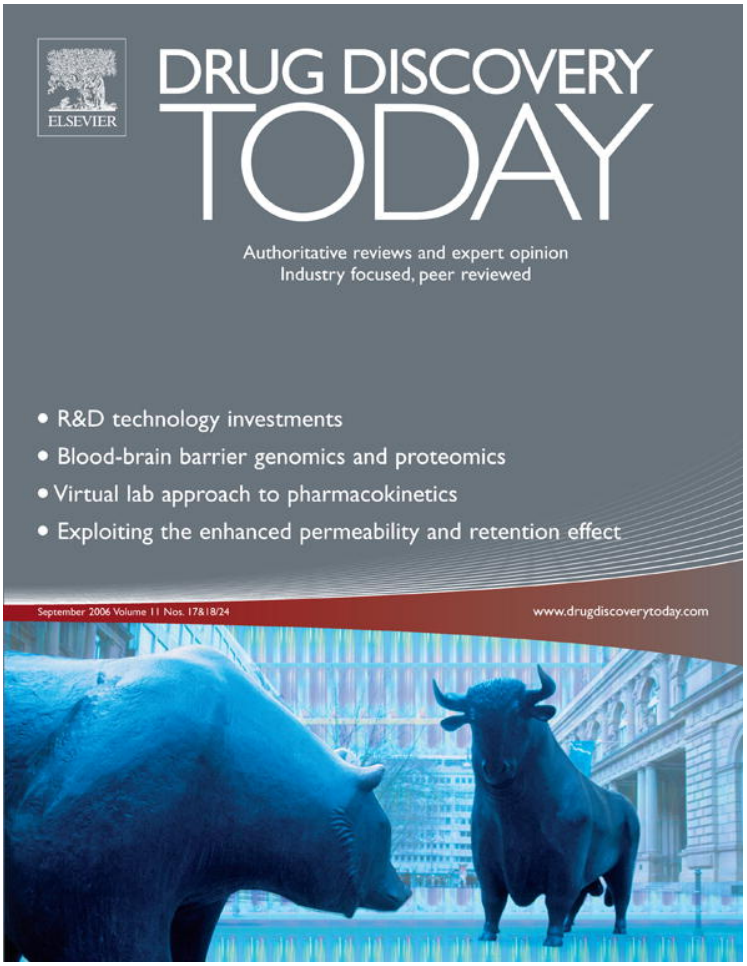

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Cytomics as a new potential for drug discovery

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At the single-cell level in conjunction with data-pattern analysis, high-content screening by image analysis or flow cytometry of clinical cell- or tissue-section samples provides differential molecular profiles for the personalized prediction of therapy-dependent disease progression in patients. The molecular reverse-engineering of these molecular profiles, which is the exploration of molecular pathways, backwards, to the origin of the observed molecular differentials, by systems biology has the potential to detect new drug targets in knowledge spaces, typically inaccessible to traditional hypotheses. Furthermore, predictive medicine, by cytomics in stratified patient groups, opens a new way for personalized (or individualized) medicine, as well as for the early detection of adverse drug reactions in patients.

Systems biology and new drug targets

After physiology- and target-driven strategies [1], hypothesis-dependent modeling of intracellular and intercellular molecular networks [2] by systems biology [3–6] has emerged as a concept with great promise for drug discovery.

Systems biology is used to model gene regulatory [7], protein–protein, protein–DNA, metabolic [8–10] or cell signaling networks [11], in an often 'bottom-up' effort that extends from the establishment of molecular networks to the molecular functionality of organelles, cells and cell systems. One of the aims of systems biology is to integrate biomolecular interactions and regulations into mathematical models until there is sufficient understanding of molecular mechanisms at the cellular level [12–14]. This will help to generate valid *in silico* predictions concerning cell behavior, for example in the presence of chemical, microbial or environmental perturbations.

Significant difficulties in generating *in silico* models for the prediction of cell behavior arise from: the unknown function of many genes and gene products; the unexplained molecular mechanisms regarding how 20,000–30,000 genes give rise to >100,000 proteins; and the steep increase of model complexity upon inclusion of more and more parameters. 'Top-down' approaches, working at the organism, organ, tissue or cell level down to the biomolecule level, do not have the same problems [15–18]. This concept is also in discussion for the discovery of new drug targets [19,20].

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Cell models

In drug discovery, a common feature in HTS and high-content screening (HCS) by image analysis [21] or flow cytometry [22] concerns the use of cell models [19,20,23] for target identification or target validation. However, standardized and comparatively easy to use cell models might not necessarily reflect the cellular conditions in the patient [24]. This prompts the use of human tissues or primary cell lines for target discovery and target validation [25]. Systems biology, as well as cell model approaches, is sometimes focused on particular molecular targets, for example specific druggable proteins [25]. The early narrowing of focus could, however, miss the detection of new principles for drug discovery.

Cytomics and new drug targets

The basics of cytomics

Cytomics (http://www.genomicglossaries.com/content/omes.asp) is the multimolecular single-cell analysis of cell-system (cytome) heterogeneity, and is combined with exhaustive bioinformatic knowledge extraction from the results of all analyzed cells in a sample (system cytometry) [26]. This enables the generation of

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maximum information on the molecular cell phenotype. It is possible to analyze suspended cells, cells in tissue samples or adherent culture, or other biological samples. Flow cytometry or image analysis provides multiparametric molecular information on thousands of single cells within a few seconds, and this generates an impression of the typical expression heterogeneity of molecular parameters in various cell types.

The cell-system heterogeneity arises, to a significant extent, from the complex interactions of cells within sophisticated regulatory networks. It contains a high degree of molecular information on functional and structural regulatory circuits that the cytomics approach tries to access in close to *in vivo* conditions. The heterogeneous expression (within a cell population) of cell parameters leads to the formation of multidimensional cell clusters, when analyzed by multiparameter single-cell image analysis or flow cytometry; and the hypothesis-driven parameter selection and data acquisition (at the broadest possible scale) is followed by a hypothesis-free differential data analysis of the multidimensional cell clusters – from all of the information collected. The comparison, for example, between cell-cluster properties of healthy individuals and those of patients in various disease stages provides discriminatory molecular profiles upon informative selection of the analysis parameters.

Reverse analysis towards the origin of the differential behavior of the differential molecular profiles (molecular reverse engineering) carries the potential for a more detailed understanding of disease processes and therapeutic effects. Single-cell imaging and flow cytometric analysis, assay procedures, and data-evaluation software are continuously developing, favoring their uses in HTS and HCS studies of patient cells. Many of these new technologies require only small quantities of cellular material for analysis, this enables predictions on disease progression of patients alongside typical clinical diagnostic or therapy-accompanying procedures.

Cytomics can be started by using large quantities of existing clinical, flow cytometric and clinical-chemistry data that can be analyzed in metastudies – because the disease outcome of patients under therapy is usually known after a certain time [27–29]. The goal of such studies is to enrich existing discriminatory data patterns to a maximum, and data patterns can be merged between institutions, provided comparable reference groups (e.g. healthy normal individuals or therapy-responder patients, with data on the same molecular parameters) are available without showing significant differences, against each other, upon data-pattern classification.

Application to drug discovery

The concept of systems biology reverse engineering [30,31] appears to be of great interest for the molecular reverse engineering of the discriminatory differential molecular profiles [27,32], because it is probable that new drug targets can be identified in this way.

Using differential molecular profiling, the investigation of molecular cell phenotypes of diseased or disease-affected cytomes is of particular importance for the exploration of disease processes [33]. The reasons for this are: cells represent the elementary functional units of organisms; diseases emerge as consequences of molecular aberrations in cells and cytomes; and molecular cell phenotypes carry disease-specific molecular profiles or signatures for diagnostic and predictive purposes – as the sum of genotype and lifelong exposure to external or internal influences.

Owing to the high diversity of genotype and exposure patterns among individuals, data collection in patients with complex diseases (e.g. allergies, malignancies, diabetes and neurodegenerative diseases) provides a personalized close-up insight into the molecular diversity of disease processes. Single-cell, single-patient oriented molecular profiling [33] provides high-resolution data at the level of the smallest respective functional unit, the cell. This concept has the potential to discriminate between parameters that are directly involved in the disease process, as well as those molecular alterations that are dependent on genotype or exposure, which are variable transit points in the emergence of an existing molecular profile of the disease (Figure 1). The cellular approach facilitates the rational search for new molecular drug targets, from diseased patients with diverse genotypic and exposure backgrounds.

The omics-driven, multiparameter data collection in diseased or disease-associated cytomes, such as inflammatory cells or immune cells, proceeds under a comparatively simple hypothesis – differences between disease states can exist at the transcriptome, proteome or metabolome level. The single-cell or array-data collection accumulates a significant number of molecular differentials between, for example, diseased and healthy individuals, or between progredient- and stationary-disease patients.

In the search for new drug targets, two advantages of collecting cellular data directly from patients are, first, disease-induced molecular differentials are observed at the same level where upon newly developed drugs will later act and, second, the study of perturbation-induced differentials in model systems, by systems biology, is only required during the molecular reverse engineering phase. The algorithmic data-pattern analysis does not depend on preexisting *in silico* cell models, nor on mathematical assumptions concerning observed parameter distributions [34,35]. Data-pattern analysis can be immediately used to explore disease-induced differential molecular profiles on a large scale, including the future prediction of patient status depending on the envisaged therapy.

Predictive medicine by cytomics

An important value of data-pattern analysis is its potential for personalized predictions on therapy-dependent disease progression in everyday medicine [27]. This is done by relating the differential molecular profiles (from multiparameter single-cell analysis) at diagnosis to disease progression (or outcome) for individual patients in a therapy-dependent way.

At therapy onset, accurate predictions on disease progression for individual patients represent an important goal for personalized (also known as individualized) therapy in everyday medicine. So far, according to Kaplan-Meier statistics, only the therapy-related prognosis of stratified patient groups is usually available, for example in acute myeloid leukemia (AML) [36] or in diffuse large B-cell lymphoma (DLBCL) [37]. Under the prognosis view, it has been identified from earlier studies that a certain fraction of patients will react to therapy whereas others will not, but it is not possible to identify responder and non-responder patients in stratified patient groups before therapy onset. This is unsatisfactory because many patients are not adequately treatable from the onset of disease.

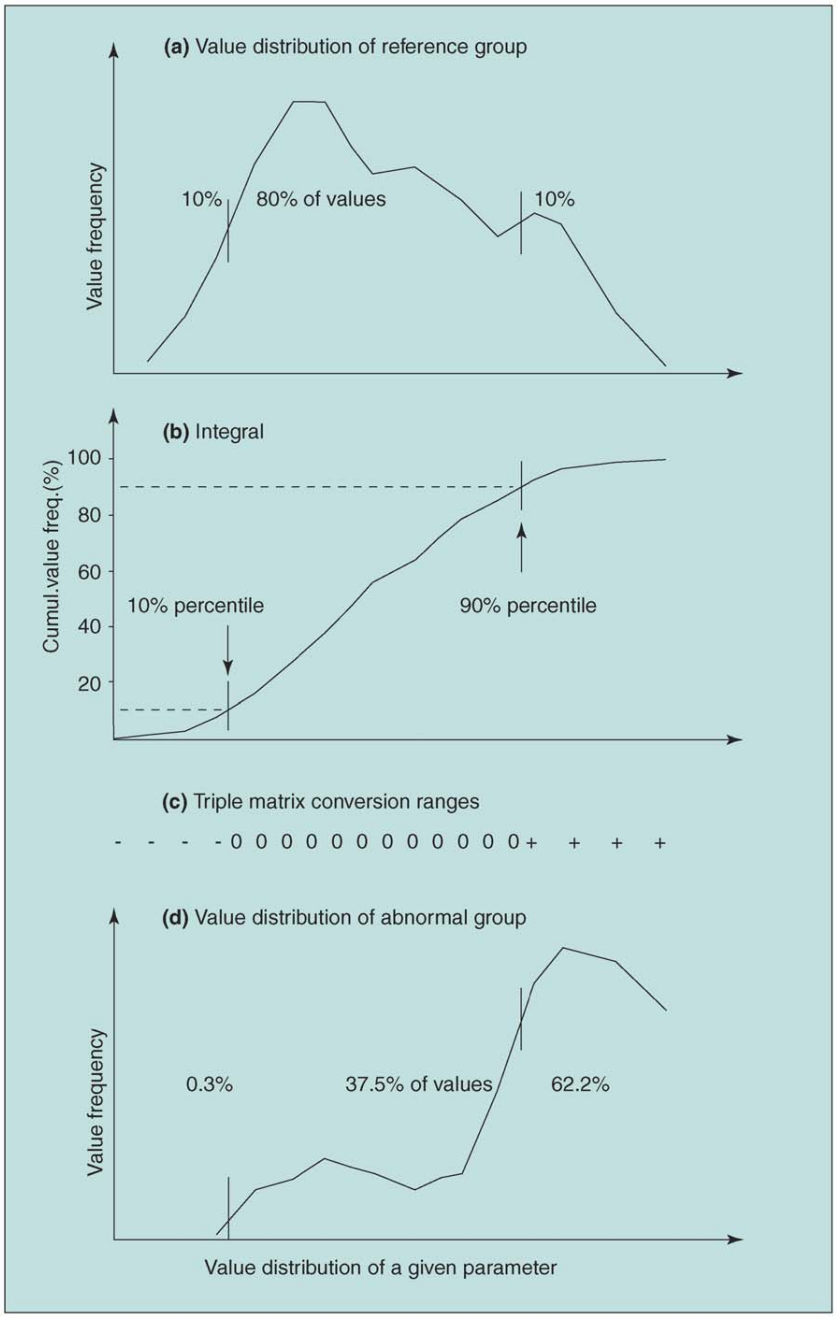

FIGURE 1

Conversion of numeric parameter values into triple matrix data-pattern characters for the sensitive detection of differences between parameter distributions. Initially, the numeric value distribution of a measured parameter for a reference group of patients **(a)** is redisplayed as an integral, the cumulated value frequencies, **(b)** – to determine lower and upper percentile thresholds (e.g. the 10% and 90% percentiles). This provides the triple matrix conversion ranges **(c)** for the numeric values of the reference and abnormal groups of patients, or experiments, for this parameter. Although 80% of the reference group values are transformed into (0), and 10% into (−), and 10% into (+) characters (a), 37.5% of the abnormal group values are converted into (0) with 0.3% being converted into (−) and 62.2% into (+) characters **(d)**. The enrichment of (+) characters, and the relative loss of (0) and (−) characters, in the abnormal group represents a standardized measure for differences between parameter value distributions without any requirement for mathematical assumptions about underlying value distributions, such as, for example, linear or logarithmic Gaussian distributions. The triple matrix transformation is similarly done for all parameters of a given multiparameter dataset. In a subsequent step the most discriminatory parameters of the multiparameter triple matrix data pattern are iteratively determined as disease classification masks, representing the most frequent triple matrix characters for reference (as well as for the single or multiple abnormal patient groups of the dataset).

Predictive medicine by cytomics [27], through differential molecular profiling (data-pattern analysis) of multitudes of single cells by image analysis or flow cytometry, as well as of cell extracts or biological fluids (e.g. blood plasma or serum) or effusions by nucleic acid or proteomics array, [28,29] therefore represents hope for the future application of therapies. However, this is only the case for the susceptible patients of stratified patient groups or for determining therapeutic resistance in individual patients. Cytomics predictive medicine adresses therapy-related disease progression and it is different from genomics predictive medicine [38], where the potential for later disease occurrence is evaluated from the presence of genetic markers. The presence of genetic markers might, however, not be indicative of later disease, for example missing exposure to disease-inducing influences.

Molecular profiling by data-pattern analysis

Statistics or hierarchical data clustering typically provides information on the average molecular properties of stratified patient groups in relation to therapy outcome (prognosis), whereas data-pattern analysis [34,35] addresses the personalized prediction of a patient's therapy-dependent disease progression in stratified patient groups (personalized medicine). In this way both approaches have the potential to complement each other efficiently.

Data patterns for the prediction of disease progression typically contain between 10 and 30 measured (or calculated) parameters, each containing values of: (−), representing diminished; (0), representing unchanged; or (+), representing increased. The selected parameters (data columns) are iteratively determined by metaanalysis of a training set of treated patients from available datasets that presently contain up to 100,000 data columns per patient.

The numeric parameter values are first converted into the triple matrix characters (−), (0) and (+) by the automated determination of lower and upper percentile thresholds (typically 5% to 95%, 10% to 90%, 15% to 85% and 20% to 80% percentiles) for all measured parameter distributions of the patient reference group (Figure 1), which might represent healthy individuals, stationary patients or survivors. Using these thresholds, the numeric parameter values of the remaining patients, for example patients with progressive disease and non-survivors, are also converted into triple matrix characters. This generates a standardized triple matrix database for each pair of previously mentioned percentile thresholds, containing all patients to be classified.

Parameters that do not discriminate between the disease stages in question are subsequently removed from consideration by an interactive procedure [34,35]. The final selected discriminatory triple matrix data patterns (disease classification masks) of the patient training set have to predict correctly the therapy-related individual disease progression (e.g. progressive versus stationary disease) for >95% of the patients to qualify for the term predictive. As a test of classification robustness, the triple matrix data patterns (patient classification masks) of the unknown patients from a validation set are positionally compared with the disease classification masks of the training set. The classification results have to be similar to those of the training set to ensure that the classifier is suitable for practical use.

For a given disease condition, classifiers can be developed for various possible therapies – to select the most efficient therapy at any given moment. The search for the best therapy can be repeated during the course of treatment to optimize the therapy of individual patients, and unsuitable therapies can be excluded.

Personalized predictions for adverse drug reactions, as well as for the potential occurrence of diseases such as asthma, allergies or diabetes, in members of risk families seem equally possible. By extending the scope of, and searching for, molecular differentials in more and more parameters, for example at the level of transcription, translation and posttranslational modification, it should be possible to enrich parameters for a given disease condition gradually, closer and closer to the molecular origins of disease processes, thus increasing the predictive accuracy beyond 99%.

Discriminatory data patterns (disease classification masks) consist of an accuracy part, containing typically between seven and ten (or more) parameters (Figure 2), to assure that the probability for random occurrence of patterns is <1%. Parameters in patient classification masks that are positionally non-coincident with the overall, most coincident, disease classification mask represent the multiplicity part of the data patterns, providing the necessary variability for the individual combinations of genotypic and exposure influences that can occur in individual patients. The accuracy part of the patient classification masks is tentatively considered as information about the association level of these parameters, with the observed disease symptoms.

The non-coincident parameters could be useful to explore common molecular access pathways for a similar disease state, for example by low exposure at high genetic susceptibility compared with high exposure at low genetic susceptibility. It is probable that the multiplicity signatures of the data patterns, for both types of patients, are different despite similar disease symptoms. The systematic analysis of patient classification masks could then potentially enable one to better understand disease induction processes in complex diseases such as malignancies or allergies.

The advantage of this approach is that the data patterns are found by data-driven evaluation, which is hypothesis-free. In this way, new knowledge spaces, inaccessible to traditional hypotheses, can be systematically explored for new drug targets.

Furthermore, the approach seems useful in the search for distributed drug targets that exist, for example with aspirin (acetylsalicylic acid) where partial inhibition of several targets [39] such as acetylation of cyclooxygenases (COX-1, COX-2) [40], inhibition of COX-2 transcript synthesis [41], adenosine liberation [42] and other mechanisms, rather than the total inhibition of a single molecular target, provides the desired therapeutic effects. The available high information densities from HCS experimentation on patient cells could facilitate this approach in the future.

Data-pattern analysis is not restricted to cellular data because results from chip arrays [29], serum mass spectroscopy [43,44], clinical chemistry [45] or clinical data [28] are equally treatable for molecular profiling by data-pattern analysis.

Transcriptomics, proteomics and metabolomics at the single-cell level

At present, a substantial variety of automated single-cell measurements can be assessed, such as the metabolic state of viable cells by flow cytometry [46] or laser scanning cytometry [47]. The determination of mRNA transcripts [48], or of mRNA expression profiles, from a few dozen laser dissected cells (or even potentially in single cells) [49] is as possible as the specific assembly of proteins

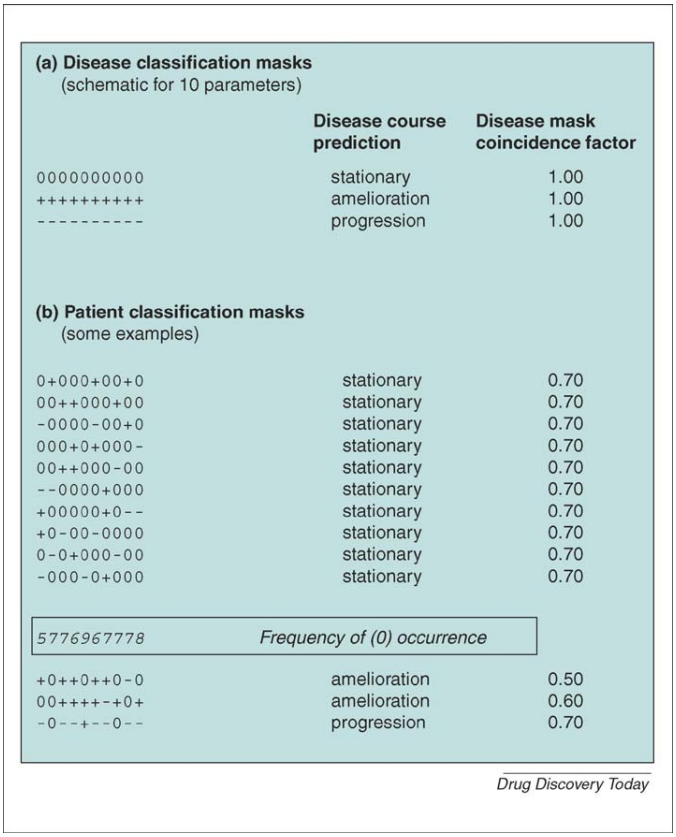

FIGURE 2

Data-pattern classification. Data-pattern classification is characterized by **(a)** high accuracy and low risk of random coincidence between patient masks and disease classification masks with probabilities of 0.0017% ($1/3^{10}$) for random coincidence of ten parameter masks and 0.046% ($1/3^{7}$) for seven parameter masks. They are also classified by **(b)** high multiplicity of patient classification masks for partial coincidence, with the closest fitting disease classification mask being seven out of ten positionally coincident parameters. $(10! \times 2^3) \div (7! \times 3!) = 960$ possible patient classification masks for each disease state as a potential result of genotype and exposure influences on molecular cell phenotype or other parameters. Unknown patients are classified according to the highest positional coincidence of the patient classification mask with any one of the disease classification masks. The disease mask coincidence factor indicates how well patient masks agree with the selected disease masks. The vertical frequency of coincidence of the various parameters in patient classification masks with the disease classification masks is considered evidence for the closeness of parameters to the molecular disease process. Grouping of patient classification masks according to mask similarity, like the patients in line two and five of the group of stationary patients, might be a way to identify patient groups with similar genotypic and/or exposure patterns (as a prerequisite for further molecular exploration).

(toponomics) in tissue sections or tissue arrays from paraffin-embedded or fresh tissues [50–52]. The simultaneous determination of up to 17 proteins by flow cytometry [53] is equally possible. Quantitative single-cell molecular and morphological data can be automatically extracted from fluorescent images that are generated by the use of unsupervised segmentation algorithms during HTS and/or HCS screening [54–56]. In cases were single-cell analysis is not sensitive enough, laser microdissection [57] of given numbers of closely related cells has the potential to deliver relatively homogeneous material for chip arrays, multiplex bead assays [46] or mass spectroscopy [58].

The concept of reverse engineering in systems biology [30,31] seems to be of great interest in the molecular reverse engineering of discriminatory differential data patterns [27,32], because it is probable that new drug targets can be identified in this way. Once the discriminatory data patterns (disease classification masks) have been derived from patient studies, the reverse engineering of the individual parameters of these disease classification masks (Figure 2) by systems biology can be started using composite cell models [19,20], primary human cell cultures [25] or other cell models [23]. Relying, from the beginning, more or less exclusively on cell models (i.e. hypothesis-driven abstraction from the clinical reality) brings with it the substantial risk of clinical failure for new drugs.

Clinical studies will also be of importance for the personalized identification of non-responder patients [28,29], initiating alternative therapies early in an effort to assure maximum therapeutic efficiency with minimum adverse drug reactions.

Present drawbacks

Despite significant potential for predictive medicine and drug discovery, cytomics is still at its very beginning. Only relatively limited numbers of scientists are presently involved in such studies, mainly because there is no specific funding. However, this situation might change after significant funds have been allocated to the MetaCyt program at Purdue University (http://metacyt.indiana.edu). Another issue concerns the relations between the pharmaceutical drug discovery field and the clinical environment where, typically, clinical studies with new therapeutic agents are performed rather than searching for new targets in patients. Instrument and software development are rapidly progressing, but clinical image analysis and flow cytometry instrumentation is preferentially oriented towards quantitative visual evaluation and hand- or script-operated computer evaluation of measured data, rather than towards the rigorous extraction of the entire information of multidimensional data spaces (as required for unsupervised automated data-pattern classification).

The various problems all seem solvable because no major drawbacks for the cytomics concept have, so far, emerged, even during all the evaluations of accessible clinical data from various medical disciplines. Focused attention at the funding agency level and raising the number of scientists in this attractive transdisciplinary field are, however, required to profit practically from the potential of cytomics for predictive medicine and drug discovery.

Conclusions

The availability of entire genome sequences, in combination with advanced instrumentation, for fast measurements of molecular parameters in multitudes of single cells, allows quantitative determination of the molecular heterogeneity of high numbers of single cells from patients – by simultaneous multiparameter image analysis or flow cytometry within tissues or cell suspensions containing various cell types in a range of functional states. The resulting information can be differentially analyzed in patients in various disease states, providing the potential of cytomics to investigate not only molecular pathways in cell systems as a pathway structure but also the regulatory state of these pathways in disease, pinpointing interesting molecular targets for future drug discovery.

The multiparameter differentials indicate the overall regulatory difference between patients in various disease stages as an

integral result of genotype and cumulated specific exposure influences, and as direct disease manifested in diseased, or sometimes also in disease-associated, cells such as lymphocytes, monocytes, granulocytes or their precursors. The determination of multiparameter differentials is largely independent of a detailed initial knowledge on specific disease mechanisms. This is a significant advantage for the field of drug discovery in complex diseases, for example in rheumatoid diseases, allergies or malignancies, where comparatively little molecular pathway knowledge is available.

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