dimensional data spaces so far only two or three parametric analyses have been established for the investigation of solid tumors and will be outlined below. Unlike in hematopoietic disorders, where clinical sample material is easily available, for solid tumors in general only minute samples are available which routinely are analysed by conventional histopathology. However, as will be shown by some non-oncological examples, the capacity of a slide-based technology in the analysis of tissue samples in principle has been established and soon will be transferred to oncological issues.

This specific clinical situation in solid tumors explains why the brilliant progress achieved by the cytomics approach in hematopoietic diseases has not yet been transferred to solid cancers.

10.3.1 Predictive Medicine in Solid Tumors

Strictly speaking, an assay that allows the prediction of the clinical course in a patient with a solid tumor has to be implemented as early as possible within the diagnostic work-up. From the clinician's point of view the first and most important issue is nothing more than to either verify or rule out the presence of a malignant tumor. This should be possible with samples available on an out-patient basis obtained by minimal- or non-invasive procedures. In theory there could be cases where the malignant phenotype is expressed only in a minority of cells which would be missed by a random sample such as a fine-needle aspiration biopsy. Keeping this in mind therefore a negative result hardly ever will be taken as a proof of absence of malignancy. However, in fact routine histopathology does not include an entire work-up of the whole specimen neither; instead, "representative" sections are screened. Actual, there is no proof that the sections in fact are representative other than the subjective judgement of the pathologist.

Exfoliative swabs on the other hand are confronted with the argument that the cells accessible by this method, i.e. the superficial layers of the lesion, are not the cells relevant for the course of the disease which rather is determined by the basal layers that infiltrate through the basilar membrane. However, from a clinical point of view just a stable surrogate marker of the disease would suffice. Besides, it is rather theoretical to argue that the superficial cells themselves do not infiltrate into the surrounding healthy tissue; in any way they serve as representatives of the basal cells. The only exception is tumors growing under intact mucosa hidden by intact epithelium as in sarcomas or some lymphomas. These tumors are hard to detect by any diagnostic procedure and make up only a minor part of malignancies.

In some anatomical regions such as the larynx the physiological function is tightly coupled to anatomical integrity. In tumors of the parotid gland incision biopsies are obsolete due to the risk of damaging the facial nerve and of spreading tumor cells in the surrounding tissue which would be hazardous even in the most common benign solid tumor, the pleomorphic adenoma. In cases like these minimal sampling is mandatory in order to avoid loss of quality of life due to diagnostic manoeuvres. "Deep" biopsies including basal layers in general induce a loss of function in these anatomical regions.

From a practical point of view predictive assays can be divided into assays that establish the diagnosis by minimal- or non-invasive ways on the one hand and into assays that predict the further clinical course of an already diagnosed disease on the other hand.

10.3.1.1 Diagnostic Cytomic Assays

As lined out above slide-based cytometry is an ideal tool to analyse hypocellular specimens. This has been exploited to determine the DNA-ploidy of tumor cells. Automated slide-based cytometry allows to analyse a multiple of cells that are

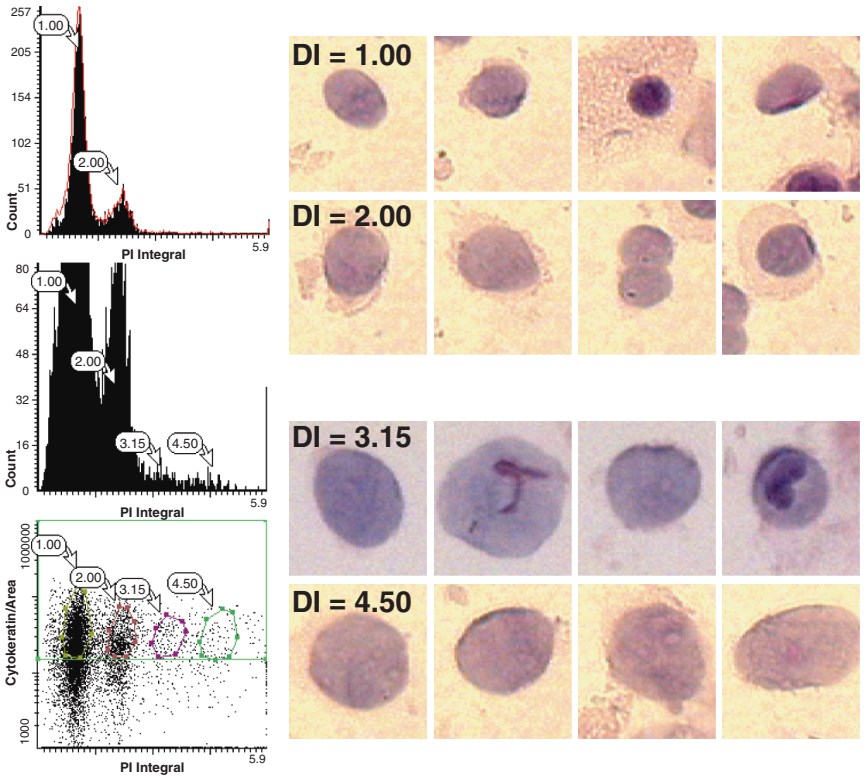

Fig. 10.3 Slide-based cytometry. Slide-based cytometry of a fine-needle aspirate taken from a solid parotid gland tumour. Cells were stained with anti-cytokeratin and PI as a stoichiometrical DNA-dye. DNA-ploidy of epithelial cells is determined taking leukocytes as an internal standard (DNA-index = 1.00). Note the minimal population of cells with a DNA-index of 3.15 and 4.50 making up less than 1% of all cells. Histology confirmed an adenocarcinoma. For detailed method see Gerstner et al. (2003) (reprinted with permission from Gerstner et al. 2008)

routinely selected by image cytometry, i.e. 20,000–100,000 *vs.* 150–300. This guarantees that also minor cell populations of less than 1% are included into the analysis that easily could be missed by manual screening with direct visualisation (Fig. 10.3). It has been shown that slide-based cytometry gives better prediction of the histopathological diagnosis than conventional cytology (Gerstner et al. 2003, 2005, 2006). In some cases, the correct diagnosis could be established weeks before an appropriate histopathological sample could be obtained (Remmerbach et al. 2001, 2004).

The combination of HPV-genotyping and determination of the DNA-ploidy with conventional cytology has been proven beneficial in the early detection of carcinomas in cervical smears (Bollmann et al. 2005a) and was shown to have the potential to predict the further clinical course (Bollmann et al. 2006).

In general, slide-based cytometry can yield quantitative and objective cytomics data where conventional cytology only can offer subjective judgements. The applications outlined above highlight only some peculiar examples.

10.3.1.2 Predictive Cytomic Assays

Prediction of the future clinical course of cancer patients by cytomic analyses date back to the 1990s (Hemmer and Schön 1993; Hemmer and Prinz 1997; Hemmer et al. 1997; Hemmer et al. 1999): in oral tumors it could be shown that DNA-aneuploid tumors have a higher rate of locoregional metastasis advocating resection of the locoregional lymph nodes in DNA-aneuploid tumors even in cases without clinical and radiological signs of metastatic disease. Since DNA-aneuploid tumors also have a higher rate of local recurrence closer follow-up is advised.

In patients with superficial bladder carcinoma slide-based cytometry has been successfully implemented in the follow-up using samples obtained from voided urine (Bollmann et al. 2005b).

10.3.2 Future Aspects

The more expertise with slide-based assays is gained the more complex samples are analysed. Several (non-)oncological applications are outlined below but it is expected that these assays soon will be transferred into oncological issues.

Slide-based cytometry allows to analyse tissue sections. This has been performed on sections from lymphoid organs: based on nuclear fluorescent staining the different microanatomical compartments of the lymphoid follicles, i.e. the mantel zone and the germinal centre, could be analysed separately and the relative content of specific subtypes (e.g. CD8+-lymphocytes) could be mapped to these compartments. The germinal centre could be further subdivided into the dark and the bright region containing the respective cell types (Gerstner et al. 2004). This concept of tissometry (Ecker and Steiner 2004; Ecker et al. 2006) has been further developed on brain sections – which have significant lower cellular density – aiming to analyse the three-dimensional interaction of different cell types within the tissue architecture (Mosch et al. 2006). This concept has recently been further pushed forward by developing a versatile hardware (Kim et al. 2007). Applications on lymphatic tissue are those most sophisticated (Harnett 2007). On prostate tissue sections the quantitative detection of α-methylacyl-CoA racemase has been exploited to allow automated classification of the tissue (Rubin et al. 2004).

Although these assays are still viewed sceptically by histopathologists they will in future be more and more relevant for clinicians who aim to base their decision about further diagnostic and therapeutic steps on objective parameters. This is not only the case in malignancy but is true for all somatic diseases.

For example, therapeutic intervention in organ transplantation is oriented on functional parameters. However, slide-based analysis is useful in thorough characterisation of graft-rejecting leukocytes in transplant rejection and therefore can help to better understand the underlying cellular mechanisms (Ecker and Steiner 2004). On this basis, therapy could be based on individual cytomic data.

The so far most detailed analysis of cellular interactions in the tissue has been achieved by a technology termed Multi-Epitop-Ligand-Kartographie MELK (Schubert 2003). A single section is repeatedly stained for an antigen with a fluorochrome, analysed, and bleached to remove the signal, followed by another cycle of staining-analysis-bleaching, and so on. Data of all analyses are combined according to the fixed x–y-co-ordinates of the slide. Since the natural micro-environment of the cells is not disrupted (as in flow cytometry in order to obtain a single-cell suspension) but the architecture of the tissue is kept this approach allows to generate hierarchical clusters of interacting proteins according to their spatial location, termed the toponome (Schubert 2006. The maximum number of antigens analysed on the same slide so far is 100 (Friedenberger et al. 2007).

It is expected that these assays will soon be applied to issues in the oncology of solid tumors: to characterise and quantitatively determine tumor-infiltrating leukocytes, to analyse the tumor invasion front where the tumor and its host have the crucial interaction, and to judge on residual tumor cells after primary radiochemotherapy.

The slide-based approach on cytomics analysis additionally allows a detailed absolute immunophenosubtyping, i.e. the measurement of absolute numbers per volume of a given specific cell type such as the number of CD8+ cytotoxic cells per liter blood (Laffers et al. 2007). In breast cancer, the quantification of circulating tumor cells after therapy has been established as a predictor for the outcome and therefore can be used in the decision about adjuvant therapy (Lobodasch et al. 2007). In future, detailed analysis of the functional capacity of these cells might develop significant input into the therapeutic regimen in other solid tumors as well.

However, quantitative data on cells can also be obtained by analysing the tissue in toto without taking a biopsy applying multispectral imaging. The idea of this technology has first been applied at the Landsat-program of the National Aeronautics and Space Administration (NASA) for earth imaging (Harris 2006). The molecular composition of a specimen interacts with the electromagnetic radiation. Therefore, the molecular phenotype of a cell is reflected by its specific spectral signature. Since the architecture of the tissue is composed by cells and their molecular products, the type of tissue can be classified by its spectral signature as well. This principle was drafted in 1998 (Farkas et al. 1998) and since then it has been developed to different biomedical applications: It has been applied to histological sections (Levenson and Mansfield 2006) where it distinguished metastasising cells in lymphoid tissue or infiltrating tumor cells in breast cancer. Without taking biopsies invasively it has been applied for non-invasive classification of pigmented naevi; in fact, this application was established even earlier. In order to verify multispectral classification the naevi were subsequently resected; histopathology confirmed exactly those regions within the naevi were malignant melanoma

developed (Farkas and Becker 2001). This concept could develop tremendous impact on routine diagnostic work-up, especially in oncology: any inner or outer body surface accessible for visualisation could be imaged by multi- or hyperspectral imaging. The topological discrimination would be limited only by the optical resolution of the imaging tool and could in principle be scaled down to the single cell level. Beyond non-trivial problems concerning the hardware that so far have not been resolved the only limitations of this approach seem to be that the lesion should be at the inner or outer surface. However, novel imaging modalities could even break this barrier: for example, optical coherence tomography (OCT) gives optical sections non-invasively up to 2 mm deep into the tissue (Armstrong et al. 2006; Bibas et al. 2004; Kraft et al. 2007). Novel developments (μOCT) give subcellular resolution allowing to differentiate even single nuclei (Pan et al. 2007; Wang et al. 2007) without a cut or a single drop of blood.

10.4 Conclusions

The single cell approach in flow and slide-based cytometry with its currently rapid methodological progress has a significant clinical and research potential, especially when entire multiparameter data sets are subjected to outcome driven data mining.

The data pattern classification algorithm evaluates individual patient pattern against previously learned disease classifications patterns. This differs from statistical classifiers where the coherence of patient patterns is typically lost by introduction of the individual parameter values into data clusters resulting in lower resolution for pattern differences during the learning process. Statistical ambiguities concerning the best achievable classification as well as the limitation to prognostic conclusions for patient groups but not for individual patients make algorithmic data pattern classifiers attractive for clinical purposes. They provide by principle individualised predictions at the optimum discrimination conditions while the potential for subsequent statistical analysis of the selected parameter patterns is maintained.

Flow cytometry as the first single cell high-throughput technology has at present a certain lead with regard to predictive medicine by cytomics since a wealth of data sets and evaluations are available. With the fast progress of automated image segmentation in fluorescence microscopy the situation is likely to change. The difficulty to standardise molecular quantification in microscopy, is outweighed by the possibility to collect intracellular morphological as well as cellular 2D and 3D neighbourhood information as a further potential for knowledge extraction and mathematic modelling in system cytomics.

Molecule specific multiparameter fluorescence staining, high-throughput and high-content single cell measurements in conjunction with multiparameter data classification will open the way for generalized *disease course prediction* for patients for the practice of medicine, similarly as microscopic single cell observation of initially textile colour stained histological sections by Virchow (Virchow 1858) has enabled histopathological *disease diagnosis*.

References

Armstrong WB, Ridgway JM, Vokes DE et al (2006) Optical coherence tomography of laryngeal cancer. Laryngoscope 116:1107–1113

Bibas AG, Podoleanu AG, Cucu RG et al (2004) 3-D optical coherence tomography of the laryngeal mucosa. Clin Otolaryngol 29:713–720

Bland JM, Douglas GA (1998) Survival probabilities (the Kaplan–Meier method). BMJ 317: 1572–1580

Bollmann R, Bánkfalvi A, Griefingholt H et al (2005a) Validity of combined cytology and human papillomavirus (HPV) genotyping with adjuvant DNA-cytometry in routine cervical screening: results from 31.031 women from the Bonn-region in West Germany. Oncol Rep 13:915–922

Bollmann M, Heller H, Bánkfalvi A et al (2005b) Quantitative molecular urinary cytology by fluorescence *in situ* hybridisation: a tool for tailoring surveillance of patients with superficial bladder cancer? Br J Urol 95:1219–1225

Bollmann M, Várnai AD, Griefingholt H et al (2006) Predicting treatment outcome in cervical diseases using liquid-based cytology, dynamic HPV genotyping and DNA cytometry. Anticancer Res 26:1439–1446

Caspersson T, Lomakka G, Svensson G et al (1955) A versatile ultramicrospectrograph for multiple-line and surface scanning high resolution measurements employing automized data analysis. Exp Cell Res 3:40–51

Ecker RC, Steiner GE (2004) Microscopy-based multicolor tissue cytometry at the single-cell level. Cytometry A 59A:182–190

Ecker RC, Rogojanu R, Streit M et al (2006) An improved method for discrimination of cell populations in tissue sections using microscopy-based multicolor tissue cytometry. Cytometry A 69A:119–123

Farkas DL, Du C, Fisher GW et al (1998) Non-invasive image acquisition and advanced processing in optical bioimaging. Comput Med Imaging Graphics 22:89–102

Farkas DL, Becker D (2001) Applications of spectral imaging: detection and analysis of human melanoma and its precursors. Pigment Cell Res 14:2–8

Feulgen K, Rossenbeck H (1924) Mikroskopisch-chemischer Nachweis einer Nucleinsäure vom Typus der Thymonucleinsäure und die darauf beruhende elektive Färbung von Zellkernen in mikroskopischen Präparaten. Hoppe Seyler Z Physiolog Chem 135:203–248

Friedenberger M, Bode M, Krusche A et al (2007) Fluorescence detection of protein clusters in individual cells and tissue sections by using toponome imaging system: sample preparation and measuring procedures. Nat Protoc 2:2285–2294

Gerstner AOH, Lenz D, Laffers W et al (2002) Near-infrared dyes for six-color immunophenotyping by laser scanning cytometry. Cytometry 48:115–123

Gerstner AOH, Müller AK, Machlitt J et al (2003) Slide-based cytometry for predicting malignancy in solid salivary gland tumors by fine needle aspirate biopsies. Cytometry B (Clin Cytometry) 53B:20–25

Gerstner AOH, Trumpfheller C, Rasc P et al (2004) Quantitative histology by multicolor slide-based cytometry. Cytometry A 59A:210–219

Gerstner AOH, Thiele A, Tárnok A et al (2005) Preoperative detection of laryngeal cancer in mucosal swabs by slide-based cytometry. Eur J Cancer 41:445–452

Gerstner AOH, Thiele A, Tárnok A et al (2006) Prediction of upper aerodigestive tract cancer by slide-based cytometry. Cytometry A 69A:582–587

Göhde W, Dittrich W (1970) Simultane Impulsfluorimetrie des DNS- und Proteingehaltes von Tumorzellen. Z Anal Chem 252:328–330

Harnett MM (2007) Laser scanning cytometry: understanding the immune system *in situ*. Nat Rev Immunol 7:897–904

Harris AT (2006) Spectral mapping tools from the earth sciences applied to spectral microscopy data. Cytometry A 69A:872–879

Hemmer J, Schön E (1993) Cytogenetic progression and prognosis in oral carcinoma: a DNA flow cytometric study in 317 cases. Int J Oncol 3:635–640

Hemmer J, Prinz W (1997) Comparison of DNA flow cytometry and fluorescence *in situ* hybridization with a set of 10 chromosome-specific DNA probes in four head and neck carcinomas. Cancer Gent Cytogenet 97:35–38

Hemmer J, Thein T, van Heerden WFP (1997) The value of DNA flow cytometry in predicting the development of lymph node metastasis and survival in patients with locally recurrent oral squamous cell carcinoma. Cancer 79:2309–2313

Hemmer J, Nagel E, Kraft K (1999) DNA aneuploidy by flow cytometry is an independent prognostic factor in squamous cell carcinoma of the oral cavity. Anticancer Res 19:1419–1422

Kim KH, Ragan T, Previte MJR et al (2007) Three-dimensional tissue cytometer based on high-speed multiphoton microscopy. Cytometry A 71A:991–1002

Kraft M, Lerßen K, Lubatschowski H et al (2007) Technique of optical coherence tomography of the larynx during microlaryngoscopy. Laryngoscope 117:950–952

Laffers W, Schlenkhoff C, Pieper K et al (2007) Concepts for absolute immunophenosubtyping by slide-based cytometry. Transfus Med Hemother 34:188–195

Levenson RM, Mansfield JR (2006) Multispectral imaging in biology and medicine: slices of life. Cytometry A 69A:748–758

Lobodasch K, Fröhlich F, Rengsberger M et al (2007) Quantification of circulating tumor cells for the monitoring of adjuvant therapy in breast cancer: an increase in cell number at completion of therapy is a predictor of early relapse. Breast 16:211–218

Meistrich ML, Göhde W, White RA (1978) Resolution of x and y spermatids by pulse cytophotometry. Nature 274:821–823

Mittag A, Lenz D, Gerstner AOH et al (2006) Hyperchromatic cytometry principles for cytomics using slide based cytometry. Cytometry A 69A:691–703

Mittag A, Bocsi J, Laffers M et al (2009) Technical and methodological basics of slide-based cytometry. In: Sack U, Tárnok A, Rothe G (eds) Cellular diagnostics. Basics, methods and clinical applications of flow cytometry. Karger, Basel

Mosch B, Mittag A, Lenz D et al (2006) Laser scanning cytometry in human brain slices. Cytometry A 69A:135–138

Pan YT, Wu ZL, Yuan ZJ et al (2007) Subcellular imaging of epithelium with time-lapse optical coherence tomography. J Biomed Opt 12:050504

Perfetto SP, Chattopadhyay PK, Roederer M (2004) Seventeen-colour flow cytometry: unravelling the immune system. Nat Rev Immunol 4:648–655

Remmerbach TW, Weidenbach H, Pomjanski N et al (2001) Cytologic and DNA-cytometric early diagnosis of oral cancer. Anal Cell Pathol 22:211–221

Remmerbach TW, Weidenbach H, Hemprich A et al (2004) Earliest detection of oral cancer using non-invasive brush biopsy including DNA-image-cytometry: report of four cases. Anal Cell Pathol 25:159–166

Repp R, Schaekel U, Helm G et al (2003) Immunophenotyping is an independent factor for risk stratification in AML. Cytometry B (Clin Cytom) 53B:11–19

Rimm DL (2006) What brown cannot do for you. Nat Biotechnol 24:914–916

Rosenwald A, Wright G, Chan WC et al (2002) The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. N Engl J Med 346:1937–1947

Rothe G, Kellermann W, Valet G (1990) Flow cytometric parameters of neutrophil function as early indicators of sepsis- or trauma-related pulmonary or cardiovascular organ failure. J Lab Clin Invest 115:52–61

Rubin MA, Zerkowski MP, Camp RL et al (2004) Quantitative determination of expression of the prostate cancer protein α-methylacyl-CoA racemase using automated quantitative analysis (AQUA). Am J Pathol 164:831–840

Schubert W (2003) Topological proteomics, toponomics, MELK-technology. Adv Biochem Eng Biotechnol 83:189–209

Schubert W (2006) Cytomics in characterizing toponomes: towards the biological code of the cell. Cytometry A 69A:209–211

Schwemmler W (1982) The endocytobiotic cell theory and the periodic system of cells. Acta Biotheor 31:45–68

The International Non-Hodgkin's Lymphoma Prognostic Factors Project (1993) A predictive model for aggressive non-Hodgkin's lymphoma. N Engl J Med 329:987–94

Valet G, Warnecke HH, Kahle H (1986) 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) Proceedings of the international symposium on histometry. Academic, London, pp 58–67

Valet G, Valet M, Tschöpe D et al (1993) White cell and thrombocyte disorders: Standardized, self-learning flow cytometric list mode data classification with the CLASSIF1 program system. Ann NY Acad Sci 677:233–251

Valet G (1997) Cytometry, a biomedical key discipline. 3. System cytometry, a new research strategy. In: Robinson JP (ed) Purdue cytometry CD series, vol. 4 (ISBN: 1-890473-03-0), http://www.cyto.purdue.edu/cdroms/flow/vol4/8_websit/valet/keyvirt1.htm#system

Valet GK, Roth G, Kellermann W (1998) Risk assessment for intensive care patients by automated classification of flow cytometric data. In: Robinson JP, Babcock GF (eds) Phagocyte function. Wiley-Liss, New York, pp 289–306

Valet G, Kahle H, Otto F et al (2001) Prediction and precise diagnosis of diseases by data pattern analysis in multiparameter flow cytometry: Melanoma, juvenile asthma and human immunodeficiency virus infection. Methods Cell Biol 64:487–508

Valet G (2002) Predictive medicine by cytomics: potential and challenges. J Biol Regul Homeost Agents 16:164–167

Valet G, Arland M, Franke A et al (2002) Discrimination of chronic lymphocytic leukemia of B-cell type by computerized analysis of 3-color flow cytometric immunophenotypes of bone marrow aspirates and peripheral blood. Lab Hematol 8:134–142

Valet G, Repp R, Link H et al (2003) Pretherapeutic identification of high-risk acute myeloid leukemia (AML) patients from immunophenotype, cytogenetic and clinical parameters. Cytometry B Clin Cytom 53B:4–10

Valet G, Höffkes HG (2004) Data pattern analysis for the individualised pretherapeutic identification of high-risk diffuse large B-cell lymphoma (DLBCL) patients by cytomics. Cytometry A 59A:232–236

Valet G (2005a) Human cytome project: a new potential for drug discovery. In: Real Academia Nacional de Farmacia (ed) Las Omicas genomica, proteomica, citomica y metabolomica: modernas tecnologias para desarrollo de farmacos. Madrid, pp 207–228

Valet G (2005b) Cytomics, human cytome project and systems biology: top-down resolution of the molecular biocomplexity of organisms by single cell analysis. Cell Prolif 38:171–174

Valet G (2006) Cytomics as a new potential for drug discovery. Drug Discov Today 11:785–791

Virchow R (1858) Die Cellularpathologie in ihrer Begründung auf physiologische und pathologische Gewebelehre. August Hirschwald, Berlin

Wang Z, Lee CS, Waltzer WC et al (2007) *In vivo* bladder imaging with microelectromechanical-systems-based endoscopic spectral domain optical coherence tomography. J Biomed Opt 12:034009

Wirdefeldt K, Gatz M, Pawitan Y et al (2005) Risk and protective factors for Parkinson's disease: a study in swedish twins. Ann Neurol 57:27–33