Cytomics: From Cell States to Predictive Medicine

Chapter 16

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ABSTRACT

Cytomics, the systematic study of biological organization and behavior at the cellular level, has developed out of computational imaging and flow cytometry and promises to provide essential data for systems biology. The ability to perform high-content and high-throughput imaging and analysis to reveal complex cellular phenotypes will not only further our understanding of how cells and tissues carry out their functions but also provide insight into the mechanisms by which these functions are disrupted.

Advances in flow, chemical, and tissue cytometry extend the applicability of cytomics to tissues, cytological smears, and blood and other body fluids. As such, cytomics not only provides a new framework for spatiotemporal systems biology but enriches personalized or individualized medicine. This can take the form of individual disease course predictions for therapy selection purposes as well as identification of discriminatory bio-parameter patterns.

I. INTRODUCTION

Systems biology aims at the understanding of the integral functionality of single cells, organs, or organisms by molecular analysis and mathematical modeling (Ideker et al. 2001; Kitano et al. 2002; Hood et al. 2003, 2004). This task is significantly more complex for organisms than for single-cell systems such as bacteria or yeast. Organismic complexity derives from the diversity of genotypes among individuals, via variable exposure histories to environmental influences in numerous specialized organs. Cell states are characterized by significant internal hetero-

geneity according to cell cycle, functional status, size, and molecule content—with a heritable variation in the baseline gene expression (Morley et al. 2004). This inherent variability may be constrained by a limited number of cell states or fates, as described in the contribution of Huang.

Cytomics, the multimolecular quantitative analysis of the heterogeneity of cells and cell systems (cytomes), in combination with exhaustive bioinformatics knowledge extraction from analysis results (Valet 2002; Chitty 2005), aims to provide comprehensive, accurate, and systematic data. These qualities have been defined as the cornerstones for measurement technologies in systems biology (Kitano 2001). High-content and high-throughput methodologies are essential characteristics of cytomics for both single cells and tissues (Ecker et al. 2005, Boyce et al. 2005).

Currently, the concept of cytomics profits from advances in areas such as location proteomics, flow and tissue cytometry, screening assays, and cell and tissue arrays. Such advances move us toward a broad, systematic collection of information for clustering and cataloging cells according to their molecular, organelle, and morphometric phenotypes. Cataloging cell states by assessing a wide spectrum of quantities, which may be seen as state variables, is not necessarily driven by particular hypotheses—a property cytomics shares with other "-omics" methodologies. Realization of this concept have been successfully applied to the generation of profiles of drug activity, using a hypothesis-free molecular cytology (Perlman et al. 2004) and signaling network analysis (Sachs et al. 2005).

This chapter reviews various approaches in basic biological research and medicine for generating quantitative, flow, and image-based data for a comprehensive profiling and structural state space analysis. Analysis of changes of the cellular phenotype due to specific experimental perturbations are reviewed elsewhere in this book. Cytomics-related image analysis of subcellular protein distributions, compartments, cells, and tissues is in many areas specific to the imaging technology employed. However, for data mining statistical tools well known in bioinformatics are employed to classify and subsequently catalog cell states, whereas statistical correlations can span across levels of biological organizations.

As an example, cytomics data may be correlated with gene expression data to identify significant molecular markers, but may also enable creation of a bridge between cellular phenotypes and emerging physiological processes in the sense of an integrated physiology approach. Furthermore, cytomics provides a framework for the development of computational models of cells in support of a spatio-temporal systems biology.

II. COMPUTATIONAL IMAGING IN CYTOMICS

A. Single-cell image analysis

One of the most important outcomes of the Human Genome Project is the realization that there is considerably more biocomplexity in the genome and the pro-

teome than previously appreciated (Herbert 2004). Not only are there many splice variants of each gene system, but some proteins can function in entirely different ways (in different cells and in different locations of the same cell), lending additional importance to the single-cell analysis of laser scanning cytometry and confocal microscopy. These differences would be lost in the mass spectroscopy of heterogeneous cell populations. Hence, cytomics approaches may be critical to the understanding of cellular and tissue functions.

Fluorescence microscopy represents a powerful technology for stoichiometric single-cell-based analysis in smears or tissue sections. Whereas in the past the major goal of microscopy and imaging was to produce high-quality images of cells, in recent years an increasing demand for quantitative and reproducible microscopic analysis has arisen. This demand came largely from the drug discovery companies, but also from clinical laboratories. Slide-based cytometry is an appropriate approach for fulfilling this demand (Tarnok and Gerstner 2002). Laser scanning cytometry (Gerstner et al. 2002; Tarnok and Gerstner 2002; Megason et al. 2003) was the first of this type of instrument to become commercially available, but today several different instruments are on the market (Jager et al. 2003; Molnar et al. 2003; Schilb et al. 2004).

These types of instruments are built around scanning fluorescence microscopes that are equipped with either a laser (Tarnok and Gerstner 2002; Schilb et al. 2004) or a mercury arc lamp as the light source (Bajaj et al. 2000; Molnar et al. 2003). The generated images are processed by appropriate software algorithms to produce data similar to flow cytometry. Slide-based cytometry systems are intended to be high-throughput instruments, although at present they have a lower throughput than flow cytometers. These instruments allow multicolor measurements of high complexity (Gerstner et al. 2002; Ecker and Steiner 2004) comparable to or exceeding that of flow cytometers.

A substantial advantage over flow cytometry is that cells in adherent cell cultures and tissues can be analyzed without prior disintegration (Smolle et al. 2002; Kriete et al. 2003; Ecker et al. 2004; Gerstner et al. 2004). In addition, due to the fixed position of the cells on the slide or in the culture chamber cells can be relocated several times and reanalyzed. Even restaining and subsequent reanalysis of each individual cell is feasible. Because a high information density on the morphological and molecular pattern of single cells can be acquired by slide-based cytometry, it is an ideal technology for cytomics.

Although at present not realized, the information density per cell can be increased further by implementing technologies such as spectral imaging (Ecker et al. 2004), confocal cytometry (Pawley 1995), fluorescence resonance energy transfer (FRET) (Jares-Erijman and Jovin 2003; Ecker et al. 2004; Peter and Ameer-Beg 2004), near-infrared Raman spectroscopy (Crow et al. 2004), fluorescence lifetime imaging (FLIM) (Murata et al. 2000; Peter and Ameer-Beg 2004), optical coherence tomography (Boppart et al. 1998), spectroscopic optical coherence tomography (Xu et al. 2004), and second harmonic imaging (Campagnola et al. 2003). All of these technologies mark the progress in optical bio-imaging.

In the future, developments in imaging resulting from a family of concepts that allows image acquisition far beyond the resolution limit (down to the nm range) are expected. These include multiphoton excitation (Manconi et al. 2003), ultrasensitive fluorescence microscopes (Hesse et al. 2004), stimulated emission depletion (STED) microscopy (Hell 2003), spectral distance microscopy (Esa et al. 2000), atomic force microscopy (AFM) and scanning near-field optical microscopy (SNOM) (Rieti et al. 2004), and image restoration techniques (Holmes and Liu 1992). Using laser ablation in combination with imaging, even thick tissue specimens can be analyzed on a cell-by-cell basis (Tsai et al. 2003).

B. Innovative preparation and labeling techniques

Biomolecular analysis techniques such as bead arrays (Lund-Johansen et al. 2000; Tarnok et al. 2003), layered expression imaging (Englert et al. 2000), single-cell polymerase chain reaction (PCR) (Taylor et al. 2004), tyramide signal amplification (Freedman and Maddox 2001), biomolecule labeling by quantum dots (Parak et al. 2003), magnetic nanobeads (McCloskey et al. 2003), and aptamers (Ulrich 2004) open new horizons of sensitivity, molecular specificity, and multiplexed analysis. With additional tools—such as laser microdissection (Taylor et al. 2004), laser catapulting (Burgemeister et al. 2003), and fast electric single cell lysis (Han et al. 2003)—single cells can be rapidly isolated and further subjected to genomic or proteomic analysis (Burgemeister et al. 2003; McClain et al. 2003; Taylor et al. 2004) or single-cell capillary electrophoresis (Han et al. 2003).

The dimensionality of measured molecular cell data can be substantial, especially when repeated six- or eight-color staining protocols are performed on many different cell populations (Lenz et al. 2003; Ecker et al. 2004; Mittag et al. 2005) and their spatial interrelationships within a tissue are taken into account (Smolle et al. 2002; Ecker and Steiner 2004; Gerstner et al. 2004). The data density is multiplied if high-density single-cell analysis such as SNOM, AFM (Rieti et al. 2004), and STED (Hell 2003)—combined with single-cell genomics (Burgemeister et al. 2003; Taylor et al. 2004) or proteomics—(Han et al. 2003; McClain et al. 2003) is added.

A highly multiplexed yet hypothetic model for cytomic analysis of biological specimens could work as follows. Viable cells may be initially stained for cell functions (e.g., intracellular pH, transmembrane potential, intracellular Ca^{2+}), followed by fixation to remove the functional stains and restaining for specific extra- or intracellular constituents such as antigens, lipids, or carbohydrates, including, specific nucleic acids. Serial optical analysis will permit for every individual cell the 3D-reconstruction of its exact localization within the network of other cells in a tissue, together with the molecular morphology of its cell membrane, nucleus, organelles, and cytoplasm (including the parameterization of 3D shapes).

Serial histological sections taking stereological aspects of tissue architecture into account (Mandarim-de-Lacerda 2003) could serve as a basis for the standardized analysis of proximity and interaction patterns for intracellular structures such as

nucleus and organelles, as well as for different cell types within the tissue architecture (which can even include time as a parameter for 4D intravital microscopy [Mempel et al. 2004]). Microscopic image capture and analysis systems using their spatial relocation capacities will increasingly permit such staining sequences. Further genomic and proteomic characterization of single cells will yield substantial input into our understanding of cell development and function in the histological context, as further outlined in the section following.

C. Location proteomics

Systems biology researchers seek to build accurate predictive models of complex biological systems, typically incorporating information about events involving different types of biological macromolecules and occurring on different length and time scales. This requires the creation of systematic frameworks for representing this information and large-scale projects to acquire it (creating the "parts lists" for building models). A critical requirement for the success of such large-scale projects is being able to automate not only sampling, specimen preparation, and data collection but also data analysis.

1. Automated classification of subcellular location patterns

A particularly important category of information for building systems models is the location of proteins and other biological molecules within cells. Because fluorescence microscopy is the most commonly used method for determining the subcellular location of proteins, an important initial question was whether automated analysis of subcellular patterns in fluorescence microscope images was feasible. This question was answered by the demonstration that five subcellular patterns could be distinguished in 2D images of Chinese hamster ovary cells (Boland et al. 1998) and that 10 subcellular patterns could be distinguished in HeLa cells (Murphy et al. 2000; Boland and Murphy 2001).

The dramatic variation in cell size, shape, and orientation exhibited by cultured cells combined with the extensive variation in position of organelles within cells suggested that approaches involving direct (pixel-by-pixel) comparisons with a library of cell images of known patterns would not provide accurate assignment of new images to one of those patterns. Instead, a feature-based approach was used in which each image is represented by a set of numerical features that capture various aspects of the pattern without being overly sensitive to rotation or translation within the sample plane.

These features have been systematically described and combined into sets of subcellular location features (SLFs). Initial work on distinguishing 10 patterns in HeLa cells achieved an average accuracy of 83% on individual cells using feature set SLF5 and a neural network classifier (Boland and Murphy 2001). Subsequent work has improved this accuracy to 92% using feature set SLF16 and a majority-voting ensemble classifier (Huang and Murphy 2004).

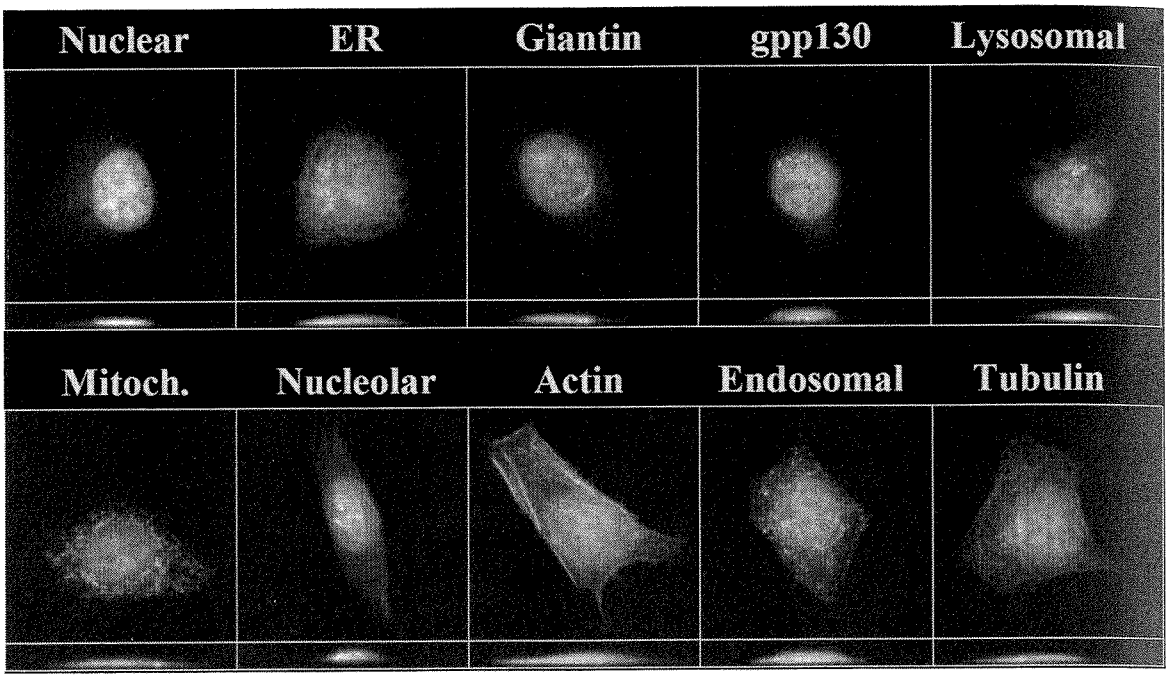

Figure 16.1. Representative 3D images of 10 subcellular patterns that can be distinguished with high accuracy by automated classifiers. The distribution of a DNA probe is shown in red, that of total cell protein in blue, and that of specific organelle markers in green. The paired images are maximum value projections along the *z* or *x* axis. *(Picture copyright Carnegie Mellon University.)* (see color plate 12).

An important conclusion from this work was that patterns that cannot be distinguished by visual examination could be discriminated by the automated systems (Murphy et al. 2003). In particular, two Golgi proteins that cannot be distinguished better than random guessing by visual examination can be recognized with accuracies of 82 to 90% using SLF16 (Huang and Murphy 2004). Discrimination of the similar lysosomal and endosomal patterns by the automated system is also 5 to 6% better than that achieved by visual examination.

Because macromolecules are distributed in three dimensions within cells, not just two, the accuracy of classification of 3D images obtained by confocal microscopy was also investigated. An initial accuracy of 91% using feature set SLF9 and a neural network classifier was obtained for the same 10 patterns previously studied in HeLa cells (Velliste and Murphy 2002), and this accuracy was subsequently improved to 98% using feature set SLF17 (Chen and Murphy 2004). Example images of the 10 patterns are shown in Figure 16.1.

2. Automated microscopy and pattern classification

These results demonstrate that the fundamental problem of recognizing the major subcellular patterns in 2D and 3D images has been solved. However, practical experience shows that the automation of the data acquisition process (including autofocusing and detection of structurally consistent and homogenously stained cells) still imposes limitations to achieve highest classification accuracy. As the technol-

ogy evolves, the next step can be taken in applying these methods to characterize entire proteomes, and we have coined the term *location proteomics* to describe this approach (Chen et al. 2003).

One way in which this can be done is to collect images of many different proteins and assign each protein to one of the major classes. An important recent test of an automated approach has been performed using expression in MCF7 cells of 11 GFP-tagged proteins via transfection (Conrad et al. 2004). The average accuracy reported was 82%, but this average included recognition of a separate "artifact" class (created by visual inspection of the training images). The accuracy obtained for assignments to the 11 protein patterns was 73%.

The corresponding higher error rate if compared for similar analysis in HeLa cells could be due to any of a number of differences between the studies, including cell type, the use of overexpressed fusion proteins versus endogenous proteins, the magnification, and the use of different feature sets. But most importantly, the additional challenges of accurate automated autofocusing and cell segmentation must be considered as well. Nonetheless, current results are encouraging for the use of automated microscopy, especially in that the accuracy of classification of a particular protein can be improved by combining results from more than one cell (Boland and Murphy 2001).

3. Clustering of proteins by location pattern

An alternative to assigning proteins to "known" subcellular location patterns is to use unsupervised learning methods to identify the statistically significant patterns observed and group proteins by them. The principle is to represent each protein pattern using the SLFs but to use cluster analysis to group them rather than to classify them. This approach was demonstrated using 3D images of a number of proteins in 3T3 cells (Chen et al. 2003). This study used cloned cell lines expressing randomly-chosen proteins fused internally with GFP using CD-tagging (Jarvik et al. 2002).

A recent study of 90 of these clones tested different ways of measuring distance between proteins in the feature space, as well as different clustering approaches (Chen and Murphy 2005). The results indicated that the clones formed 17 distinguishable clusters that provide greater refinement than visual description of the patterns using standard terms. The consensus tree obtained, along with example images from various clusters, is shown in Figure 16.2.

4. Imaging protein kinetics

The results summarized previously were all obtained using static images that represent the steady-state distribution of proteins. A major upcoming challenge will be the acquisition and incorporation into location proteomics of information on the kinetics with which proteins move in these steady states, as well as the kinetics with which those states change due to the cell cycle, environmental changes, onset of disease, or addition of drugs. Time-lapse imaging techniques allow consistent pro-

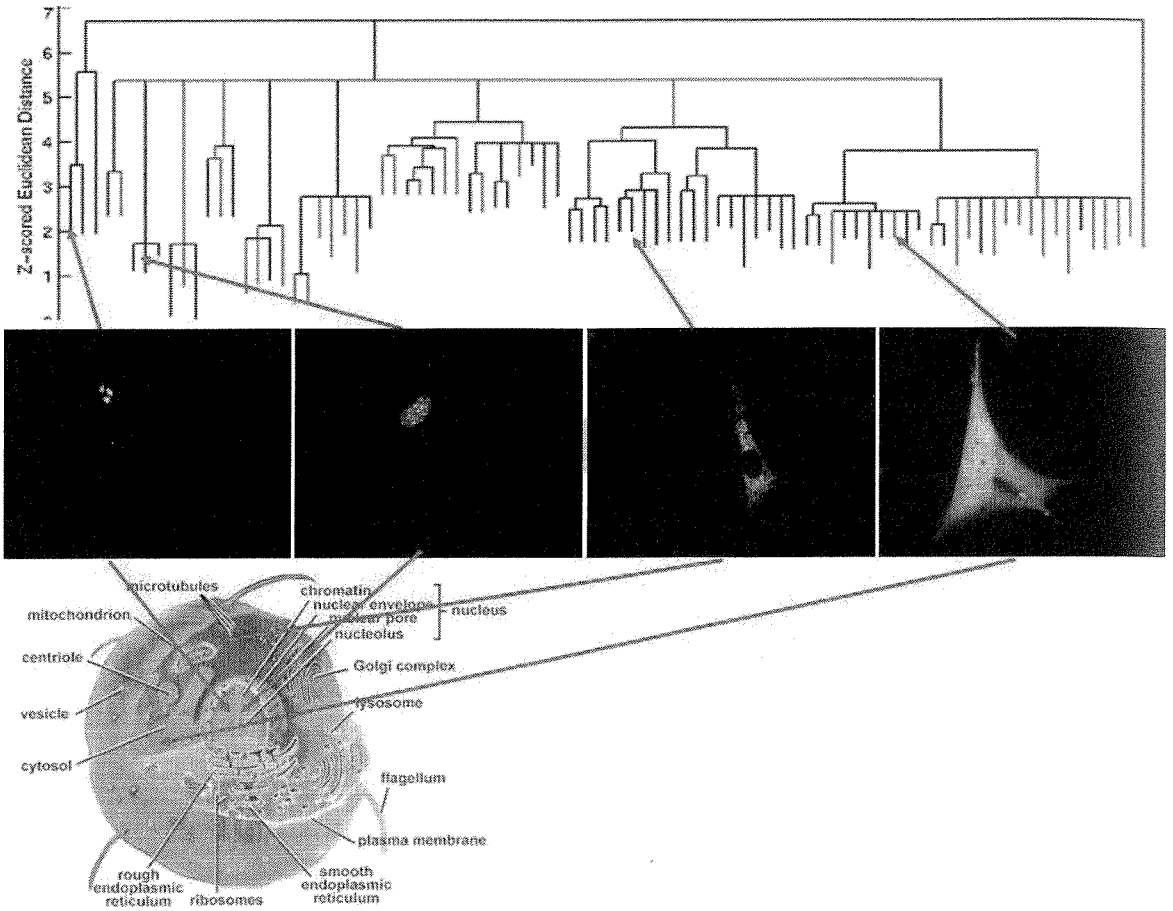

Figure 16.2. Consensus subcellular location tree for 3T3 cell lines obtained by CD-tagging. 3T3 cells were infected with a retroviral construct carrying a GFP coding sequence surrounded by splicing donor and acceptors sites (CD-tagging). Clones expressing randomly-tagged proteins were isolated and the tagged gene identified by RT-PCR (Jarvik et al. 2002). At least 10 3D images of live cells were obtained for each clone using spinning disk microscopy (Chen and Murphy 2003), and the clones were grouped as described in the text and as by Chen and Murphy (2005). The degree of dissimilarity between any pair of clones can be found by measuring the vertical distance from one of them to the highest node along the minimal path to the other clone, plus the vertical distance from that node to the other clone (as discussed in the text, this distance reflects the separation between the two clones in the SLF feature space). The names of the proteins are not shown due to space limitations. Examples of images from various branches of the tree are shown. The full tree with names and images of all clones is available at *http://murphylab.web.cmu.edu/services/PSLID/tree.html* (see color plate 13).

filing of changes in the cell state. FRAP and FRET (and the tracking protein complexes with q-dots) are powerful methods for studying dynamics. Relocation of protein species reveals important functional activities on state-space transients. An example is the release of cytochrome c from the mitochondria into the cytosol that mediates apoptosis (Goldstein et al. 2000), or the dynamics of histone binding to chromatin in living cells (Mistell et al. 2000).

5. Location proteomics and generative models

It is anticipated that the methods described previously will be used for large-scale studies to characterize the subcellular distribution of proteins in a number of cell

types. Important areas of additional research involve enabling patterns that are a mixture of other patterns to be "unmixed" and making it possible to describe the distribution of any protein using generative models that can be incorporated into simulations to create distributions of many proteins within cells *in silico*.

D. Cytomics analysis in tissues

The introduction of robotic microscopy in concert with robust machine vision software that can discern the histomorphology of multicellular arrangements in tissues in a comprehensive fashion has extended cytomics into the tissue domain. A multicellular high-throughput, high-content analysis of tissues (sometimes termed tissomics [Ecker and Tarnok 2005; Kriete and Boyce 2005]) can be used to support and confirm histopathological assessment of tissues, allowing a more complete quantitative evaluation of phenotypical cellular responses and the identification of structural markers of tissue normality, injury, and disease. Specific applications include basic biomedical research, pathobioinformatics, investigative toxicology, drug target development, and tissue engineering.

1. High-throughput imaging

Complete imaging of a histological glass slide (20 × 50 mm) at 20× microscopic magnification can generate up to 3,000 individual digital color images, which new types of ultrafast scanners can image within minutes (Weinstein et al. 2001). The resulting image montages of several GB in size represent entire histological slides or tissue arrays. The enormous amount of data generated by this new class of microscopic scanners is stored in databases. Secondary representations, by subsampling or data compression, mainly serve viewing or control purposes and have been developed as part of adequate solutions for the handling and mining of such large data sets.

2. High-content image analysis

The analytical task of cytomics in tissues is a fully automated analysis of tissue profiles without user intervention, which can be challenging given variations caused by the prevailing methods of tissue preparation and staining. Robust analysis procedures that rely on the topology of cells and tissue structures, and new object-oriented approaches, are preferred solutions that have distinct advantage over the prevailing pixel-oriented methods (Price et al. 2002; Kriete et al. 2003). Understanding tissues as a hierarchy of larger anatomical constructs, consisting of different cell types in different phases of development, that further contain cell organelles and cell nuclei is key for object segmentation (Kriete and Boyce 2005).

These entities, once identified, provide a rich source for a hypothesis-free geometric intensity and field-specific characterization. The identification of significant components that change with disease state or treatment may be found by multivariate statistics in the course of further data analysis. The method is extendable to include specific stains and biomarkers, as well as tissue microarrays or tissue cultures.

III. DATA ANALYSIS

A. Data mining, differential analysis, and discrimination

Because cytomics investigates individual cells, it is possible to separate (or gate) cells into different state or response categories (Boolean classification). Cells are grouped and catalog based on a number of cellular features from one or multiple probes, typically originating from one level of observed granularity or resolution (horizontal analysis according to biological hierarchy). This includes overall fluorescence intensity, rate of rise/fall (for kinetics), area, object pixel statistics (average intensity, min and max), and variation of pixel intensity within ROIs (granulation algorithm).

Classes of cells can be color-coded for easy visualization, and average measurements over a subset of cells can be taken. A "well" classification is then applied based on the number of cells in each well that meet a user-defined threshold. In turn, a response "heat" plate-map that readily highlights cellular trends or compound hits can be generated. This process enables us to identify features that best reflect specific biological responses and that are therefore good screenable parameters.

Multiparametric single-cell analysis by flow or image cytometry can provide significant amounts of data that may seem difficult to distribute and analyze (Hood et al. 2004). Solutions to handle biological image data over the Web have been suggested but have had limited application (Lindek et al. 1999), whereas analytical procedures may relate cytomics data with biomedical literature and bioinformatics databases (Abraham et al. 2004).

Differential data pattern analysis (Valet and Hoeffkes 2004) provides a means of analyzing multiparametric data of various types in parallel in a nonhierarchical way. such data from flow and image analysis, chip arrays, clinical chemistry, and clinical data can be simultaneously processed in a manner similar to that of predictive medicine by cytomics (Valet 2002). Disease-induced differentials in patients versus normal individuals, stationary disease patients, or survivors are analyzed in this approach instead of differentials from perturbed model systems that may not exactly reflect the human situation (Horrobin 2003).

The algorithmic procedure is summarized as follows. Numeric data columns are transformed into triple matrix characters (–) = decreased for values below a lower percentile threshold, into (0) = unchanged when between lower and upper threshold, and into (+) = above an upper percentile threshold. The resulting triple matrix database is classified in a learning situation for samples of patients from different classification categories (such as healthy versus diseased, progressive versus stationary disease, and survivor versus non-survivor patients). Individual triple matrix columns are temporarily removed from the learning process in a sequential way to assess their individual contribution to the classification result.

At the end of the learning process only data columns having improved the initial classification remain in the discriminatory bio-parameter patterns comprising typically between 10 and 30 parameters. The bio-parameter patterns can be further

used for the exploration of molecular disease pathways and in the search for new drug targets. In this way, single-cell- and single-individuum-oriented analyses provide a maximum of discrimination because no averaging over heterogeneous entities occurs during data acquisition and bioinformatic evaluation (Szaniszlo et al. 2004).

Principal component analysis is another way of reducing the complexity of the data, in particular if cytomics data are merged with other bioinformatic data sets from the same cells and tissues, such as gene expression profiles (Kriete et al. 2003). An alternative is Fisher discriminant analysis (FDA), which was used previously to demonstrate improved differentiation of treatment groups if chemical data are combined with multicellular phenotypical data (Kriete et al. 2005).

Nature-induced bio-parameter perturbations or differentials (such as between diseased versus healthy, progressive versus stationary disease, or survivor versus non-survivor patients) can be directly analyzed instead of generating hypothesis-driven systematic perturbations in model systems. Individualized disease course prediction for patients is possible in this way (Valet 2002), without the prerequisite of fully understanding the entire molecular network of disease-associated cell system changes. Discriminatory data patterns are obtained by multiparameter data analysis.

These data patterns can be further investigated by a molecular reverse-engineering strategy (Valet 2005) to understand disease-inducing molecular pathways or to find new drug targets. It is advantageous for this concept that many data sets are already available as starting material from current or past clinical studies in which patients are routinely followed for diagnostic or therapeutic purposes.

B. System-wide data correlations

Multicellular profiles can be correlated statistically with gene expression profiles. Foundations for this (vertical) analysis crossing different levels of biological hierarchy are multi-sample comparisons, assuming that changes on one level of biological organization consistently alter the phenotype and function on a higher physiological level.

As an example, Spearman's rank order correlations have revealed significant monotonic relationships that illuminate important connections between structural features in tissue composition and gene expression levels (Kriete et al. 2003). Similarly, a hierarchical clustering analysis based on a jackknife correlation demonstrated correlations between groups of genes with tissue cytometric markers (Kriete and Boyce 2005). As such, cytomics-related techniques provide covariants that can be used to enrich gene expression analysis (Boyce et al. 2005).

IV. DISCUSSION

Cells represent elementary building units of cell systems, organs, and organisms, and diseases are caused by molecular changes in cells and cell systems. Consid-

ering the heterogeneity of human cell systems, single-cell analysis (Szaniszlo et al. 2004) is important in resolving a maximum of compartmentalized molecular heterogeneity; for example, to discriminate changes in diseased or disease-associated cells from nonaffected bystander cells. Technical progress broadens the number and quality of available cell state variables, such as cytometry using microfluidic chips (Palkova et al. 2004; Wu et al. 2004) and capillary electrophoresis (Dovichi and Hu 2003; Arkhipov et al. 2005). Cell microgenomics expression profiles (Taylor et al. 2004)—as well as single-cell proteomics (Dovichi and Hu 2003) and metabolomics (Palkova et al. 2004; Wu et al. 2004; Arkhipov et al. 2005)—also become accessible.

An important concept of systems biology subsists in the application of multiple differential perturbations on biological cell systems to observe their molecular reactivity with the aim of mathematical modeling to understand the mechanisms of the observed alterations. The prediction of the reactivity for biological systems under predefined conditions represents a further goal. Cell arrays and microwell infection assays on cultured cells in conjunction with RNAi allows screening of the morphological phenotypical states in a high-throughput fashion. At present, a suggested comprehensive mapping of all proteins in the cell or in cell compartments by using high-resolution electron tomography (Baumeister 2004) is still limited, and the required resolution has to be improved. Light microscopic imaging techniques in conjunction with fluorescence markers, as described here, are therefore the preferred technique and can be more easily applied in a medical environment.

Single-cell techniques overcome the problem of averaged cellular information in cell homogenates or extracts where it cannot be decided whether observed changes derive from all cells or only from a particular cell subpopulation. The analysis of humoral body compartments such as blood plasma or serum, urine, or cerebrospinal fluid as a further alternative provides only secondary information by cell-derived molecules. Metabolites from cellular disease processes may have been altered in the meantime, or they may not become apparent in humoral compartments for lack of secretion or owing to fast renal or biliary excretion.

It may be contended that the single-cell approach will frequently not be feasible because not all cells of a given sample can be analyzed (as, for example, in smears, biopsies, or histological sections). Experience shows that it is not obligatory to analyze all cells of a given sample before one can derive relevant conclusions. It is frequently sufficient to analyze a representative fraction of diseased cells as well as reference cells. This will be shown by the subsequent examples. Mechanical disaggregation of tissues at 0 to 4°C for cell function analysis by flow cytometry destroys between 90 and 95% or more of all cells.

Furthermore, a relative enrichment of epithelial and inflammatory cells occurs because fibroblasts or smooth muscle cells have been largely destroyed. More than 90% of cancer patients are correctly identified from flow-cytometrically identified molecular cell properties (Valet et al. 1984; Liewald et al. 1990). This indicates that a representative fraction of cancer cells and normal epithelial reference cells has

survived despite the fact that the cellular composition of the samples has changed and that the tissue architecture was lost during cell preparation.

The result is not surprising because diseases represent molecular changes in cells and cell systems. The analysis of diseased cells or disease-associated inflammatory and immune cells should therefore by itself contain the relevant molecular information about the actual state (diagnosis) and the future development (prediction) of a disease, irrespective of the original position of the analyzed cells in an organ. A further reservation concerning single-cell analysis is that cell properties may be altered during preparation for analysis (Hood et al. 2004). Deep-freezing of tissues, immediate cell fixation, or cell preparation between 0 and 4°C for functional studies, however, minimizes such risks.

Valuable information is obtained, for example, from the functional analysis of oxidative status or oxidative burst in inflammatory immune cells such as lympho-, mono-, and granulocytes. Such disease-associated cells can be measured in tissues but advantageously also in the peripheral blood, where high-speed multiparameter flow-cytometric single-cell analysis is possible and provides individualized predictions or risk assessments for intensive-care patients (Valet et al. 1998, 2001). We can conclude that molecular alterations by cell preparation or staining steps cannot be generally excluded. They do, however, definitely not impair the determination of clinically relevant molecular cell parameters.

V. CONCLUSIONS

The value of the single-cell/single-individual analysis concept resides in its clinical value for the individual patient as well as in the bio-parameter patterns being of interest for molecular reverse engineering by systems biology. The backward molecular analysis may provide information on specific molecular pathways responsible for disease formation and reveal new drug targets. A specific focus of cytomics is in location proteomics, which uses quantitative readouts for functional genomics.

Detailed knowledge of the location, concentration, and activation of proteins and other biological molecules and valuable information can be obtained by studying cell behaviors in a systematic fashion in parallel with ongoing proteomics projects. Information on specific biomarkers and proteome changes associated with function, disease, and age can be valuable for diagnostics or therapeutics before a complete mapping of the proteome is available.

Diseases are typically diagnosed by clinicians from clinical symptoms or clinical chemistry parameters, or by pathologists from the evaluation of the altered microscopic morphology in tissue sections or in cytological samples. Single-cell image or flow-cytometric analysis extends the diagnostic knowledge level by the description of molecular cell phenotypes and may detect alterations at a stage where no morphological correlate is yet detectable.

Such measurement may also address therapy-related future disease courses of individual patients as a clinically promising new feature (predictive medicine by

cytomics) (Valet et al. 2001; Valet 2002; Valet and Tarnok 2003). A human cytome project has recently been proposed (Valet and Tarnok 2004; Valet et al. 2004) to particularly focus on the development and management of clinically complex diseases such as malignancies, infections, diabetes, allergies, rheumatoid diseases, asthma, myocardial infarction, stroke, and others.

The translational research concept laid out is deductive for the selected analytical parameters, but inductive during the data evaluation phase because the information of all quantifiable variables and cells is investigated for its discriminatory potential. In this step, most of the non-differential information in state space is typically eliminated as irrelevant during the algorithmic data-sieving phase.

The remaining discriminatory information may uncover new molecular knowledge otherwise unreachable by traditional hypothesis formulation. It also provides initial focus points for modeling efforts. It is unknown how much molecular knowledge is required to model, for example, disease susceptibility or future disease courses in individual patients. However, cytomics now opens the possibility of constraining bottom-up forward engineering (Collins et al. 2003)—as in network or spatiotemporal modeling—with precise data from a cellular level of biological organization.

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