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Potential of a Cytomics Top-Down Strategy for Drug Discovery

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Abstract: It takes about 10 to 15 years and roughly 800 mln $ to bring a new drug to the market. Only 10% of drug molecules entering clinical trials succeed and only 3 out of 10 drugs generate enough profit to pay back for the investment.

Drug targets may be searched by hypothesis driven modeling of molecular networks within and between cells by systems biology. However, there is the potential to simplify the search for new drugs and drug targets by an initial top-down cytomics phase. The cytomics approach i) requires no detailed a-priori knowledge on mechanisms of drug activity or complex diseases, ii) is hypothesis driven for the investigated parameters (genome, transcriptome, proteome, metabolome a.o.) and iii) is hypothesis-free for data analysis. Moreover it iv) carries the potential to uncover unknown molecular interrelations as a prerequisite for later new hypothesis driven modeling and research strategies.

A set of discriminatory parameter patterns (molecular hotspots) describing the cellular model (mechanism of drug action) can be identified by differential molecular cell phenotyping. Hereby, the immediate modeling of existing complexities by bottom-up oriented systems biology is avoided.

The review focuses on the fast technological developments of molecular single cell analysis in recent years. They comprise a multitude of sensitive new molecular markers as well as various new image and flow cytometric high-content screening methods as facilitators of the cytomics concept. New bioinformatic tools enable the extraction of relevant molecular hotspots in description of cellular models, being required for the subsequent molecular reverse engineering phase by systems biology.

Keywords: Cytomics, drug discovery, high-content analysis, data mining, high- throughput screening, cytometry.

A. THE PROBLEM – WHERE WE ARE IN THE DRUG DISCOVERY PROCESS

Pharmaceutical and biotech companies try to develop new drugs that have a high chance to reach the market and to fund their research. The current disease models used in drug discovery and preclinical development have difficulties to predict failure in drug development (clinical phase I to III and IV) in 80–90% of drugs entering clinical trials.

It requires about 10 to 15 years and between US$ 500–800 million to bring a new drug to the market [1, 2]. Only a 10% overall success rate of drug molecules entering clinical trials [3] is typically reached. In addition, only 3 out of 10 drugs generate enough profit to pay back for the investment [4]. One of the reasons for this is that currently used disease models show a correlation deficit to clinical reality, because of the underestimation of the complexity and variability of clinical disease processes in man [5]. To improve the overall efficiency and profitability new technologies and parameter screening approaches supporting drug discovery and development are being introduced in the following.

The emerging potential to gain detailed quantitative data from biological specimens has become increasingly important in the new fields of high-content and high-throughput single-cell analysis for systems biology and cytomics [6, 7]. Genomics, proteomics and metabolomics provide important technical contributions to cell biology but become limited when single or scarce cells are examined or fast cellular processes have to be followed kinetically [8].

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B. CYTOMICS

It is important to keep in mind that single cells constitute the elementary building units of living organisms. Cytomics as bioinformatic knowledge extraction from molecular cell phenotype analysis of many single cells of cell systems (cytomes), tissues, organs, and organisms by image or flow cytometry [9] provides a new potential to unwind the cellular biocomplexity of organisms starting from the cellular level.

In analogy with other -omics like genome/genomics, proteome/proteomics, metabolome/metabolomics, the scope of cytome/cytomics concerns heterogeneous cellular systems. Cytomics is the broadest approach of any cell-based -omics and harbors among others (cellular) metabolomics, lipidomics, location proteomics and toponomics [6]. The functional heterogeneity of cytomes results from both the genome and external environmental influences. Cytomics can be considered as a discipline that links genomics and proteomics to cell and tissue phenotype and function, as modulated by external influences. Of special importance is the cell-by-cell basis of cytomics analysis. This approach allows resolving heterogeneous systems by avoiding the loss of information that characterizes bulk technologies where average values are obtained from large number of cells or from tissue homogenates [9]. The cytomics approach reflects the reality that cells and their inter-relationship and not genes or biomolecules represent the elementary function units of organisms.

Typical cytomes are the system of leukocytes in the peripheral blood or the cell system of an organ. For drug discovery such cytomes are difficult to obtain in large entities. That is why appropriate cellular models like hepatocytes are being introduced and are treated as representative cell sys-

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tems (cytomes) consisting of single cells in variuos states of proliferation, differentiation and activation [9]. Cellular populations, however, are observed to respond heterogeneously to perturbations. In response to a stimulus, cells undergo changes seen as repertoire of underlying states of functional significance [10]. In the era of stem cell technology new cellular models like iPS (induced pluripotent stem cells) are at reach and are good candidates for establishment of stable cell models for drug screening [11]. Multidimensional (3 to 4 dimensional) analysis of cellular factors as the consequence of cellular dynamics, heterogeneity of structure and function resulting from genome and impact of environment [12] and phenotypic background [8] have to be taken into account when searching for new drugs binding to proteins [1] and affecting cellular responses.

Top-Down Strategy

Cytomics investigations use a top-down strategy (Fig. **1a**) with ***hypothesis-driven*** parameter selection in combination with ***hypothesis-free*** exhaustive knowledge extraction. While a given hypothesis can be proven or disproven using multiparametric measurements by focusing only on a restricted number of measured parameters, the evaluation of all collected cell data in a data driven fashion (discovery science) enables the hypothesis-free exploration of unknown multiparametric data and knowledge spaces for discriminatory parameters. A new round of hypothesis driven parameter selection and further experimentation may result from the first evaluation [13].

The top-down approach realizes that cells and not genes or biomolecules represent the elementary self reduplicating function-units of organisms and that diseases are caused by molecular alterations in cells or cytomes as consequence of genotype and exposure to external or internal influences [13].

Parameter Selection Dilemma

The overall aim of the cytomics approach is to reduce costs and increase the speed of the drug discovery process. This is obtained by reducing redundancy of the selected informative parameters but keeping the highest possible information density. Due to the hypothesis-free knowledge extraction the minimum number of required parameters selected for the final discriminatory data set is not predefined.

It is hard to theoretically predefine the exact (minimum) number of parameters which have to be analyzed for an unbiased drug selection. The fundamental problems of drug screening are low specificity for the target selection, which refers to the loss of false negative drugs, combined with low sensitivity, which refers to selection of false positive drugs. Therefore, the main drug discovery problems are: false positive lead-drug selection (low sensitivity) and the loss of potential valuable drugs due to lack of investigating specific

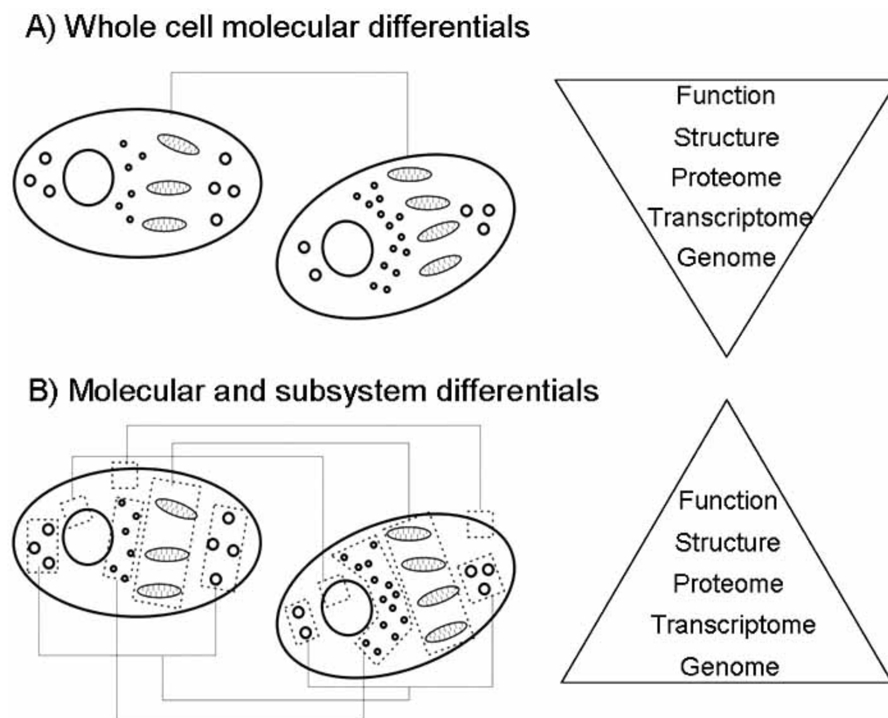

Fig. (1). Comparison of (**A**) top-down (cytomics) and (**B**) bottom-up strategies (systems biology) for data mining.

A. Multiparametric molecular cell status and reactivity like: proliferation, cell death, differentiation, cell movement or metabolic parameters (pH, membrane potential) are assessed by the top-down cytomics strategy. Thus entire cells (cytome, cellular networks, balanced systems) instead of biomolecules as elementary units are processed to collect the heterogeneity of parameter expression in individual cells to access the underlying regulatory information rather than to explain the behavior of the average or typical cell. Afterwards molecular reversed engineering may be used to search molecular pathways and target molecules from discriminatory data patterns.

B. The search and selection of molecular parameters by bottom-up strategy is based on the hypothesis-driven assessment of particular molecules within a putative interaction concept. This leads to poor overall complexity understanding as a progress limiting factor.

targets or cellular models (low specificity) [14]. Here the cytomics approach may be the solution.

Initial testing for informative parameters (relevant for drug screening) may include all possible (accessible and testable) parameters (proteins, genes, transcripts, cell death, proliferation, and cell function), best in their spatio-temporal relationship. It is obviously not feasible to measure all possible parameters on a cell system due to high costs, technical challenge and low throughput. Therefore strategies have to be developed to scrutinize for those parameters and parameter combinations bearing the highest discriminatory power (i.e. the molecular "hot spots") and thereby reducing the number of required cellular parameters to be analysed to a minimum. The additional advantage of multiparametric measurement is that within a single analysis a multitude of drug actions can be interrogated thereby again reducing costs and time.

Such strategies have been proposed by us for the panel development for the predictive medicine by cytomics approach [15] and could be applied likewise for the development of drug discovery test systems. In the following we will briefly describe the necessary steps to be taken for predictive medicine by cytomics that can serve as a model for the drug discovery by cytomics procedure. This strategy typically comprises three levels (see also Fig. **2**):

1) At the first level, the ***explorative phase***, a high number of parameters is analyzed on a relatively low number of samples such as healthy individuals vs. diseased patients. In drug discovery the groups tested would be in analogy drugs without (negative control) vs. drugs with (positive control) known biological effects. The positive control may consist of different classes of drugs with differing biological effects (e.g. inducing apoptosis and/or cell activation and/or modulation of translation or transcription). Parameter selection to discriminate these test drugs should not only be hypothesis driven but has to be set broader in order to be able to detect the unexpected [10].

   After exhaustive hypothesis-free mathematical data-mining, out of this wide range of tested parameters (variables) those are selected that can discriminate the investigated classes of drugs by >95% [16]. Multivariate mathematical tools for parameter selection and reduction are typically discriminant analysis, principal component analysis, Classif1, neuronal networks and others [16-18]. In this first phase, the parameter selection and reduction should be done at low stringency in order to leave more testable parameters for the next study level than essentially needed for optimal group discrimination.

2) In the second level of ***test development***, the first evaluation phase, the selected parameters from the 1st level are applied to a larger test group of drugs. The intention of this phase is to test validity and reliability of the test system and to further optimize and reduce the parameter number to the absolutely essential. At this point also "blinded" drugs should be included and tested how well they can be identified by the optimized panel. Using the multivariate test for the unsupervised learning of discriminatory patterns the blinded (but functionally known) drugs should be identified correctly [16].

3) The third level of test development is the ***final validation*** of the assay on a large number of functionally known compounds and also a large number of blinded but known compounds. The intention of this third phase is the final testing and fine-tuning of the panel. If the third phase yields the expected result of ~100% correct prediction of the blinded drugs then the cytomic test is ready to go for the unknown samples.

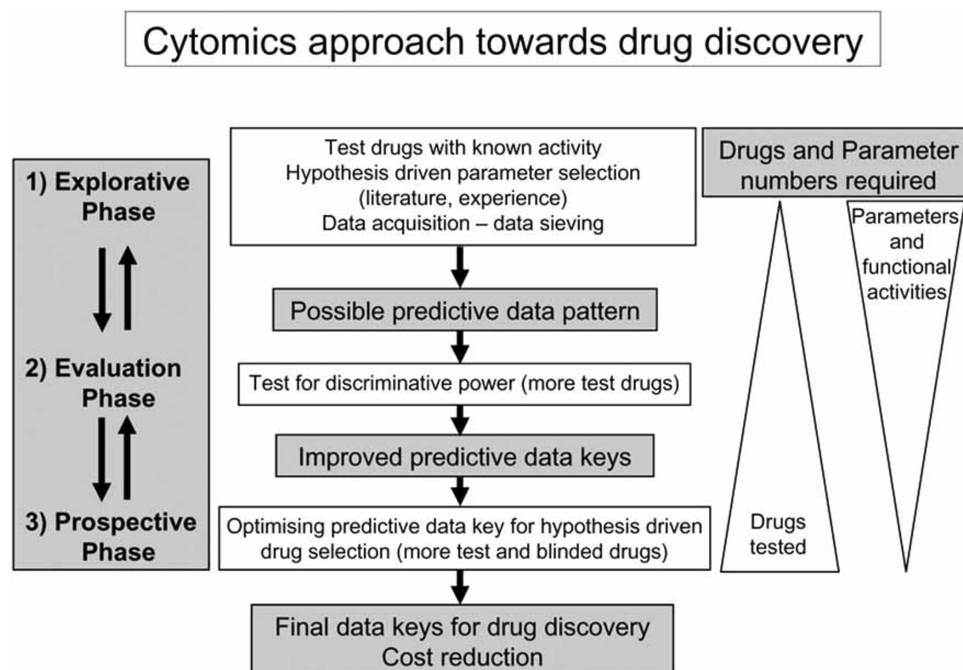

Fig. (2). Flowchart showing how cytomics approach supports drug discovery.

As an example from our preclinical studies, we performed cytomics to discriminate pediatric patients with an uneventful clinical course after cardiac surgery from those who overreacted based on preoperative blood sample analysis. Initially, we started with several hundreds of data values per sample. Using the above approach we were able to cut down the number of required parameters to nine or even less and still clearly discriminate patient groups with an overall correctness of nearly 100% [19]. Blinded blood samples in the second phase were correctly identified with around 90%.

Consequently, these development and optimizing steps lead to drug discovery process acceleration from tedious high-content or med-content and low-throughput multiparameter assays to a few parameters-based high-throughput drug activity screening process. Importantly, as all acquired data and results are databased aided by machine learning the test system is permanently improving its specificity and sensitivity.

The lack of discriminatory functional parameters not included into the primary parameter selection set may be one reason for a poor discriminatory capability of a data classifier. Also, selection of an inappropriate cellular model will limit drug function detection because the selected cellular model is not representative for a specific drug activity. Having not found the target for a drug in a cellular model a drug candidate will be eliminated. Cell-based disease models in which the molecular diversity of the human cytome could be taken into account will improve the predictive value of drug discovery [1, 20-23]. Assembling a database of cellular processes by establishing a human cytome project would help to improve our understanding of disease processes and their spatial and temporal dynamics at the cellular level [13]. This information could stimulate drug discovery by definition of the new valuable targets for drugs [13].

The cytomics approach limits rejection of valuable parameters thanks to its unbiased data mining process. Moreover, cytomics allows elimination of those parameters that are not informative to the drug selection process thereby decreasing redundancy, time of analysis and costs. As an example, in cell cycle analysis combined measurement of cyclin B1 and the mitotic index does not improve discriminatory power for a drug that perturbs cell cycle as both are aspects of one process and one of them may be eliminated from the final set of parameters [24].

A specific aspect of the cytomics approach is that the data mining process requires a defined, repetitive set of cell responses (cell death, change in morphology a.o.) for the proper learning process of the classifier. Therefore, these responses are not taken into account for the selection process of unknown activities and drugs may remain unrecognized during screening. However, the initial parameter selection can be improved by using different cellular models depicting different cellular functions which may be putatively affected by the drug [25-27]. To this end one can facilitate and enrich parameter selection by new approaches for cell status description [10, 23].

Perspective – Multicenter Drug Evaluation

Whichever assay or technology is used, the increased amount of parameters requires appropriate standardization procedures and controls for reliable data acquisition and analysis. Such methods have been recently reviewed for flow cytometry and similar approaches apply for image cytometry and high-content screening [28, 29]. Standardization as well as regular quality control is pivotal in drug discovery in order to make the data obtained during several cycles of cell analysis or analyzed in different laboratories comparable. They are of course of central relevance in developing standard operational procedures (SOPs) and making an analytical process GLP and GMP compatible.

Final evaluation of drug candidate efficacy has to be performed by international consortia trials. This requires standardization and quality control of instruments and laboratory procedures for comparison of the results [30, 31]. Such standards for inter-laboratory portability have been already introduced for multiparameter cytometric analyses of cell phenotype and function [32]. These efforts meet well with the demand for the standardized, reliable data collection and processing of cytometric data in drug discovery [33, 34]. The SOPs for the parameters selected by cytomics data mining applied in multicenter studies will prove their usefulness for drug efficacy evaluation [35].

C. SYSTEMS BIOLOGY APPROACH

The molecular biocomplexity of organisms may alternatively to cytomics be investigated bottom-up from genes to biomolecules, and organelles to cells, tissues and organs [36], by systems biology (Fig. **1b**).

Systems biology aims to describe quantitatively how properties of biological systems can be understood as functions of the characteristics of and interactions between their macromolecular components [37]. Whereas traditional biochemistry focused on isolation and characterization of cellular components, the challenge for systems biology lies in the integration of this knowledge and the knowledge about molecular interactions in the cell. So systems biology is, like cytomics, a holistic view of biological systems. Computer models (“virtual cell”) play an important role in this integration and may render in-silico prediction of drug function.

Systems biology uses computational analyses to surf through large volumes of data, to identify unique sets of molecules involved in particular cellular responses, and to develop statistical relationships between molecules that suggest causal interactions [38]. Once such data are acquired, the crucial and obvious challenge is to determine how these, often disparate and complex, details can explain the cellular process under investigation. The ideal way to meet this challenge is to integrate and organize the data into a predictive model [38, 39].

To develop a full understanding of the mechanisms underlying a cell biological event, however, the quantitative physicochemical details must be elucidated. As a first prerequisite, this requires measurements of the concentrations, locations, biochemical reaction rates and membrane transport kinetics for the involved molecules. Such data can come from traditional biochemical methods, but are also available through direct *in vivo* measurements on live cells, using modern microscope imaging with fluorescent probes and

indicators and electrophysiological techniques such as patch-clamp single-channel recording [38]. The model can also be used to perform 'virtual' experiments in advance of a real experiment and can simulate the behavior of molecules that are not easily visualized experimentally.

A "Virtual Cell" biological model is composed of three parts in an expanding tree structure: (1) a single 'physiological model' that captures the mechanistic hypothesis; (2) one or more 'applications', where experimental conditions, geometry and (3) modeling approximations are introduced to pose a concrete mathematical problem; and one or more 'simulations', where each represents a numerical solution to the mathematical problem posed by an application [40].

Cytomics and systems biology approach for drug discovery are approaches that strongly interrelate and support each other [13, 18]. The hypothesis-free cytomics approach serves with the detected molecular hot-spots as data inventory for cell modeling. In turn, systems biology provides hypothesis for targets of novel drugs and helps to set up and optimize functionalities that have to be investigated for a specific class of drugs.

We advocate integrating a top-down approach, where measurements on the complete system are used to derive fluxes in a detailed structural model, with a bottom-up approach, consisting of the integration of molecular mechanism-based detailed kinetic models into the structural model.

D. STATE OF THE ART TECHNOLOGY – TOWARDS SINGLE CELL DECIPHERING

With the rapid developments in the different 'omics' fields, our knowledge of cells and their molecular components has dramatically increased. Although many cellular components have not yet been identified, we are rapidly approaching a complete description of several cellular systems. Corresponding with such an increase in our detailed knowledge of the cell we can also expect an increase in the level of details at which our models describe such systems. Ultimately, such models could describe a complete cellular system at the reaction level, i.e. containing all enzymes, their mRNA levels and metabolite concentrations [41].

Combining data streams collected at different levels of biological organization such as molecular, cellular, and physiological responses offers the chance to a system-wide view in biology [42] that is to combine "-omic" studies with functional analysis [43]. High-content, quantitative analysis and network construction can be performed without a priori knowledge of pathway connectivity warranting circumvention of unknown pathways with loss of valuable and essential information [43, 44].

From the point of view of developing a cytomics drug-discovery platform we face a multi-step process including several preparation steps for drug activity evaluation (see Table **1**). A final protocol however requires not only good and standardized preanalytics but furthermore the application of the appropriate high-technology platforms.

State-of-the-art multicolor flow and image cytometry combined with powerful novel dyes and detector molecules allows validating candidate genes and proteins at the single cell level combined with a detailed phenotypic and functional characterization of cell subsets. The integration of appropriate bioinformatics solutions with multiparametric analysis on the level of cell phenotype and then down to genotype predestine top-down approach to promote the drug discovery [45]. Thanks to the availability of plethora of fluorescent markers and the multiplicity of fluorescence detectors interfaced to the dedicated instrumentation, cytomics assays may be multiparametric, polychromatic, hyperchromatic and multiplexed [44, 46-48].

Selecting the Right Dyes

High-throughput automated fluorescence imaging of biological processes in living cells is currently technically challenging, and requires robust and simple fluorescent labeling techniques. The fluorescent reporters need to be specific, they should interfere as little as possible with the biological process being visualized and they must not perturb the global physiological conditions of the cells. Most importantly, the labeling and detection procedures have to be quantitative and highly reproducible so that different experiments that have been carried out at different times can be compared, and that image data can be evaluated using automated phenotypic analysis — an absolute requirement for large-scale projects [49].

With the right selection of a combination of different fluorescent detecting molecules with different colors as well as site-specific structural and functional targeting we are able to combine the quantitation of different functional aspects of cellular response in a single experiment. Fluorescent tags such as quantum dots [50], a plethora of fluorescent proteins [51], and switchable molecular colors (PS-CFP, PA-GFP) [52] give the substantial advantage for imaging selectively

Table 1. Steps Required for Drug Activity Evaluation

Step	Assays	References
1	Identification of specific target cells or cells susceptible to drugs or xenobiotics	[9, 11, 23, 67]
2	Detection and quantification of toxicity	[23, 27, 106]
3	Characterization of drug and xenobiotic mechanisms	[23, 24, 112]
4	Multiplexed analysis of soluble analytes	[7, 32, 88]
5	High-throughput and high-content screening	[49, 91, 111]
6	In-fluxo kinetic analysis by flow cytometry	[7, 29, 34]

labeled cells and their interaction *in-vitro* and *in-situ* with an excellent signal-to-noise ratio. Once we are able to stain the molecular targets with a myriad of available fluorescent molecules, single-cell-based optical analysis will be more specific and quantitative [53].

Development of fluorescent, organelle-targeted probes has been driven by an interest in discovering new dyes that excite and emit in the visible spectrum, and that possess specific subcellular localization features so that they can be used as organelle markers or physiological biosensors [54, 55]. The group of Allan Waggoner developed recently protein reporters that generate fluorescence from otherwise dark molecules (fluorogens) [56]. They presented eight unique fluorogen activating proteins (FAPs) that have been isolated by screening a library of human single-chain antibodies using derivatives of thiazole orange and malachite green. These FAPs bind fluorogens with nanomolar affinity, increasing green or red fluorescence thousands-fold to brightness levels typically achieved by fluorescent proteins. Visualization of FAPs on the cell surface or within the secretory apparatus of mammalian cells can be achieved by choosing membrane permeant or impermeant fluorogens extending the possibility to image live cells and analyze their subcellular locations.

The emergence of powerful probes and dyes as well as fluorescence microscopy techniques such as fluorescence recovery after photobleaching (FRAP) [57], fluorescence resonance energy transfer (FRET) [58], total internal reflection fluorescence (TIRF) [59], fluorescence correlation spectroscopy (FCS) [60] and fluorescence uncaging [61], have made fluorescence microscopy an indispensable tool for cell biological investigation. They particularly, have opened opportunities for quantitative measurement of molecules in-vivo. Although most of the above technologies are presently still medium to low through-put, large efforts are being made to increase sample analysis for large scale screening [62].

Emerging label free technologies may once replace the need of tagging and thus perturbing biological systems. Such technologies on the horizon are a.o. Raman spectroscopy, impedance cytometry, and near infrared spectroscopy [63-65]. Newly developed or modified instrumentation for optical imaging based on reflectance, two-photon, and multispectral imaging, can detect and localize cellular signatures of cancer *in vivo*, without the use of contrast agents or extrinsic dyes [66]. However, these assays are still even more time-consuming than traditional high-content fluorescence microscopy and far from being applicable in high-throughput assays.

Cells and Models

Depending on the drug's activity being tested different cell and tissue models and sample preparations are applicable [23]. The cell type analyzed also determines which instrumentation is suitable for analysis (adherent or suspended cells or tissue; live cells or fixed cells). Large-scale projects using high-throughput microscopy have applied almost exclusively fixed-cell assays so far [49]. Unfortunately, such endpoint experiments do not provide any temporal information and results might be misinterpreted if, for example, the final state of the examined cells is an indirect consequence of a number of sequentially occurring events. Experiments using live-cell assays and high-throughput time-lapse microscopy can overcome this problem, and they provide much more detailed phenotypic information than fixed-cell assays [67-69]. Finally, site directed cell modification and perturbation by targeted gene modification [70] or iPS are good putative models for drug discovery [11].

Single cell arrays are nowadays available for cells in suspension (e.g. leukocytes) to deposit ten-thousands of individual cells in microchambers for further high-content *in vivo* or *in vitro* phenotypic and functional high-content analysis [71-76]. Cell culture systems consisting of different microscale cell cultures that are inter-connected by a fluidic circulation can be used in high-throughput imaging systems to monitor drug response system in different cell lines simultaneously and may one day mimic whole organisms [77].

High-Content High-Throughput Technologies

Currently, the development of the concept of cytomics goes hand in hand with the establishing of high-content and high-throughput methodologies for flow and image cytometry [20] screening assays [24, 74, 78]. New and emerging technologies focus on the single-cell level to address demand for precise high-content multiparameter analysis (see Table **2**). In recent years, we have witnessed the development of large scale quantitative cellular research as in High-Content Screening which is the promise for high-content and high--throughput analysis [79].

Inherent to cytomics are the use of sensitive, scarcely invasive, fluorescence-based multiparametric methods and the event-integrating concept of individual cells to understand the complexity and behavior of tissues and organisms. These methods comprise: flow cytometry (FCM) [44], confocal microscopy [78, 79], laser scanning cytometry (LSC) [6], high-content screening bioimaging [80], automated scanning fluorescence microscopy [81]. As the single-cell level is of

Table 2. Constituents of Multiparameter Single-Cell Analysis

Step	Levels of single cell quantitative analysis	References
1	Quantitation of as many cellular constituents on the single cell level as possible	[47, 74, 89]
2	Quantitative structural analysis of cellular constituents and their interrelations	[93, 95,110]
3	Quantitation of molecular interactions in cells e.g. FRET	[58, 62,100]
4	Quantitation of cell function properties	[7, 27, 54]
5	Quantitation of the interrelationship of cells	[6,12, 107]

interest rapid cell identification and selection is required by fluorescence activated cell sorting [44], laser capture microdissection [82] or optical tweezers [83], the laser-enabled analysis and processing (LEAP) technology [84] or single-capillary gel electrophoresis for single cell proteomics [85].

Historically, FCM is the first single cell based high-content technology. It is extremely powerful and versatile for quantitative analysis of soluble compounds, molecules of prokaryotic and eukaryotic cells in basic science, biotechnological, environmental and clinical studies [86, 87, 88]. Although state of the art FCM can measure up to 12 colors per cell simultaneously and some groups are even able to measure up to 17 labels at a time [28, 44], still these high-content experiments are fairly complicated to handle and are cost intense due to expensive exotic dyes that have to be used. An alternative approach was developed by the EuroFlow consortium: Here multiple multi-color (up to 6 colors) panels with different antibody combinations are measured per sample and then mathematically combined to a meta-measurement yielding virtually infinite number of colors [89].

Regarding the number of individual cells that can be anlysed in a short amount of time (up to 50,000/second) FCM is high-throughput. But, the number of different samples that can be analyzed in a certain time-frame is medium-throughput. However, several improvements are being made to increase the sample throughput, making FCM a high-throughput high-content instrument. This includes automated sample loading from microtiterplates, high-throughput machines [90, 91], cellular barcoding [92] and robotics.

However, regarding high content analysis microscopic technologies are superior to FCM. With the appropriate technology we are theoretically enabled to quantitate virtually all compounds of individual cells including their intracellular localization [47, 72, 74, 93]. These instrumentations enable for screening many drug targets and actions at the same time in the identical specimen. Their major limitation hitherto is its limited throughput which may be 5-10% of that of a FCM.

As an example - a photonic microscopic robot technology is capable of tagging and imaging hundreds, and possibly thousands, of different molecular components, e.g. proteins, of morphologically intact fixed cells and tissue. Data, assembled in a toponome (all-protein network on a single cell level) dictionary of the cell, led to the development of a new concept for target and drug lead discovery [94]. The currently available quantitative high-content microscopic techniques have been comprehensively reviewed by others [49, 95] and show that different instrumentations are nowadays on the market that are more dedicated either to life cell analysis or for high-content screening.

The simultaneous determination of many colors can be realized by means of state-of-the-art technologies like spectral imaging [96]. Multispectral imaging, which spectrally characterizes and computationally eliminates autofluorescence and discriminates dyes by spectral deconvolution, enhances the signal-to-background dramatically; revealing otherwise obscured targets [97]. Cytomics requires both, accurate architectural segmentation as well as multiplexed molecular imaging to associate molecular phenotypes with relevant cellular and tissue compartments [98]. Multispectral imaging can assist in both these tasks, and conveys new utility to brightfield-based microscopy approaches. Multiple molecular details may be obtained by polychromatic analysis of different cells and their function can be simultaneously recorded [99].

Slide-Based Cytometry

New developments in image-based and slide-based cytometry (SBC) enable to study cellular processes in great detail, so that a multitude of structural and functional information can be extracted from cells [8, 100]. It can combine both, single-cell genomics and proteomics, with structural analysis [93, 95].

With the increased number of measurable and quantifiable characteristics of a cell, in addition with its morphological evaluation, multiplexed cell analysis by SBC is a powerful analytical and diagnostic tool. Its most important impact can be expected in drug discovery in the pharmaceutical research [1, 101]. So far up to 8 colors could be simultaneously measured by LSC [46]. However, hyperchromatic cytometry by SBC sets new frontier for multiparameter analysis practically without limits [47]. This approach comprises a set of consecutive steps including: polychromatic cytometry, iterative restaining, differential photobleaching, photoactivation, photodestruction. One of these approaches, namely iterative restaining has been adapted and is being used now by several groups for the high-content analysis of individual cells or subcellular locations of proteins [47, 72, 74, 93].

A specific feature of SBC, not covered by FCM, is the quantitative analysis of tissue sections. Steiner *et al*. demonstrated that quantitative immunophenotyping of leukocytes in tissue sections is possible by confocal microscopy [102]. Gerstner *et al*. [103] showed quantitative three- and four-color immunophenotyping of tonsil sections, and Mosch *et al*. [104, 105] established quantitative two- and three-dimensional analysis of the distribution of nuclei and neurons in brain tissue sections by LSC. Recently, approaches have been proposed towards the 3D-cytometry of tissue sections and in tissue cultures termed Tissomics. It assures that cells are in their natural environment while looking at effects of drugs [78, 106-110]. Microscopy-based multicolor tissue cytometry (MMTC) represents one of the first commercial tissomics instruments to quantify tissues at the single-cell level with automated processing of multicolor-labeled tissue samples [107].

Recently, automated whole slide imager that is capable of fully automated simultaneous fluorescent sample detection, autofocusing, multichannel digitization, and signal correction [81, 111] became available. It allows automated object recognition and scanning of the whole tissue sample thereby substantially speeding-up the process of image acquisition and analysis. But even so, image cytometry is with 100-1,000 cells per second relatively slow regarding cell throughput as compared to FCM with >10,000 cells/second, meaning that speeding up the tests is a demand.

The Challenge of Fully Automated Image and Data Analysis

Traditional visual and quantitative evaluations of gated 2D or 3D cytometric histograms like in FCM collect only a very limited amount of the available information, and one is never certain whether the really relevant information has been extracted. Experience has also shown that it is not easy to provide quality controlled consensus strategies for multi-parameter data evaluation [30, 112, 113]. There is also little pre-existing interpretative knowledge on very complex multiparameter data spaces. Essential information may therefore be lost, simply due to lack of awareness. As a consequence, more sophisticated unbiased multidimensional data mining techniques, rather than pattern recognition by an expert and reduction of dimensionality approaches, is required [114, 115].

The increasing number of cellular parameters being analyzed in imaging as well as screening from chemical compound libraries requires automation. The acquired images can be subdued to automated image recognition. To this end new approaches were proposed to facilitate this process. They focus mainly on characterization of cellular and subcellular shapes, and subcellular protein location by new software developments [116-122]. Each of these approaches applies automated learning resulting in software capable of machine vision [123, 124]. This is aided by systematic collection of information to cluster and catalogue cells according to their molecular, organelle, and morphometric phenotype [125]. Consequently, analysis of multi-parametric measurements needs to be supported by sophisticated feature recognition which is part and parcel of image analysis [126, 127] (see chapter: parameter selection dilemma). Application of the above mentioned automated image recognition tools will lead also to the determination of targets for novel drugs.

To realize the full potential of these cytomic data for elucidating cellular mechanisms of drug activity, software-modeling tools such as the “Virtual Cell” are destined to become as indispensable to a cell biology laboratory as the microscope. All these developments in turn enable for rapid drug screening and multiparametric cell analysis with unbiased drug selection and are the basis for the systemic approach of the analysis of biological specimens to enhance the outcome in clinical diagnosis and in drug discovery programs [127, 128].

CONCLUSIONS

It is essential to work towards better quantitative understanding of the dynamics of cellular processes in multiple cell types to improve drug discovery and development. Cell based disease models taking into account the spatial and temporal molecular diversity of the human cytome seem capable to improve the predictive power of drug discovery and especially to bring down the high attrition rates in drug development.

Future instruments and/or work flow will enable the analysis of cellular contexts at the single-cell level for the whole organism over high-throughput single-cell analysis in tissues (tissomics, toponomics). This analysis can be combined with single-cell high-content metabolic investigation for lipidomics [129, 130] and metabolomics, intra-cellular location proteomics [94] down to single-cell proteomics [72] and genomics, leading to the merge of genomics, proteomics, cytomics and systems analysis [43, 131]. This will lead to faster and more precise drug discovery with lower frequency of false positive but, in our view more importantly, less false negative new drugs tested.

ABBREVIATIONS

FAP = Fluorogen activating proteins

FCM = Flow cytometry

FRET = Fluorescence Resonance Energy Transfer

iPS = induced pluripotent stem cells

LSC = Laser scanning cytometry

SBC = Slide-based cytometry

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