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NEW HORIZONS IN FLOW CYTOMETRY BY CYTOMICS AND BIOINFORMATICS. A MINIREVIEW.

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ABSTRACT. Cytomics is the study of cell systems (cytomes) at a single cell level. It combines all the bioinformatic knowledge to attempt to understand the molecular architecture and functionality of the cell system (Cytome). Much of this is achieved by using molecular and microscopic techniques and flow cytometry that allow the various components of a cell to be visualised as they interact i*n vivo*.
Key words: flow cytometry, -omic, cytome, cytomic, bioinformatics, microscopy, human cytome project.

"OMICS"

The term "omic" is derived from the latin suffix "ome" meaning mass or many. Thus "omics" studies are like other studies except that they involve a mass (large number) of measurements per endpoint rather than one or a few.

The goal of "omic" approaches is to acquire comprehensive, integrated understanding of biology by studying all biological processes to identify the different players (e.g., genes, RNA, proteins and metabolites) rather than each of those individually. "Omic" approaches currently attempt to address specific biological questions, often without need for prior understanding of a biological basis. With the development of current and future technologies, "omic" research might aim to explain more complex systemic questions and become a tool in diagnostics and drug development.

Omics is the large-scale study of genes (genomics and epigenomics), transcripts, (transcriptomics), proteins (proteomics), metabolites (metabolomics), lipids (lipidomics) and interactions (interactomics), and is both the foundation and the driving force for systems biology (Hood et *al.*, 2004). These all -omics are summarized in Table 1.

These and other "omics" retain the analytical requirements and problems associated with older or classical analytical approaches and add additional challenges due to mixture complexity and the number of biomarkers measured from a single sample.

Omics strives to identify, quantify and characterize all of the components in cellular systems with spatiotemporal resolution, and thereby dissect the intracellular pathways and networks. Currently, major technologies for genomics, epigenomics and transcriptomics include DNA sequencing and microarrays (planar-, bead- and fiber-optic arrays); for proteomics, mass spectrometry (MS) and protein arrays; and for metabolomics, MS and NMR. The above-mentioned major technologies have undergone dramatic advances inautomationand miniaturization. The logical limit to the ever-increasing miniaturization of omics technology is the comprehensive spatiotemporal analysis of genes, transcripts, proteins, metabolites and interactions in single cells and their subcellular compartments.

CYTOMICS

Molecular cell systems research (cytomics) aims at the understanding of the molecular architecture and functionality of cell systems (cytomes) by single-cell analysis in combination with exhaustive bioinformatic knowledge extraction. In this way, loss of information as a consequence of molecular averaging by cell or tissue homogenisation is avoided.

Cytomics is the study of complex and dynamic cellular systems starting with the single cell as its reference point. From this vantage position, molecular events within the boundaries of the cell can be explored together with a higher-level recognition of how cells populate, interact and behave within cellular networks (Jain K.K., 2006, Valet G., 2005). Cytomics can reveal the 'informative cells' within a cell-based assay and clarify an endpoint readout using an expanding range of cytometric technologies of increasing throughput capacity (Herrera *et al.* 2007). At the other end, the large-scale acquisition and synthesis of multiparameter data from individual cells can provide an understanding of the behaviour of the system as a whole (i.e. the relevant cytome) and such data could be used to fuel predictive models in systems biology approaches.

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Table 1.

-OME	FIELD OF STUDY	COLLECTION OF	PARENT SUBJECT	Notes
Bibliome	Bibliomics	Scientific bibliographic data		
Connectome	Connectomics	Structural and functional brain connectivity at different spatiotemporal scales	Neuroscience	
Cytome	Cytomics	Cellular systems of an organism	Cytology	
Exposome	Exposomics	An individual's environmental exposures, including in the prenatal environment	Molecular genetics	A proposed term and field of study of the disease-causing effects of environmental factors (the "nurture" component of "nature vs. nurture").
Exposome		Composite occupational exposures and occupational health problems	Occupational safety and health	The proposers of this term were aware of the previous term as used above but proposed to apply the term to a new field.
Exome	Exomics	Exons in a genome	Molecular Genetics	
Genome	Genomics (Classical genetics)	Genes (DNA sequences/ Chromosomes)	Genetics	"Genome" refers to the set of all genes in an organism. Today, both definitions are used, depending on the context
Glycome	Glycomics	Glycans	Glycobiology	
Interferome	Interferomics	Interferons	Immunology	Also a database of the same name.
Interactome	Interactomics	All interactions		The term "interactomics" is generally not used. Instead, interactomes are considered the study of systems biology
Ionome	Ionomics	Inorganic biomolecules	Molecular Biology	
Kinome	Kinomics	Kinases	Molecular Biology	Proteins that add a phosphate group
Lipidome	Lipidomics	Lipids	Biochemistry	
Mechanome	Mechanomics	The mechanical systems within an organism		
Metabolome	Metabolomics	Metabolites		All products of a biological reaction (including intermediates)
Metagenome	Metagenomics	Genetic material found in an environmental sample	Molecular Biology	The genetic material is assumed to contain DNA from multiple organisms and therefore multiple genomes, hence the inclusion of the prefix meta-.
Metallome	Metallomics	Metals and metalloids		
ORFeome	ORFeomics	Open reading frames (ORFs)	Molecular Genetics	
Pharmaco-genome	Pharma-cogenomics	SNPs and their effect on pharmacokinetics and pharmacodynamics	Pharmacogenetics Genomics	
Phenome	Phenomics	Phenotypes	Genetics	
Physiome	Physiomics	Physiology of an organism		
Proteome	Proteomics	Proteins	Molecular Biology	
Regulome	Regulomics	Transcription factors and other molecules involved in the regulation of gene expression	Molecular Biology	
Secretome	Secretomics	Secreted proteins	Proteomics	Subset of the proteome consis- ting of proteins actively exported from cells.
Transcriptome	Transcriptomics	mRNA transcripts	Molecular Biology	

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The cytomics concept has been significantly advanced by a multitude of current developments. Cytomics, for example, may be considered a new name for cytometry. Cytometry as a technologically oriented science is focused on the multiparametric determination of molecular or morphologic cell parameters by flow or image cytometry. Only a fraction of the available information is usually extracted by visual evaluation of multiparametrically gated histograms or by quantification of marker positive or negative cells. In this situation, the conceptual dimensions of cytomics **(**Valet *et al.,* 2004**,** Valet &Tarnok, 2003) emerge.

Cells constitute the elementary building units of organisms. Diseases are induced by systematic changes of certain molecular cell phenotypes in the background of the full heterogeneity of cells and cell systems (cytomes).

Further, molecular cell phenotypes evolve in individuals during their lifetimes due to genotype and exposure to external or internal influences. Differential single-cell screens of diseased versus healthy cytomes using hypothesis-driven parameter panels uncover disease-induced cell phenotype changes. Cytomics are operationally defined as the multimolecular cytometric analysis of cell and cell system heterogeneity in combination with exhaustive bioinformatic knowledge extraction from all measured cells (Chitty M). Cytomics enables the correlative analysis of differential individual cellular information with regard to future (prediction) or current (diagnosis) disease states of patients. Disease-associated cytomes are typically investigated, but no detailed a priori knowledge on specific disease-inducing mechanisms is required. This is powerful because it permits one to uncover molecular networks of as yet unknown functionality during the exhaustive knowledge extraction phase of this inductive approach.

A considerable conceptual difficulty currently exists in understanding the corroborative action of the 30,000 to 40,000 genes of the genome by *bottom-up* analysis from the genome level via the proteome, the metabolome (Chitty M) including all the low-molecular-weight molecules, the organelle compartments up to the level of cells, cell systems, organs, and organisms (Collins *et al.,* 2003). The *top-down* approach by cytomics as an alternative represents an efficient and simplifying research strategy that often leads to new hypotheses. Patient-derived molecular data patterns of 20 to 40 gene products are typically obtained from cytomics data pattern differentials. These data patterns are of immediate relevance for everyday medicine because they provide therapy-related, individualized, disease-course predictions for patients **(**Valet &Tarnok , 2003 **).**

Cytomes, i.e. cell systems are composed of various kinds of single-cells and constitute the elementary building blocks of organs and organisms. Their individualized (cytomic) analysis overcomes the problem of averaged results from cell and tissue homogenates where molecular changes in low frequency cell populations may be hidden and wrongly interpreted. Analysis of the cytome is of pivotal importance in basic research for the understanding of cells and their interrelations in complex environments such as tissues and in predictive medicine which is a prerequisite for individualized preventive therapy.

Cytomics aims to determine the molecular phenotype of single cells. Within the context of the -omics, cytomics allows the investigation of multiple biochemical features of the heterogeneous cellular systems known as the cytomes.

Cytomics can be considered as the science of single cell-based analyses that links genomics and proteomics with the dynamics of cell and tissue function, as modulated by external infuences. Inherent to cytomics are the use of sensitive, scarcely invasive, fuorescence-based multiparametric methods and the event-integrating concept of individual cells to understand the complexity and behaviour of tissues and organisms. Among cytomic technologies, fow cytometry, confocal laser scanning microscopy and laser capture microdissection are of great relevance.

Other recent technologies based on single cell bioimaging and bioinformatic tools become important in drug discovery and toxicity testing, because of both high content and high-throughput. The multiparametric capacity of cytomics is very useful for the identifcation, characterization and isolation of stem cell populations. In our experience, flow cytometry is a powerful and versatile tool that allows quantitative analysis of single molecules, biotechnological, environmental and clinical studies.

The dynamic nature of cytomic assays leads to a real-time kinetic approach based on sequential examination of diferent single cells from a population undergoing a dynamic. Finally, cytomic technologies may provide in vitro methods alternative to laboratory animals for toxicity assessment. Inherent to cytomics are the use of sensitive, scarcely invasive, fuorescence-based methods and the integrating concept of individual cell analysis to understand the complexity and behaviour of tissues and organisms. Due to the availability of large number of fluorescent markers and the multiplicity of fuorescence detectors interfaced to the dedicated instrumentation, cytomic assays may be multiparametric, polychromatic and multiplexed.

Fluorescence-based measurements may be qualitative and quantitative and can be obtained as the result of single end-point measurement or kinetic, sequential measurements. While these features are common to all cytomic technologies, there are important specific diferences depending on whether the quantitative cell fuorescence data are extracted together with cell morphology in image-based cytomics (Eils & Athale, 2003) or from fuorescence-pulse analysis in flow-based cytomics (O'Connor *et al.,* 2001). Among the current cytomic technologies, flow cytometry (FCM),

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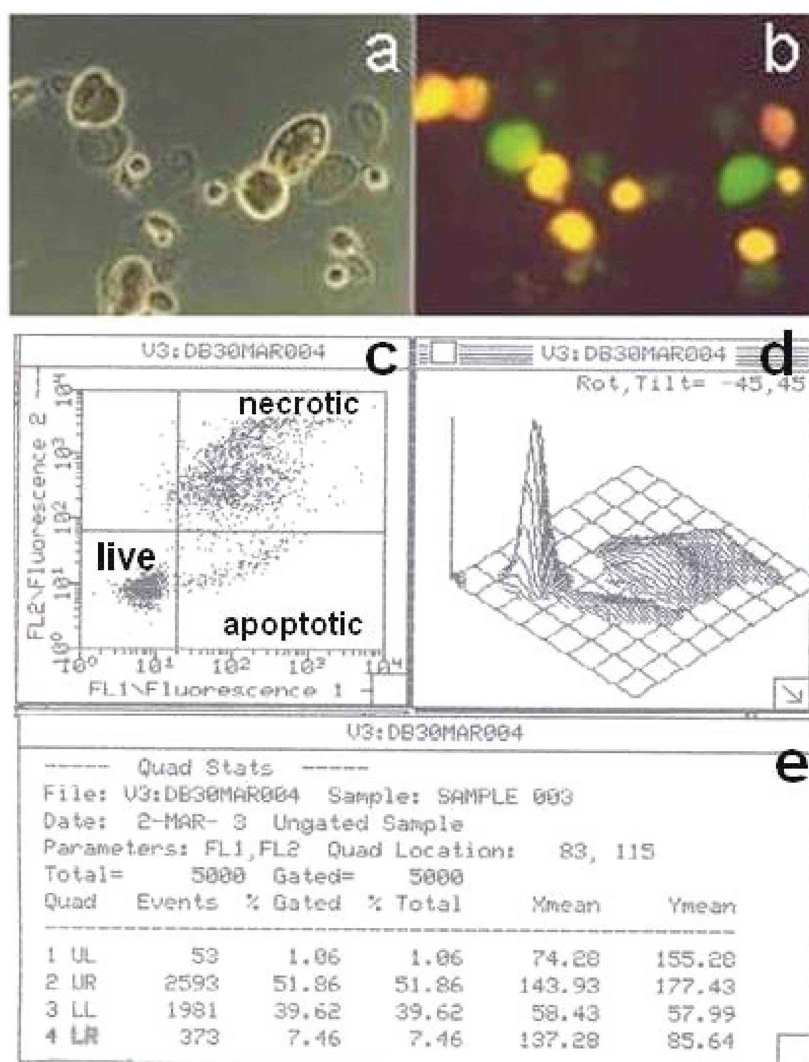
confocal laser scanning microscopy (CLSM), spinning-disk confocal microscopy (SDCM) and laser scanning cytometry (LSC) are of established relevance. Other cytomic technologies based on single-cell based image analysis and powerful bioinformatic tools (high-content screening bioimaging, HCSB) have been recently introduced for drug discovery and toxicity testing, as they can provide both high-content and high-throughput analysis. Finally, laser capture microdissection (LCM), a preparative technique for obtaining pure cells from specific microscopic regions of tissue sections, can also be considered among cytomic technologies.

Flow cytometric analysis. This methodology requires that cells (or microscopically biological particles) are in suspension. FCM allows the simultaneous quantification of multiple fluorescence emissions in the same cell, arising from fuorescent markers, and scattered light related to morphology, revealing key cellular functions or structures (O'Connor *et al.,* 2001). The velocity of analysis can be up to thousands of single-cells per second and individual cells from heterogeneous subpopulations can be physically isolated on the basis of their fluorescence or light scatter properties.

The multiparametric capacity of FCM permits to quantify the efects induced by the exposure to a toxic agent, providing a direct proof of cellular susceptibility or resistance. The advantages of FCM derive from its multiparametricity that provides multiple, simultaneous targets to assess cell lesion or death in selected cell populations, either as end-point or kinetic measurements (Fig.1).



Quad	Events	% Gated	% Total	Xmean	Ymean
1 UL	53	1.06	1.06	74.28	155.28
2 UR	2593	51.86	51.86	143.93	177.43
3 LL	1981	39.62	39.62	58.43	57.99
4 LR	373	7.46	7.46	137.28	85.64

Fig. 1. Cellular heterogeneity in apoptosis of nucleated erythrocytes of Rana sp. analysed by cytometry: a- optical microscopy, b- fluorescence microscopy of double stained annexine-V-FITC(green) / propidium iodide(rouge), c- flow cytometrc quadrant analysis of annexin-V-FITC/propidium iodide double-stained of normal nucleated erythrocytes and 3D analysis of cellular heterogeneity (d), e –statistical multiparametric analysis obtained after flow cytometric analysis.

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One of the specific objectives of Cytomic is to explore innovative tools and cell systems to identify new endpoints and strategies that anticipate better human and animal toxicity (Fig.2). Incorporation of cytomics to this project may define new endpoints of in vitro cytotoxicity to be incorporated into the predictive model or to provide new alerts and correctors of toxicity (Bratosin *et al.,* 2011).

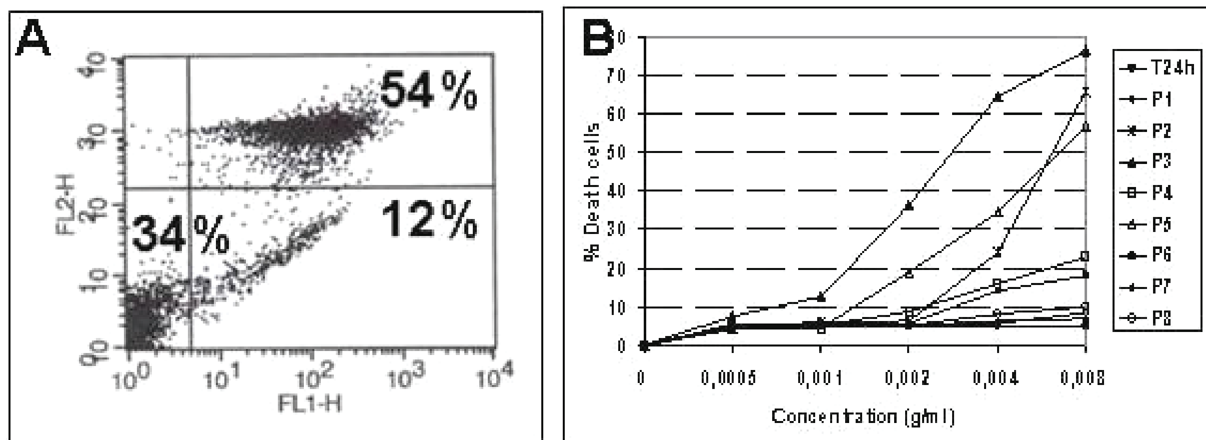

Fig. 2. Flow cytometric quadrant analysis of Annexin-V-FITC/propidium iodide double-stained of normal nucleated erythrocytes (To and T24h) and exposed at 0,008 g/ml nanomaterials (P1 to P8). Abscissae: log scale green fluorescence intensity of annexine-V-FITC (FL-1). Ordinates: log scale red fluorescence intensity of propidium iodide (FL-2). Low left quadrant: viable cells (annexin-V and propidium iodide negative cells); low right quadrant: apoptotic cells (annexin-V positive and propidium iodide negative cells); upper right quadrant: dead cells (annexin-V and propidium iodide positive cells). % refers to the cell percentage of each population. (A) and curves dose-response to calculate EC_{50} conforming to % of death erythrocytes determined by Annexin V-FITC and propidium iodide double-labelling.
Abscissae: concentration of nanomaterials. Ordinates: % of death cells refers to the % of total cells (100%) less % of viable cells (low left quadrant: viable cells (annexin-V and propidium iodide negative cells) at different concentrations.

Confocal fluorescence microscopy. Confocal fluorescence microscopy is advantageous to conventional fuorescence microscopy, eliminates out-of-focus blur and allows producing serial optical stacks from thick specimens (z-axis resolution). Confocality may be applied to image single cells from fixed or living preparations labeled with appropriate fluorescent probes (Fig.3).

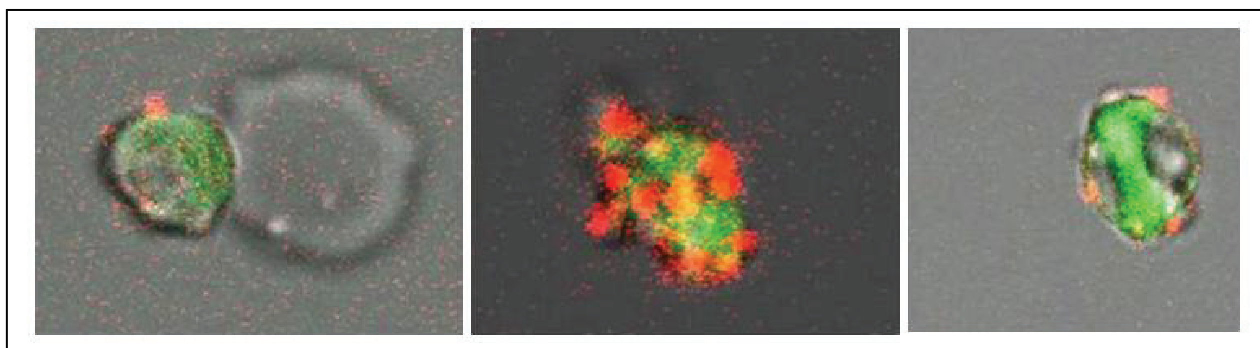

Fig. 3. Confocal microscopic analysis of human RBC senescent population double stained for caspase-3 activity using CaspGLOW™ (green colour) and for phophatidylserine externalization using annexin-V-PE (red colour). Pictures clearly showing the cytosolic location of caspases -3 and morphological change of discocyte shape to shrunken shapes.

This technique allows sophisticated cell information based on spatial- and time-resolved fluorescence measurements and as such is being increasingly used for scientific and technological applications (Rubart, 2004). Usually, CLSM yields better image quality but the imaging frame rate is slow while SDCM may produce video rate imaging, which is required for efcient dynamic observations (Maddox *et al.,* 2003). However, the new confocal systems function like hybrids allowing obtaining both high speed and good resolution.

Laser scanning cytometry. LSC is a microscope-based, scanning cytofluorimeter that combines the advantages of flow and image cytometry. It allows multiparametric analysis performed directly by measuring the fluorescence of individual cells in solid-state preparations, such as monolayers, smears, imprints, cytospins or tissue sections in several supports including slides, dishes and multiwell plates. LSC provides increased sensitivity and specificity compared to traditional microscopic techniques and a similar structure for data analysis to flow cytometry, albeit at lower data acquisition velocity. In addition, it allows relocation of the coordinates of analyzed cells of interest following slide restaining. Finally, single cells identifed by their fuorescence measurements can be individualized from histogram or dot-plot displays and shown as single-cell pictures or combined in picture galleries for further analysis (Juan & Cordon-Cardo, 2001).

A hypothetic model for cytomic analysis of biological specimens could work as follows: Viable cells may be initially stained for cell functions like intracellular pH, transmembrane potentials or Ca^{2+} levels, followed by fixation to remove the functional stains and staining for specific extra- or intracellular constituents such as antigens, lipids or carbohydrates. After destaining, specific nucleic acids may be stained. 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. Serial optical imaging will permit 3D-reconstruction of the molecular morphology of cell membrane, nucleus, organelles and cytoplasm including the parameterization of 3D-shapes. Serial histological sections taking stereological aspects of tissue architecture into account will serve as basis for the standardized analysis of proximity and interaction patterns for intracellular structures like nucleus and organelles as well as for different cell types within the tissue architecture that can even include time as a parameter for 4D intravital microscopy.

Figure 4 (based on a model proposed earlier (Tarnok A., 2004) tries to summarize how a future instrument and/or work-flow will look like that will enable to analyze the cellular context on the single-cell level from the whole organism over high-throughput single-cell analysis in tissues (tissomics, toponomics) combined with single cell high-content metabolic investigation (lipidomics (Wenk MR., 2005 , Werner *et al.,* 2006), metabolomics) (Bocsi, 2006), intracellular pattern recognition (location proteomics) down to single-cell proteomics and genomics. Combined with data pattern analysis these approaches will lead to the complete characterization of the cytome (Valet G. 2004) with the detection of new cell types, cellular functions and interrelationships and imminent implications for individualized risk assessment in the clinical environment (Valet & Tarnok, 2003).

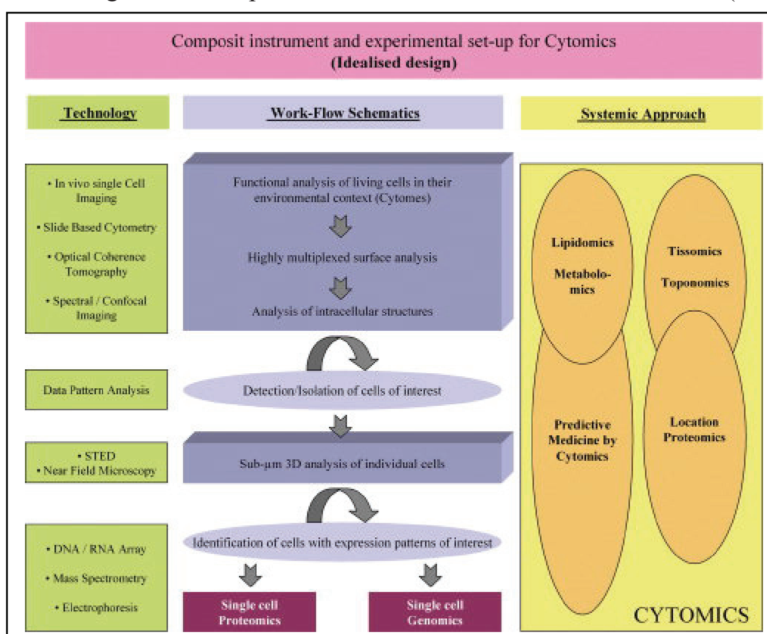

Fig. 4. Idealized slide-based cytometer for cytomics. The scheme summarizes the workflow for the complete high context dissection of biological specimens such as tissues. The analysis starts on the top with low resolution high-content analysis (e.g., by whole animal single cell imaging) and ends in the single-cell proteomic and genomic dissection. The different components of cytomics SBC and how these components can be combined in a work flow. The left column shows the different technologies and instrumentation that come to application at the different steps of the work (flow). These detection modalities may either be combined in a single instrument or are combined in a work-flow scheme. The right column shows the different Omics appearing in this article and in articles of this focus issue and their relation (after Tarnok, 2006).

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Considering the efforts being made toward sample collection, staining, measurement, and data analysis, it seems mandatory to routinely use automated, self-gating evaluation strategies to extract the entire information content of all measured cells for subsequent knowledge extraction. This means, in practice, for example in flow cytometry, that the percent frequency, means, or medians of light scatter and fluorescence signals, and light-scatter and fluorescence ratios, as well as the coefficients of variation for all parameters in all evaluation gates should be calculated and databased. An effort should be made to collect this information for more than 95% of all measured cells, to be reasonably certain that no relevant information escapes the analysis (Valet G, 2002). It is empirically advisable to use self-adapting and contiguous gates for the automated evaluation of flow cytometrically well-known cell population entities like lymphocytes, monocytes, or granulocytes, as defined by forward (FSC) or sideward (SSC) light scatter or by typical antigenic properties like the expression of CD45 antigen. The subsequent fluorescence gating can be equally automated by using standard quadrant evaluation at fixed threshold levels in gated two-parameter histograms. The evaluation should always include fluorescence-negative as well as single and double fluorescence-positive cells. It is not of primary importance at this stage that cell population boundaries be respected, since relevant information will be picked up anywhere by subsequent data sieving analysis, provided the information for more than 95% of all cells has been accessed during the information collection phase (Valet G, 2002).

Bioinformatic. Cytomics is bioinformatic knowledge extraction from a large amount of structural and functional information by molecular cell phenotype analysis of tissues, organs, and organisms at the single-cell level by image or flow cytometry (Valet G, 2005). Areas of research and diagnosis with the demand to virtually measure ‘‘anything’’ in the cell include immunophenotyping (Mittag *et al.,* 2005, Perfetto *et al.,* 2004, Gerstner *et al.,* 2006), rare cell detection (Bocsi *et al.,* 2004) and characterization in the case of stem cells (Lovell & Mathur , 2004) and residual tumor cells (Szaniszlo *et al.,* 2006, Shen & Price , 2006), tissue analysis (Steiner *et al.,* 2000, Ecker &, Steiner , 2004, Gerstner *et al.,* 2004, Megyeri *et al.,* 2005), and drug discovery (Van Osta *et al.,* 2006, Van Osta P., 2006).

Knowledge extraction after generalised information collection represents a very essential task. Collected information may easily represent several thousand data columns per set of measurements. The classification of such numbers of data columns by statistical, principal component, fuzzy logic, or neuronal network analysis is frequently beyond the capacity of typical software packages, and may require distributed computing (Snow *et al.,* 2002). Classification results by these analysis strategies may furthermore depend, to some degree, on the assumption of certain mathematical distributions of parameter values or on predefined levels of correlation coefficients (in the case of cluster analysis). Missing experimental values may have to be reconstituted or data records may have to be discarded, which may influence the final classification result. A further important complication is due to the mixed data-type format, particularly from proteomic databases. Data sieving (Valet & Hoeffkes, 2004) as an alternative nonstatistical knowledge extraction strategy does not require mathematical assumptions, missing values do not have to be reconstituted, and the analysis is suitable for parallel computation and inherently fast, because only data thresholding is required for classification.

The determination of multiparametric individual cell molecular parameters by cytometry, along with multiplex bead assays as well as cell population and single cell-based microarray technologies, generates large amounts of data.

However, a major challenge remains to efficiently and effectively extract the relevant predictive medicine parameters. Currently, this information is frequently extracted in a fragmentary way by computer-assisted identification and characterization of a few cell populations or gene clusters of interest. Alternatively, all of the available information can be screened exhaustively by multiparametric clustering (Rosenwald *et al.,* 2002, Weinstein *et al.,* 2002), data mining, or other procedures (Peltri *et al.,* 2003) for diagnostic or prognostic information-using hypothesis-driven analysis strategies. These frequently require mathematical, statistical, or other assumptions that may unintentionally bias the results. Assumption-free algorithmic evaluation concepts like data sieving (http://www.biochem.mpg.de/valet/classif1.html) as a bottom-up approach seem of particular interest for the detection of unknown molecular disease mechanisms that are inaccessible to a-priori top-down oriented hypothesis. Following identification of the important predictive data patterns, a major task will still consist of the consensus driven development of standardized predictive disease classifiers for clinical purposes.

Are histochemistry and cytochemistry “Omics”?

A plethora of new “omics” such as transcriptomics, proteomics, metabolomics (or metabonomics), pharmacogenomics, physiomics and cytomics are upon us, but can histochemistry be an “omic”? To be an “omic” a technique must take a “global” and “holistic” view of biology that addresses biological complexity head-on by synthesising multiparameter data into predictive models. Thus to be an “omic”, a histochemical technique should be as inclusive as possible in identifying as many targets as possible with equal likelihood and sensitivity of detection. Any technique capable of detecting only one or two targets is not within the spirit of an ‘omic, ruling out it seems most of histochemistry.

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Nevertheless, new developments in high-throughput histochemistry and cytochemistry are making powerful claims to the title “Histocytomics”. Histocytomics and all the other “omics” are components of the only real “omic “, Biomics”, that is, the integrated application of science into a coherent strategy for understanding biological complexity (Coulton G., 2004)

Cytomics approach has the potential to advance general health care by therapy-related individualized disease-course predictions. It also provides the potential to uncover practically relevant disease-inducing molecular pathways by biomedical cell systems biology and to identify new drug targets within such pathways. The incentive for the development of sensitive single-cell technologies for research and medicine and the provision of advanced software for analysis and standardized data management represent further important facets of this challenge.

The human cytome project.

The human cytome project is conceived as a joint cross-disciplinary effort of cytomics, systems biology, and high-throughput-oriented research involving basic, clinical, and industry scientists (Valet *et al.,* 2004., Available at:http://www.biochem.mpg.de/valet/humcyt9.ppt.)

The general applicability of differential molecular cell phenotype screening in disease favors accurate diagnostics and individualized disease course predictions for optimized patient therapy (personalized medicine) and the search for new drug targets.

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