Single-Cell Analysis in Biotechnology, Systems Biology, and Biocatalysis

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Abstract

Single-cell analysis (SCA) has been increasingly recognized as the key technology for the elucidation of cellular functions, which are not accessible from bulk measurements on the population level. Thus far, SCA has been achieved by miniaturization of established engineering concepts to match the dimensions of a single cell. However, SCA requires procedures beyond the classical approach of upstream processing, fermentation, and downstream processing because the biological system itself defines the technical demands. This review characterizes currently available microfluidics and microreactors for invasive (i.e., chemical) and noninvasive (i.e., biological) SCA. We describe the recent SCA omics approaches as tools for systems biology and discuss the role of SCA in genomics, transcriptomics, proteomics, metabolomics, and fluxomics. Furthermore, we discuss applications of SCA for biocatalysis and metabolic engineering as well as its potential for bioprocess optimization. Finally, we define present and future challenges for SCA and propose strategies to overcome current limitations.

INTRODUCTION

Omics: integrative concept that comprises the biological disciplines of genomics, transcriptomics, proteomics, and metabolomics/fluxomics

Biological noise: variations in specific biological output owing to intrinsic or extrinsic factors

Heterogeneity: physiological cell-to-cell differences in a clonal microbial population emerging from stochastic processes

Microenvironment: chemical, physical, and mechanical conditions in the microscale environment of a single cell

Biotechnology: any technological application that uses biological systems, living organisms, or derivatives thereof to make or modify products or processes for specific use

Systems biology: interdisciplinary and integrated study of complex interactions on all omics levels

Evolution generated complex cellular functions that are based on a limited number of molecules responsible for the self-sustaining capabilities of cells. The mechanistic elucidation of these biological functions has rapidly accelerated with the integration of omics research (1). However, complete revelation of mechanistic data is often not possible on the population level.

Mechanistic data are often masked in average values measured from heterogeneous populations. Although deterministic models for the prediction of average population network behavior have been successfully established, these models fail to describe cellular processes in single cells because of individual cell diversity, which is referred to as biological noise (2). An understanding of cellular mechanisms and regulatory circuits on the single-cell level is often necessary to unveil and specify the origin of phenotypic diversity within isogenic cell populations. Phenotypic heterogeneity originating from cell cycle, cell lineage, and genetic or epigenetic imprinting impedes lumping of cellular behavior (3). However, not only strictly causal and deterministic effects are involved. Gene expression and protein translation in single cells clearly underlie a significant variability between individual cells within an isogenic population and influence further cellular functions on other omics levels (4). The high complexity of such systems leads to inherent functional changes, which are the result of fluctuations in transcription or translation activity (2, 5–8). Consequently, these origins of interpopulation diversity generate a sustained library of subpopulations. It has been argued that the occurrence of subpopulations is an important measure to maintain biological evolution and that such subpopulations ensure the continued existence of the whole population by allowing it to cope better with rapid perturbations (7).

Moreover, individual cells in a population cannot be cultivated equally owing to uncontrolled microenvironments with complex cell-cell and cell-surface interactions, even in precisely controlled bench-scale chemostat cultivations. Environmental conditional shifts in bioreactors are reduced with enhanced mixing but cannot be controlled because of unknown complex equilibria in individual microenvironments. Decoupling of single cells from populations allows the experimenter to define those microenvironments and grants access to the isolated, unmasked cellular response. However, isolation and systematic perturbation of single cells in defined microenvironments is challenging, and the degree of difficulty depends strongly on the nature of the cells. The rapidly accelerating development of sophisticated lab-on-a-chip (LOC) devices allows unprecedented analytical resolution by maintaining an analytical space that is proportional to the dimension of a single cell (9).

With these novel devices, single-cell analysis (SCA) has already become a pioneering technology in omics and an integral part of biotechnology (10). SCA is prevailingly used in medical (red) biotechnology, where it has become a promising tool in important areas such as diagnostics, prognostics, and cancer therapy (11). Additionally, many potential research applications of SCA can be found in industrial (white) biotechnology. The high potential of SCA as an optimization tool for industrial biotechnology is often explained by the dependence of bioprocess productivity on heterogeneity (12); SCA also has potential as a tool for target identification in metabolic engineering and synthetic biology (13). SCA is applied in plant (green) biotechnology rather infrequently. Even well-established SCA techniques such as flow cytometry are rarely used and remain nearly unknown in plant research (14). However, excellent studies have impressively demonstrated that SCA techniques have a highly overlapping relevance for all areas of biotechnology. Driven by this observation, in this review we first summarize the currently available microfluidic devices and microreactors for SCA. Second, we highlight examples of its applications in omics and discuss the role of SCA as tool for systems biology. Finally, we explain why SCA has a high potential and is becoming a key technology for metabolic engineering.

MICROFLUIDICS AND MICROREACTORS FOR SINGLE-CELL ANALYSIS

Since the early twentieth century, several microfluidic methods for the analysis of single cells have been developed (15). Today, SCA methods can be divided into invasive SCA (chemical SCA), used to quantify the intracellular compounds of a single cell, and noninvasive SCA (biological SCA), used to quantify the responses of an intact cell. Noninvasive SCA can be further separated into non–time resolved SCA and time-resolved SCA. Time-resolved SCA can be further divided into contact and contactless single-cell trapping approaches. Microfluidic approaches for SCA are summarized in **Figure 1**.

Chemical SCA: lysis of single cells for identification and quantification of the released cell inventory

Biological SCA: a combination of simultaneous cell cultivation and spatiotemporal analysis of biological processes with the possibility of subsequent cell retrieval and subcultivation

Non–Time Resolved Single-Cell Analysis

Invasive single-cell analysis. The combined lysis and extraction of the intracellular components of a single cell and separation of the compounds by downstream processing using capillaries and LOC devices [also known as chemical flow cytometry (16)] has the major advantage that intracellular data from the genomic, transcriptomic, proteomic, and metabolic levels become accessible for analysis (17). However, the complex single-cell sample preparation required limits the throughput of analyzed cells, and thus the method cannot compete with the high-throughput analysis of noninvasive flow cytometry in terms of evaluating the heterogeneity of a population.

The method of cell destruction is important for invasive SCA because it can have a significant and often undefined impact on single-cell sample processing as well as on the structure of analyte targets. Because cell lysis depends strongly on the nature of the cell, cell type–optimized lysis approaches are required. To allow analysis of dynamic cellular processes, cell lysis must be faster than cellular process kinetics and must be able to integrate into capillary or LOC devices. In these dimensions, single-cell lysis can be performed by acoustic (18), chemical (19), thermal (20), mechanical (21), electrical (22), and optical (23) approaches.

Chemical lysis using standardized lysis buffers is most common but may influence downstream analysis, such as the polymerase chain reaction (PCR) for DNA amplification. It provides lysis times of seconds, which are slow compared with optical lysis, which happens within microseconds. Lysis buffers are broadly used because they require a minimal degree of technical complexity and specificity to cell types and analytical targets. Thermal lysis is often used for PCR approaches but is difficult to apply to downstream protein analysis owing to the risk of denaturation. Electrical, optical, and acoustic methods have high technical demands and are expensive, but electrical and especially optical methods allow high-speed lysis of single cells, which often makes them the method of choice. Chemical, thermal, and mechanical lysis methods are well suited for highly parallelized on-chip lysis of single cells. With mechanical lysis approaches, the cellular physiology remains unaffected up to the point of lysis, and they allow a fast lysis with minimal technical demands (21), but lysis is often incomplete because of vesicle formation (24). This and other general assets and challenges of the discussed approaches are summarized in **Figure 1*a***. The ultimate goal of invasive SCA is the high-throughput recording of the physiological status of single cells on all omics levels at a specific point in time. In our opinion, the attainment of this goal will require a combination of different lysis methods in a single LOC device.

Noninvasive and non–time resolved single-cell analysis. Since the development of the Coulter counter by Wallace H. Coulter in 1947, technologies for counting, examining, and sorting cells suspended in a stream of fluid have become standard methods to investigate the diversity of individual cells in populations. Noninvasive analysis of single cells moving in streams allows analysis of millions of cells per minute and enables quantification of the cells' physiological status

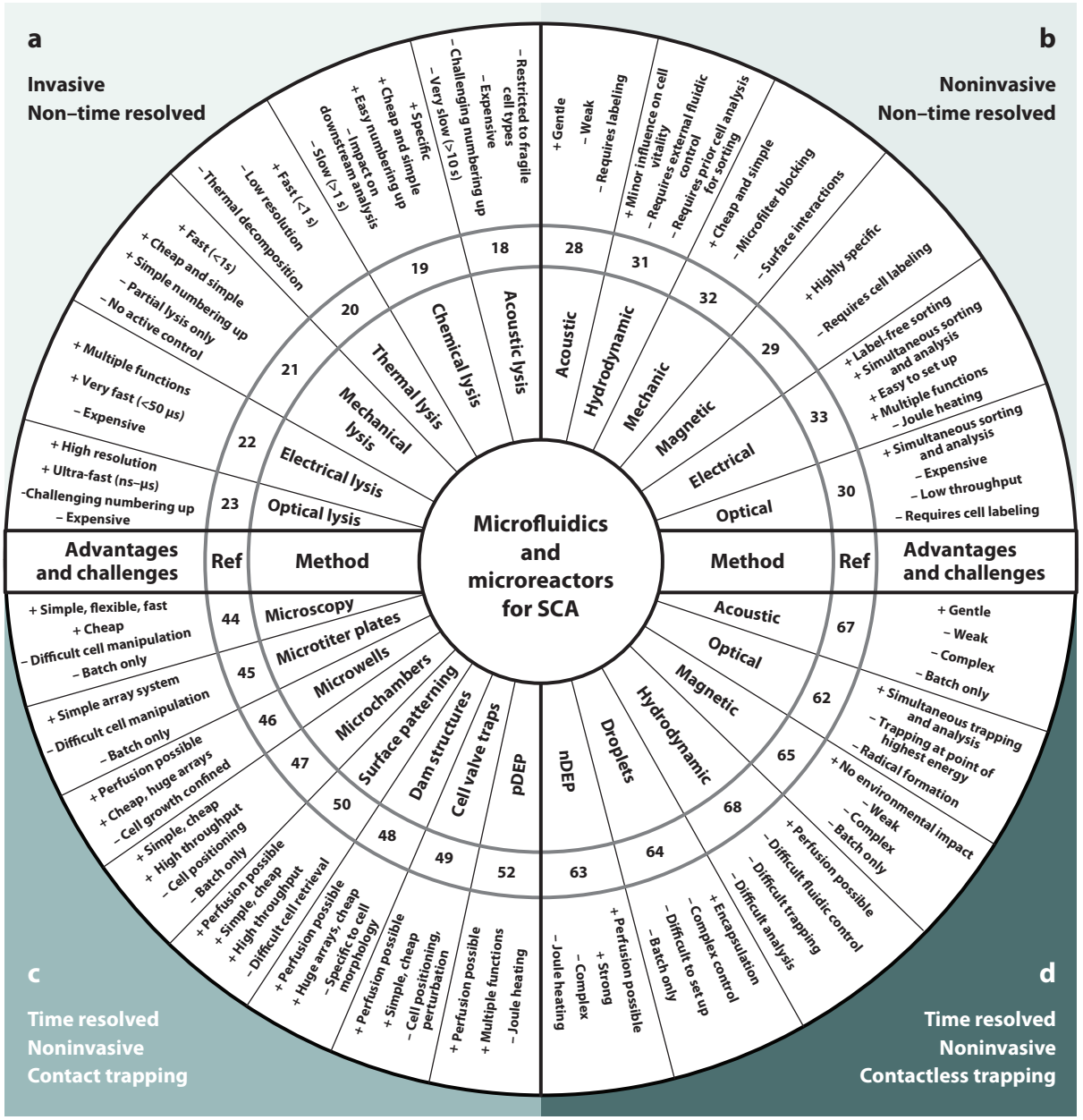


Figure 1

Overview of microfluidics and microreactors for single-cell analysis (SCA). (*a*) Lysis approaches for chemical SCA. (*b*) Non–time resolved, noninvasive micro fluorescence-activated cell sorting approaches for SCA. (*c*) Contact-based SCA approaches. (*d*) Contactless SCA approaches. Reference numbers (Ref) are given in the figure. Abbreviations: nDEP, negative dielectrophoresis; pDEP, positive dielectrophoresis.

in their natural population environment. The preferred method to quantify cell-to-cell differences in a population is flow cytometry, which utilizes standard hydrodynamic focusing of single cells in a continuous flow and analysis in a beam of light. Flow cytometry using fluorescent dyes has been used to perform kinetic investigations for more than 30 years (25). One of the biggest advantages of flow cytometry for the analysis of single cells is that it allows the simultaneous measurement of multiple fluorescent signals as well as light scatter–induced illumination of single cells or microscopic particles in suspension (26). It can be combined with sorting methods to isolate cells with specific phenotype characteristics.

The most frequently used techniques for cell sorting are fluorescence-activated cell sorting (FACS) methods that often involve complex systems. With this approach single cells can be encapsulated in small liquid droplets to facilitate high-throughput sorting, but this technology also allows cell sorting in air or immiscible liquids. If the cells are to be cultured afterward, approaches using immiscible liquids are disadvantageous because single cells must be removed from the emulsion (27). Today, sorting approaches are integrated on LOC devices to create so-called μFACS. Newly developed micro cell sorting methods employ acoustic (28), magnetic (29), optical (30), hydrodynamic (31), mechanical (32), and electrical (33) approaches.

Acoustic approaches can be applied, for example, by surface acoustic wave actuated cell sorting and allow a gentle deflection of cells. Compared with electrical approaches, acoustic methods allow only a weak deflection of cells and become challenging with smaller cells such as bacteria, which demand application of cell labeling methods (34). Application of magnetic approaches allows specific and rapid cell sorting after labeling of cells with antibody-conjugated magnetic micro- or nanoparticles (29). A drawback of this approach is the possible influence of the particles on cellular physiology. Optical μFACS approaches apply light to deflect and analyze cells for sorting. This technique requires labeling, which may influence cellular physiology, and is still limited to a throughput of a few cells per second (35). One novel hydrodynamic approach for cell sorting is the use of on-chip fluidic gating. Chen et al. (31) demonstrated that this method affected the viability of sorted HeLa cells only slightly. A challenge for hydrodynamic sorting is that it requires precise microfluidic control together with the application of multiple streams.

Mechanical approaches are based mainly on microfabricated filter structures to separate cells according to their morphology. These approaches have the advantage that they do not require staining or labeling with micro- or nanobeads that might have an impact on cellular physiology. As recently demonstrated, cell-size sorting with microfilter structures can be used to obtain cells at the same cell cycle stage (36). The challenges of microfilter structures are that they can be blocked and that intensive surface interactions during the filtration process can cause significant shear forces on sorted cells according to the throughput applied.

An effective and noninvasive selection of cells based on the cell cycle also can be achieved using negative dielectrophoresis (nDEP). The deflection force of nDEP is dependent on cell size, applied frequency, and voltage, which can be used to allow cells with a specific size or dielectric properties pass whereas further grown, larger, or still dividing cells are deflected (37). This and other electrical methods such as positive dielectrophoresis (pDEP) (33, 38), electrostatic manipulation (39), electrowetting (40), and alternating current electroosmotic flows (40) allow shear stress–free, label-free, and strong deflection as well as fast response times. Interestingly, cell sorting with DEP allows automated separation and selection for several phenotype characteristics, such as differences in morphology, electrical permittivity, or dielectric properties, which can be used as sorting criteria without the need for prior analysis (41). Using advanced fabrication technologies, effective cell sorting has been achieved even with low voltages and negligible Joule heating effects (33). However, because of Joule heating effects, applied voltages are limited for noninvasive cell sorting (42).

Micro total analysis systems (μTAS): devices that integrate multiple reaction steps on a miniaturized chip to perform complex chemical analysis or sample processing

General assets and drawbacks of current methods for cell sorting are summarized in **Figure 1*b***. Depending on the downstream analysis used, the sorting methods must fulfill specific requirements. If recovery of a population by cell deposition and subsequent cultivation is desired, the method of choice should have as little effect on the natural state of the cell as possible. Additionally, it should allow high throughput of cells and highly specific sorting as well as provide simple integration into LOC devices with a minimum of technical effort and flexible applicability for different cell types. Hybrid systems combine most of the mentioned advantages. The combination of easy setup and flexible electric sorting with highly specific magnetic sorting in particular has great potential to become a part of standardized and flexible micro total analysis systems (μTAS). Kim & Soh (43) took the first step in this direction in 2009 when they constructed a dielectrophoretic-magnetic activated cell sorter that enabled highly efficient multitarget separation of different bacterial cell phenotypes.

Time-Resolved Single-Cell Analysis

In addition to the rapid development of μFACS and invasive SCA devices, there is a focus on the development of LOC techniques to enable time-resolved SCA. These approaches are not restricted to a snapshot analysis of a single physiological status but rather allow analysis of the temporal development of living single cells (9). Most time-resolved SCA approaches are performed with classical microscopy or fluorescence detection microscopy on standard object slides. Over decades these approaches have been optimized, for example, by application of micromanipulators (44). The previously mentioned sorting technologies were used in part to drop single cells in microtiter plate wells (45). Here we focus on the technologies developed to construct micro on-chip bioreactors for single-cell trapping in a defined position using microscale geometries comparable with the dimensions of the targeted cells. Generally, LOC devices for time-resolved SCA can be divided into contact and contactless trapping approaches (**Figure 1*c*,*d***).

Devices for time-resolved single-cell analysis by contact trapping. Contact-based LOC approaches involve application of microwells (46), microchambers (47), dam structures (48), cell valve traps, pDEP (49), or simple single-cell adhesion as well as defined trapping with functionalized micropatterning on surfaces in batch or flow chambers (50, 51). Contact-based single-cell trapping approaches are easy to apply and cheap, and they allow high-throughput studies. To minimize induction of unknown phenotypes, surfaces must be selected carefully. Today, defined surfaces functionalized by micropatterning enable high-throughput array-based single-cell cultivations and are particularly desirable for long-term cultivation of single cells (52). Several physical or chemical surface modifications enable defined cell affine micropatterns (53). For example, Hardelauf et al. (54) cultivated thousands of aligned cells using thin-film polydimethylsiloxane (PDMS) prints. However, it still remains challenging to apply this approach to microbial cultivations, particularly those of bacteria. This is because some bacteria are capable of rapidly forming biofilms, which can quickly cover micropattern structures.

Besides micropatterning, microwell approaches also allow high-throughput time-resolved SCA. Love et al. (47) have impressively demonstrated the feasibility of single-cell batch-like cultivations in approximately 250,000 microwells that were sealed in microchambers for time-resolved analysis of secreted proteins from *Pichia pastoris*. Although microchambers allow single-cell batch-like cultivation, high-throughput perfusion systems can be achieved by inserting dam structures into microfluidic streams. A challenge for these approaches is that the trapping efficiency depends strongly on dam structure geometry and cellular morphology, which often undergo continuous changes during growth. The trapping geometry must be optimized for every cell type, which

makes these approaches inflexible and could lead to false conclusions for a certain population in the case of trapping only of cells with a specific shape. However, to our knowledge, dam structures in LOC devices are currently the only method to trap single motile (55) or nonmotile bacteria (48).

The mentioned challenges for dam structure approaches have been overcome using cell valve traps, which enable fixing of single cells at the microchannel wall opening by vacuum or hydrodynamic effects (56). These approaches allow highly controlled trapping (57) and removal of single cells (58). Depending on the size of the opening, this principle should work for a huge range of cell types and morphologies. Furthermore, microelectrodes allow the application of pDEP for precise contact positioning of single cells. These approaches have the advantage that the cells of interest can be selected by the experimenter or automatically (49). After immobilization via cell adhesion, the electrodes can be used either for gene transfer studies by electroporation (59) or as patch clamps for impedance analysis (60).

Devices for time-resolved single-cell analysis by contactless trapping. Although eukaryotic cell types often require hydrophilic surface interactions to survive, microorganisms and blood cells remain vital in suspension. Contactless trapping approaches can prevent unknown phenotypes induced by surface contacts. Sensitive cells such as platelets rapidly change their morphology, whereas prokaryotes are known to rapidly form their own undefined microenvironment via biofilm production when they come into contact with surfaces (61). Today contactless approaches for single-cell manipulation use optical tweezers (62), nDEP (63), and droplets (64) as well as magnetic (65), acoustic (66, 67), and hydrodynamic traps (68). Contactless single-cell trapping may be suitable to overcome the undefined impact of surfaces, but it generally has higher technical demands. Additionally, single-cell holding forces can have a significant impact on physiology.

Optical tweezers are often used for contactless single-cell manipulation and for defined positioning on surfaces. A great advantage of this technology is the simultaneous manipulation and analysis of single cells in any microchannel structure, as long as the deployed materials allow the necessary transmittance of light. The technology was used successfully for cell fusion experiments (69), but it has not been used for contactless long-term single-cell cultivation because the trapping of single cells takes place at the position of highest energy input, where light beam exposure often causes photo- and thermal damage. Another challenge of optical traps is control of the excitation of molecular oxygen into reactive oxygen species that cause oxidative damage. This problem does not appear when working under anaerobic conditions (70).

Acoustic traps should allow a gentler trapping of single cells, but to our knowledge this has been demonstrated only for cell clusters (67). However, the applied forces are too weak (0.1–1,000 pN) to hold micrometer-scale single cells in a stream of medium that provides perfusion cultivation. Ultrasonic transducers integrated into the chip are relatively large compared with the microelectrode arrays of nDEP devices, which might cause problems for numbering up of cell trapping on chip and require a complex setup to enable acoustic single-cell cultivation.

Recently, magnetic manipulation was used to trap and cultivate single labeled yeast cells on magnetic domain walls (71). Upon labeling the cells with magnetic beads, the technology allowed a highly selective and relatively strong contactless single-cell manipulation (10–10,000 pN) compared with optical (0.1–1,000 pN) or acoustic (0.1–100 pN) cell manipulation (72). Careful selection of beads allows manipulation without impact on the cellular viability, and antibody-labeled beads can be used for trapping of single cells according to their phenotype (71). The contactless magnetic manipulation requires an additional preparation step to label cells with micro- or nanobeads, which can be a drawback because it can have an impact on cell physiology.

The strongest force fields and most stable single-cell trapping are achieved by nDEP cell manipulation (20–100,000 pN) (72). This technology allows simple setups in comparison with acoustic,

magnetic and optical methods, as well as label-free and frequency-dependent manipulation. A simple ac generator can be used to control single-cell manipulation between multiarray electrode structures. In addition to sorting and single-cell cultivation, cell-trapping microelectrodes can be used for electroporation, cell fusion, and amperometric sensing. Joule heating effects can be controlled to enable defined microenvironments (63). The impact of force fields or surfaces can be excluded when using hydrodynamic traps, which trap single cells at the stagnation point of two liquid flows in a T junction (73) or crossing of microchannels (68). Active control of sorting and trapping can be arranged by microvalves within PDMS chips (68). However, precise control of two independent streams for stable cell trapping remains challenging.

In contrast to acoustic, optical, magnetic, nDEP, and hydrodynamic traps, microdroplets allow contactless, high-throughput, and batch-like single-cell cultivations (74). The processing of microdroplets with encapsulated single cells allows the analysis of accumulating undiluted secretions. Depending on droplet size and nutrient concentrations, the availability of nutrients may be limited, which is why microdroplets do not allow single-cell cultivation in constant microenvironmental conditions (75). In **Figure 1*c*,*d***, we summarize the aforementioned advantages and challenges of microfluidics and microreactors for time-resolved SCA.

Several excellent technical studies demonstrate the capability of microfluidics and microreactors for a wide range of applications from the lysis of a single cell to time-resolved analysis of hundreds or thousands of cells. In the following section we summarize how scientists are using several parts of this toolbox to answer many different types of questions within systems biology and omics research.

SINGLE-CELL ANALYSIS IN SYSTEMS BIOLOGY AND OMICS RESEARCH

The research field called systems biology is an integrative approach to unite two or more of the omics disciplines: genomics, transcriptomics, proteomics, and metabolomics/fluxomics. The ultimate goal is to obtain a complete and dynamic picture of intracellular processes and regulatory circuits with spatiotemporal resolution. To achieve this, all genes (genomics), transcripts (transcriptomics), proteins (proteomics), intracellular metabolites (metabolomics), or intracellular metabolic conversion rates (fluxomics) are identified, quantified, and characterized (10). The combination of data from different omics fields usually involves mathematical description and modeling and results in a dynamic map of all cellular functions ranging from the genome to complete, living organisms. This holistic approach is often referred to as the biology of the twenty-first century. SCA will make a decisive contribution to this rapidly developing research field by delivering functional biological data beyond the statistical average of a microbial population and by providing an undistorted view of the mechanisms of life on the simplest possible level, the cell (**Figure 2**).

Single-Cell Analysis in Genomics

The genome of a microorganism represents the blueprint for every cellular function and determines its metabolic and regulatory extent. Therefore, genomic research forms the basis of any systematic approach for the understanding of cellular functions and the rational improvement of technologically employed strains.

This principle also applies to SCA. Recent developments in genome amplification technology render genome-based identification on the level of an individual cell possible (76, 77). Nowadays, this technology is predominantly used for the exploration of the genomic diversity and variability

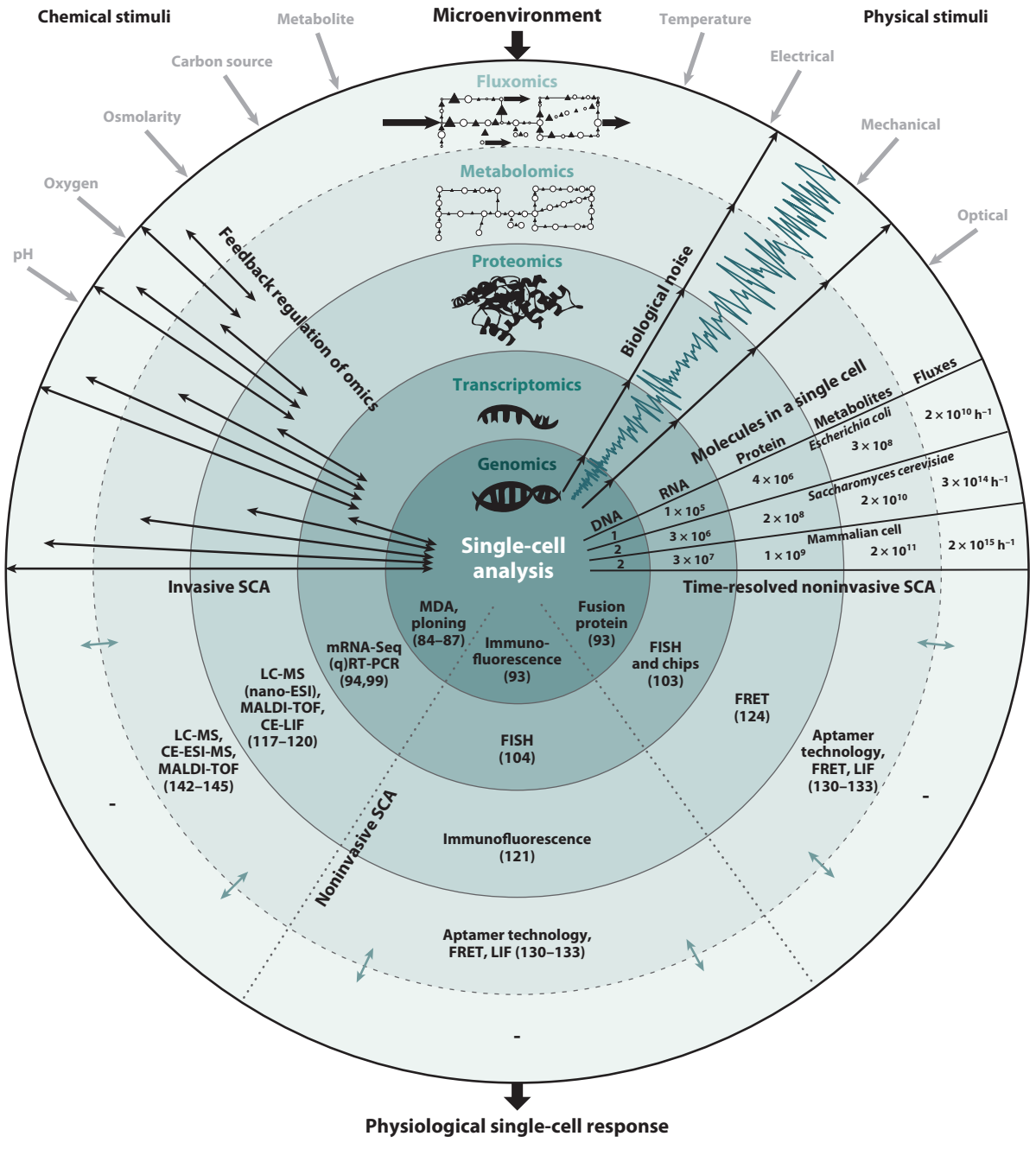
in ecosystems and is crucial for a broad variety of applications from microbial screening to climate simulation and medical diagnostics (78). A vanishingly small fraction of the total realm of microorganisms in the biosphere has been identified and classified, and an even smaller fraction of these microbes has been successfully cultured and sequenced (79). Hence, the major interest in the identification of individual organisms has led to the development of culture-independent sequencing technologies with single-cell accuracy and sensitivity. Moreover, this technology is also heading toward the identification of genetic variations in individual cells within microbial clonal populations (80).

Numerous whole-genome amplification (WGA) strategies have been investigated and developed so far. Besides single-cell WGA methods based on classical PCR, techniques such as ligation-mediated PCR (81) and degenerate oligonucleotide-primed PCR (82, 83) have recently revolutionized the field of high–molecular weight DNA amplification. This is especially true for the multiple displacement amplification (MDA) approach, which focuses on the strongly strand-displacing DNA polymerase from bacteriophage Φ29 (84–86). In contrast to PCR-based methods, MDA enables the amplification of all template material contained in a sample without a priori sequence knowledge (78). Additionally, MDA exhibits a more uniform locus representation in comparison with other high-fidelity amplification technologies. Furthermore, it features single nucleotide polymorphism (SNP) coverage, high amplification rates, and a matchless sensitivity, which predestines this technology for demanding applications such as SCA (76). Zhang and coworkers (87) achieved a breakthrough in the field of single-cell whole genome sequencing in 2006 by improving MDA technology in terms of sequence coverage and sensitivity. Real-time isothermal amplification was used to form polymerase clones, known as plones, from single cells, which were then analyzed by Affymetrix chip hybridization. Sequence coverage of more than 90% after 15 cycles of repeated shotgun sequencing on plonal DNA was achieved, and the typical MDA amplification bias could be eliminated to a large extent.

In 2006 Ottesen et al. (88) demonstrated the first successful identification via gene mapping of individual members of a small but complex ecosystem. With a microfluidic LOC digital PCR system, random genes from organisms in the microbial termite gut community were amplified and assigned to organisms. Approximately one dozen previously unknown bacterial species involved in the acetogenic reaction network in the termite gut were found. This example impressively shows that identification of organisms can be coupled directly to gene function analysis and marks a milestone toward directed environmental screening for a desired catalytic activity.

The high sensitivity of these next-generation sequencing technologies comes with the vulnerability to contamination with foreign DNA strands. Even a single foreign strand can lead to a substantial number of contamination amplicons (89). Because DNA strands can be found everywhere in laboratory reagents and surfaces, efforts have been made to prepare ultrapure reagents and to modify and miniaturize amplification approaches (77). For example, Blainey & Quake (90) evolved MDA into dMDA (digital MDA) for direct absolute quantification of DNA templates and identified contaminant high–molecular weight DNA as the sole source of MDA background. On the basis of these crucial findings, high-quality whole-genome sequencing with single cells is possible, although it requires the highest reagent purity and absolute equipment cleanliness. The distinct trend toward integrated microfluidic platforms for simultaneous cell sorting, cell handling, sample preparation, and DNA amplification clearly reflects these requirements for handling and equipment. The use of nanoliter reactors for MDA amplification has drastically reduced nonspecific synthesis (91), and microfluidic devices with combined separation and amplification have enabled parallelized whole-genome haplotyping of single human metaphase cells of four individuals with 99.8% accuracy and a SNP coverage of 96% (92). However, none of the mentioned technologies allows the investigation of dynamic biological processes within living single cells

because cell lysis prior to analysis is inevitable. Noninvasive technologies have been successfully established for single-cell genomics as well. For example, Babic et al. (93) directly visualized the horizontal gene transfer in *Escherichia coli* by means of fluorescent protein fusion. Even though the vast majority of published applications in single-cell genomics predominantly focus on medical applications for the analysis of mammalian cells, with the fast maturation of microfluidic



WGA technologies, whole genome–based identification and characterization of single cells with biotechnological relevance is around the corner.

Single-Cell Analysis in Transcriptomics

In general, transcriptome analysis delivers information about the quality and quantity of gene expression. In contrast to genome analysis, transcriptome analysis reveals the genetic information that cells actually use at a certain point in time and under certain conditions. Hence, analysis of the expression of the corresponding genes allows linking of the genotype to the observed phenotype. In addition to this major capability of transcriptome analysis, the short turnover times of transcript molecules allow dynamic resolution of the cellular response to chemical and physical perturbations. In SCA, transcriptomics is one of the most prominent and powerful analytical techniques applied, not least because of the potential to amplify the target transcripts to provide sufficient analyte amounts for unambiguous identification of the target transcript sequence. Well-established high-sensitivity PCR technologies such as reverse transcription (RT)-PCR require just a few template molecules for successful amplification of the target mRNA species. Simultaneous amplification of several hundred different mRNA species, each with an initial copy number of approximately 25, using RT-PCR has already been demonstrated (94).

At the single-cell level, all currently applicable techniques for transcriptome analysis are based on the amplification and analysis of complementary DNA (cDNA) (95, 96). Whole-transcriptome analysis was performed with extracted cytoplasm from single neuron cells followed by RNA amplification with subsequent sequencing and chipping of cDNA with Affymetrix microarrays. Those experiments showed a significant variability in the gene expression profiles of individual neuron cells as a response to fever (97).

With next-generation techniques such as mRNA sequencing (mRNA-Seq), it is possible to detect 75% more transcript species from a single mouse blastomere when compared with conventional microarray-based techniques (98, 99). The unprecedented depth and accuracy of these next-generation sequencing technologies allowed identification and quantitative estimation of thousands of new transcripts and variants expressed in mammalian cells (100). Further evolution of single-molecule sequencing is likely to facilitate direct sequencing of complete mRNA molecules from a single cell without error-prone and elaborate transcript processing (101). However, current single-molecule sequencing technologies are able to detect only 15–25% of the transcripts and still require hundreds of cells (102). Higher accuracy is likely to be achieved by new technologies that allow the repeated sequencing of a single transcript strand without damaging it.

Possibilities for application of these advanced transcriptome analysis techniques are manifold. Despite their obvious power for single-cell transcriptome analysis, all mentioned assay

Figure 2

The hierarchy of single-cell omics. Cellular physiology is determined by the microenvironment comprising all external stimuli acting on the cell, such as chemical and physical factors. The physiological response to external stimuli consists of induced changes on the respective omics levels and their interaction via feedback regulation. In addition to changes induced by external stimuli, the cellular inventory is continuously subject to alterations owing to biological noise. External stimuli and noise are generally reflected in altered (bio)molecule turnover rates and activities as well as abundances. Current analytical technologies for chemical and biological analysis of single cells on each omics level are given in the lower part of the diagram. Abbreviations: CE, capillary electrophoresis; ESI, electrospray ionization; FISH, fluorescence in situ hybridization; FRET, fluorescence resonance energy transfer; LC, liquid chromatography; LIF, laser-induced fluorescence; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; MDA, multiple displacement amplification; MS, mass spectrometry; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SCA, single-cell analysis; Seq, sequencing.

technologies share the central aspect of cell consumption upon analysis. To resolve dynamic changes in the cellular phenotype and relate them to the gene expression profile, continuous in vivo monitoring of the target cell is indispensable. Fluorescence in situ hybridization (FISH) is a feasible and noninvasive approach to this problem. Levsky et al. (103) used computational fluorescence microscopy with multiplex DNA probes (known as FISH and chips) to resolve the expression of 11 genes in human colon adenocarcinoma cells simultaneously with high spatial and temporal resolution. Le et al. (104) demonstrated another approach to dynamic transcript analysis based on fluorescence correlation spectroscopy. Transcript quantification was performed with an RNA-binding fluorescent fusion protein probe in single *E. coli* DH5α PRO cells. However, in all optical technologies for single-cell transcriptomics, the number of target genes is limited. Although this restriction can be partly overcome by using multiplexed probes, the sheer amount of genes in the total genome of a cell cannot be covered. This limits optical analysis of a single-cell transcriptome to a few target transcript sequences. Nevertheless, fluorescent probes are well suited for noninvasive analysis and high-throughput flow cytometric transcriptome analysis with combined cell sorting and subsequent analysis or subcultivation of individuals or subpopulations of interest (105).

In conclusion, applied transcriptome analysis focuses mainly on the elucidation of gene regulatory networks at the whole-genome scale. In combination with directed overexpression of process-relevant genes or with the knockout of genes involved in central metabolic pathways, the rational improvement of microbial performance in biocatalytic processes becomes possible (106). Transcription analysis at a single-cell level is thereby of special importance because of the heterogeneity of microbial populations. Gene usage and expression dynamics profiling of subpopulations allows the filling of empty spots on the map of the cellular landscape (3, 98, 107). Clearly, a major goal is the simultaneous analysis of genome and corresponding transcriptome sequences to link the gathered data directly to the cells' environmental conditions, genotype, observed phenotype, and physiology (108). Future developments and unambiguous transcriptomic data for single cells are likely to allow a deeper understanding of genetic and epigenetic mechanisms and render the development of innovative approaches possible, including the modeling, design, and improvement of biotechnological applications and processes. However, the course has been set. Now it is time to apply the well-equipped toolbox of single-cell transcriptomics to central questions of systems biology and to integrate these functional data into the entirety of all omics disciplines.

Single-Cell Analysis in Proteomics

Although genome and transcriptome together contain the required information, the phenotype of an organism itself starts to be comprehensible on the level of the proteome. Virtually all cellular processes are ruled by proteins, from the DNA polymerases that are crucial parts of all replication processes to the catalytic enzymes in metabolic pathways. The proteome comprises the entirety of all cellular proteins and thereby determines the functional capacity of a cell at a particular point in time. In general, proteomics deals with the elucidation of cellular functions directly at the proteome level. A typical proteome consists of approximately 26,000 individual protein species in human cells (109), approximately 6,000 in *Saccharomyces cerevisiae* (110), and 4,000 in *E. coli* (111). This considerable diversity is further increased by posttranslational modifications such as phosphorylation, acetylation, and glycosylation, which substantially change protein folding and influence protein-protein and protein-substrate interaction (112). However, not only protein diversity but also protein concentration is relevant for proteome analysis. Average protein numbers can vary from 100,000 copies per cell down to only 50 copies per cell for low-abundance proteins (9). Therefore, the combination of complexity, broad range of protein abundance, and minute

sample amounts places extremely high demands on analytical approaches to study the proteome of a single cell.

In return, the information content of functional proteomic data is immense. As one can easily imagine, cellular heterogeneity occurs on multiple levels, including translational regulation, post-translational modification, and protein turnover, and it entails dynamic protein expression levels and enzymatic activity. As a result, the proteome directly reflects biological noise and fluctuations in environmental conditions. The mean half-life of an average protein is approximately 43 min in budding *S. cerevisiae* (113). Average protein degradation times in other cell types are assumed to be in the same range. Thus, the proteome composition is relatively conserved in contrast to compositional changes owing to an external stimulus on the metabolome level. However, changes in enzyme activity can occur within a few minutes when regulated by fast modifications such as phosphorylation (114).

One major task of proteome research is the large-scale identification and quantification of proteins from biological samples (115). Mass spectrometry (MS) rapidly took proteomics to the next level and became an indispensable method for the analysis of complex samples (116). With ever-increasing sensitivity, specificity, and resolution, the latest MS devices are now suitable for the analysis of single cells. Rubakhin and colleagues (117, 118) demonstrated the quantitative characterization of peptides from single cells in the low femtomole range and cell-cell signaling peptides with a detection limit of 19 fmol using matrix-assisted laser desorption ionization (MALDI) time of flight (TOF) MS. Another study showed the repeated quantitative detection of more than 6,000 proteins from single pancreatic islets with a nano-liquid chromatography (LC) and nano-electrospray ionization (ESI)-equipped linear trap quadrupole-Orbitrap at attomole sensitivity (119). Despite recent achievements, complete MS-based single-cell proteomics has not been fully realized. The core challenge still lies in the integration of microfluidic devices for on-chip sample processing and sample volume reduction for drastically improved MS sensitivity and resolution. However, MS systems for SCA certainly will be established in the near future and will grant access to the extraordinarily high content of functional biological information.

In addition, capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection achieves excellent separation efficiencies and sensitivities for single-cell protein analysis, but it fails to identify unknown proteins. Nevertheless, CE-based methods are capable of delivering quantitative data with unmatched sensitivity. With CE-LIF and specific fluorescent immunolabeling of proteins, a detection limit in the zeptomole range is possible (120). Indirect quantification of enzymatic activity was also demonstrated with a CE-based method. Instead of LIF, electrochemical monitoring of released NADH was employed for the quantification of glucose-6-phosphate with a detection limit of 1.3 zmol (121). Both MS and CE-based technologies involve the lysis of the target cell, and therefore they enable neither time-resolved nor spatial analysis of the single-cell proteome.

With noninvasive single-cell enzyme assays based on optical analysis, spatial and temporal resolution of enzyme location and activity in subcellular structures is accessible. This allows different states of specific enzymatic activity to be linked to the particular stage and age or microenvironments of a cell. In the mid-1970s, Yashphe & Halvorson (122) investigated the in vivo enzyme kinetics of intracellular β-galactosidase in single cells of *Saccharomyces lactis* during the cell cycle using fluorescein-di-(β-D-galactopyranoside) as substrate. Today, numerous fluorogenic substrates are commercially available (123), and opto-based investigation of intracellular enzyme activity could be further expanded with fluorescence resonance energy transfer (FRET) (124). Until recently, measurements with FRET were limited to the detection of protease and kinase activities, but the methodology is expanding to other enzyme classes and simultaneous activity

determination. Ouyang et al. (125) demonstrated the principle with simultaneous determination of the activity of two different enzymes involved in cancer invasion and metastasis in single cells.

Another time-resolved and noninvasive approach for proteome studies is the detection of secreted protein from single cells. Kortmann et al. (126) demonstrated the quantification of green fluorescent protein (GFP) secreted from single *Schizosaccharomyces pombe* cells trapped with nDEP and cultivated under controlled microenvironmental conditions. Love et al. (47) showed that single *P. pastoris* cells stochastically secrete heterologous protein using a PDMS chip comprising 250,000 microengraved cultivation wells. The secreted protein was detected and quantified with an immunoassay microarray.

When temporal resolution of enzyme activity, quantity, and location is not required for accessing functional biological data, proteome analysis on the single-cell level via flow cytometry is the method of choice. The major limitation of this technique, its lack of time-resolved biological data, is compensated for by its ability to produce a huge amount of data in a high-throughput procedure. The enzymatic activity in flow-based assays is usually correlated to the concentration of a specific antibody-labeled enzyme or a fluorescent conversion/reaction product (26).

However, not only the enzymatic activity and enzyme quantity of a single cell are of interest. The relationship between activity and/or quantity of certain enzymes and fluctuations in transcription and/or translation activity harbors valuable functional information for systems biology on the single-cell level (127, 128). Taniguchi and coworkers (129) recently investigated the interplay of the transcriptome and proteome in single *E. coli* cells at different protein expression levels. They combined next-generation cDNA microarrays and mRNA-Seq for transcript identification and quantification with single-molecule accuracy and fluorescence-based protein quantification. At low expression levels, transcript copy number and corresponding protein amount clearly exhibited a mathematical connection. No correlation could be found for any tested gene at high expression levels.

To conclude, single-cell proteomics has matured with the rapid developments in MS and micro/nanofluidics. As already witnessed in other omics disciplines, the integration of cell cultivation, cell lysis, sample processing, separation, and direct coupling to analytical devices is the key to success in single-cell proteomics. The future of single-cell proteomics is heavily dependent on the development of MS-based technologies. MS offers the highest functional information content because of its capability not only to resolve protein quantity, but also to simultaneously identify thousands of proteins and reveal their posttranslational modifications. However, if biological analysis of a cell is desired, opto-based technologies such as FRET coupled to microfluidic devices will remain the method of choice.

Single-Cell Analysis in Metabolomics and Fluxomics

Although the investigation of genome, transcriptome, and proteome allows conclusions to be drawn about cellular functions, the fastest and most sensitive response of a cell to changing environmental conditions takes place on the metabolic level. This addresses fluxome and metabolome, the basis of cellular physiology. Recent findings imply that cells from isogenic populations simultaneously display diverse metabolic phenotypes because their physiological nature differs between cells. Using classic analytical approaches, cellular physiology can be investigated only on an average level, which is why efforts have been made to identify and measure differences between individual cells originating from the same mass cultivation.

In general, procedures for single-cell metabolic studies can be divided into noninvasive and invasive approaches. Noninvasive approaches preserve the anatomical and functional integrity of the cell and enable in vivo analysis, whereas invasive methods require extraction of analyte

molecules from the cell and imply the destruction of cellular integrity (118). Microscopic analysis of morphological appearance is considered the first attempt to detect the physiology of single cells. Apart from the detection of size, shape, and appearance, the image-based detection of enzyme kinetics is possible with fluorescent substrates (130). Today, molecular sensors capable of metabolite analysis on the single-cell level are available. Aptamer-based technologies (131), FRET sensors (132), and LIF (133) are the most prominent approaches employing molecular sensors. Because of the limitation to a few target analytes, these methods can never be extended to contribute to omics research with the required full range of coverage of the total metabolome on the single-cell level. Nevertheless, these technologies are well suited for resolving the in vivo dynamics of specific functional targets under transient conditions.

The small dimensions of the analytical space in single-cell studies necessitate the miniaturization of the separation procedure for chemical analysis as well. LC using microcolumn separation as well as CE has been applied to the analysis of single cells (134). In most studies CE is the method of choice for separation of intra- and/or extracellular metabolites in miniaturized sample volumes. For example, coupling of CE and fluorescence spectroscopy was successfully used to simultaneously identify and quantify more than 30 compounds in individual neurons from *Aplysia californica* and *Pleurobranchaea californica* (135).

The separation of analyte molecules from single-cell sampling is only part of the challenge because of the limited number of sufficient detection methods. Microelectrodes placed adjacent to single cells allow for the measurement of secreted compounds by voltammetric or amperometric detection. The feasibility of this approach has been shown for the monitoring of catecholamine release from bovine adrenal medullary chromaffin cells (136). Using this approach, the secretion of catecholamine from the trapped cells could be detected within the attomole range. An even more powerful detection method is the combination of single-cell trapping, separation via CE, and detection of analyte molecules using MS. Coupling of CE to ESI Fourier transform ion cyclotron resonance MS (ESI-FTICR-MS) was initially applied to the detection of the α and β chains of hemoglobin from a single human erythrocyte (137). Another study also showed that coupling of CE to tandem MS via ESI allows the quantification of proteins in attomole amounts from crude extracts of human blood (138). But not only proteins and peptides are detectable with this approach. Because of its high sensitivity, CE-ESI-MS has been applied to the measurement of signaling molecules in neuronal cells. One example is the detection of intracellular titers of acetylcholine, histamine, dopamine, and serotonin in cell extracts from R2 neurons and metacerebral cells of *A. californica* (139).

Even though most single-cell studies focus on the investigation of neuronal cells, MS also makes single-cell analytics feasible for chemical analysis of a wide range of cell types, including plant cells, human blood, and even single microbial cells. In situ metabolic profiling has been performed for single isolated cells and small cell populations of onion (*Allium cepa*) and daffodil (*Narcissus pseudonarcissus*) bulb epidermal cells by laser ablation ESI-MS (140). Thirty-five different metabolic species could be assigned, which allowed the determination of a metabolic profile from a single plant cell. An approach that is particularly interesting for biotechnology research is the biochemical profiling of single microbial cells. The combination of MALDI-MS with microscale sample preparation is sufficient for the detection of endogenous metabolites with a sensitivity that allows the chemical analysis of a single yeast cell (141). Using this approach, important key metabolites such as ADP, GDP, ATP, GTP, and acetyl coenzyme A could be measured with a detection limit in the attomole range. The method was tested on diluted cell extracts from *S. cerevisiae* with sample amounts corresponding to less than a single yeast cell.

In addition to the analytical challenges of single-cell metabolome analysis, sample preparation and processing are two crucial points. Obstacles also arise in the adequate preservation of the

original metabolome in the presence of enzymes with fast metabolic turnover rates and in cleaning of the sample to remove interfering cell debris, nucleotides, and proteins. However, several single-cell sampling approaches have already been pursued, for example, direct sampling of cell content with a micropipette and subsequent injection to a mass spectrometer with a nano-ESI source (142). Unfortunately, this approach is applicable only to large cell types. Currently the trend is moving toward miniaturization and integration of the classic metabolomics approach with combined microfluidic quenching, lysis, and separation of the metabolites with MS detection (143, 144). Apart from sample preparation, handling and transfer of minute sample amounts poses a considerable challenge in single-cell metabolome analysis as well.

In summary, microfluidic cultivation of a single cell with integrated microfluidic sample preparation and handling coupled to a MS detection method clearly exhibits the greatest potential to provide metabolomic data with high spatial and temporal resolution for systems biology (10, 145). Among all methods for metabolome analysis, MS plays an important role because of its ability to handle the chemical diversity of the involved compounds. Furthermore, the detection limit of modern mass spectrometers has recently reached the low attomole level, which is the required range for single-cell metabolome studies. However, the integration of sample processing steps into one device or workflow has not yet been realized. Despite the impressive progress in microfluidics and analytical technologies in recent years, many of the integrated metabolomics approaches remain at the proof-of-concept level or focus on fairly exotic metabolites with little biological relevance (145). This likely will change within the next few years, and integrated microfluidic solutions for single-cell metabolome and fluxome analysis will allow unrestricted access to relevant data for systems biology.

SINGLE-CELL ANALYSIS FOR BIOCATALYSIS

Whole-cell biocatalytic operations use the catalytic activity of enzymes within living cells for the production of biomass, cellular components, and metabolites or for the performance of highly selective biotransformations producing diverse bulk chemicals. However, increasing employment of advantageous but complex biological systems for chemical production comes with limitations on productivity that result from regulatory mechanisms originating from evolution. Evolutionary development of these mechanisms was driven by changing environmental conditions, which deliver a stimulus for mutations and a mechanism for the selection of the fittest organisms. Environmental dynamics led to the generation of highly flexible biological systems, thus ensuring survival through adaptation, but this leads to disadvantages for the selective and plentiful production of a target molecule. This evolutionary process at the population level caused inherent system instabilities, which is why most bioprocesses are realized only in batch or fed-batch formats, unlike industrial chemical processes, which preferably are run in continuous modes. Hence, the central question is how we can adjust the fuzzy regulation to a minimum of self-sustaining mechanisms and a maximum of robust product formation performance. Here we discuss two major SCA-based strategies used to face this challenge: first, the bird's eye perspective, analysis of population kinetics in terms of heterogeneity during a bioprocess, and second, analysis of the smallest catalytic unit, the single cell, by application of systematic perturbations using defined changes in microenvironmental conditions.

Non–Time Resolved Single-Cell Analysis for Bioprocess Optimization

Today, understanding the dynamics of heterogeneity in bioprocesses is considered to be the key to higher productivity and product quality (146–148). Because cell heterogeneity has a significant

impact on the productivity of bioprocesses (12), the quantification of cell-to-cell differences is recognized as a valuable tool for bioprocess optimization with increasing regularity. In particular, the design of sophisticated reporter systems such as fluorescent fusion proteins has allowed the monitoring of changes in the physiological state of a single cell via microscopy and flow cytometry (147). Díaz et al. (149) recently reviewed available reporter systems for omics analysis. Those techniques enable the detection of heterogeneity in biocatalytic processes by monitoring differences in the catalytic activity between individual whole-cell biocatalysts. The challenge of most methods is that they require relatively complex sample preparation, which makes online monitoring of bioprocesses especially difficult. This problem can be overcome by application of fully automated sample preparation for direct coupling of bioreactors to microfluidic SCA platforms. Recently, Broger et al. (147) used this approach to monitor the expression of an enhanced GFP-tagged target protein (human membrane protein) in fed-batch cultivations of *P. pastoris*, *S. cerevisiae*, and *E. coli*, using fully automated real-time, flow injection flow cytometry (**Figure 3**).

The flow injection flow cytometry system can be combined with advanced cell sorting to select hyperproducing cells during process-specific stress. The selected subpopulations might be used as inoculum for subsequent fed-batch cultivations and therefore might be a tool for strain optimization (150).

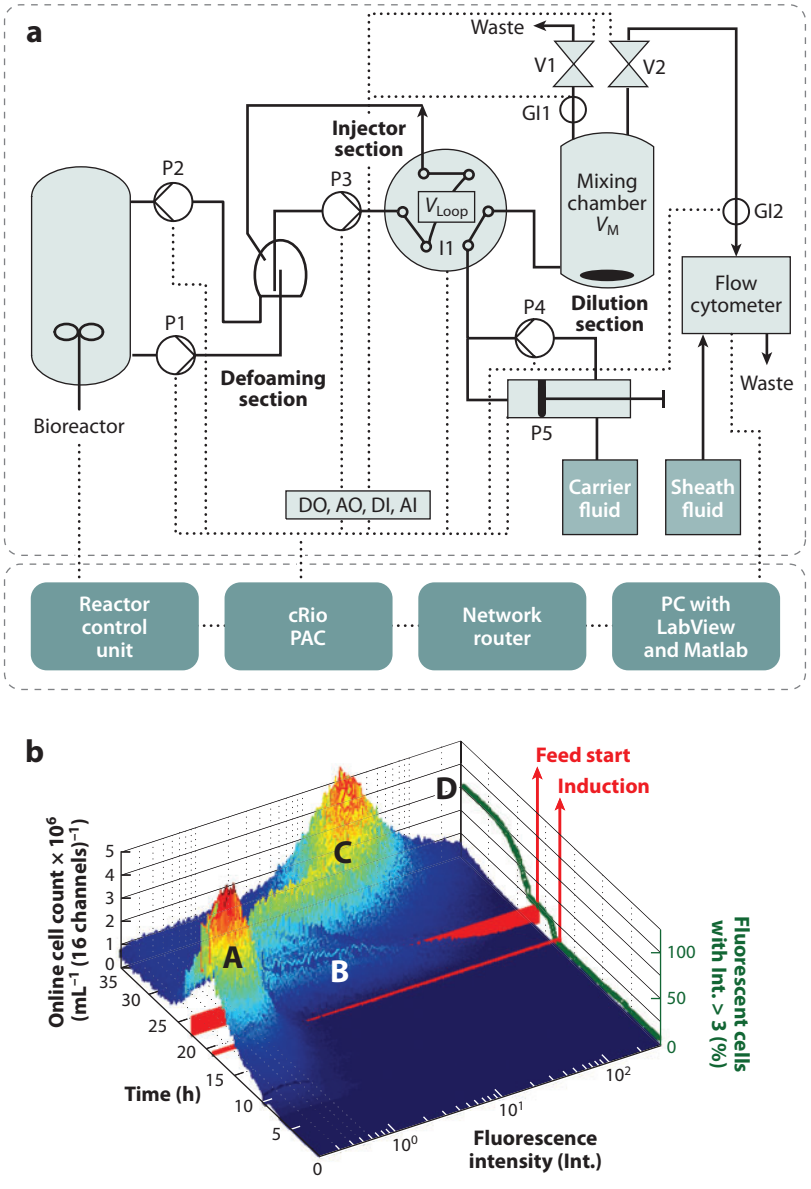
A vast amount of data on cellular heterogeneities in bioprocesses is now accessible for all different omics levels. To the best of our knowledge, even after decades of excellent developments in the analysis of heterogeneity in bioprocesses, none of the obtained data have been used for the development of optimization strategies. The complex interplay of cellular physiology with changing environmental conditions in biotechnological processes is still not entirely understood. Predictive modeling of cellular heterogeneity requires sophisticated and reliable mathematical descriptions of the related processes within the particular omics concept, including the individual cellular response to different microenvironments (**Figure 2**). Lencastre Fernandes et al. (148) have comprehensively reviewed current modeling techniques for the description of cell populations.

As already mentioned, important mechanisms within a population cause cell-to-cell differences to boost the population's fitness and enable it to better cope with rapid environmental changes (3, 12). Next to stochasticity in gene expression, cell cycle, aging, and epigenetic regulation, local microenvironments have been identified as the key reason for the development of heterogeneity in bioprocesses (148). In addition to inherent stochastic events, the interaction of the cell with the surrounding environment is a starting point for the improvement of microbial performance. Fermentation parameters such as pH, temperature, dissolved oxygen, and nutrient availability appear to be controllable on the macroscale, but local temperature and concentration gradients cannot be avoided completely in the microenvironments surrounding single cells. Now the challenge is to identify the intracellular regulatory dynamics induced by a specific microenvironment as a target for metabolic engineering. Today, time-resolved SCA allows researchers to face this challenge with novel LOC devices that enable the control of microenvironments for systematic perturbation experiments.

Balancing the Smallest Catalytic Unit, the Single Cell

Time-resolved SCA only recently has started to receive much attention for the optimization of industrial biocatalytic processes. Numerous approaches exist to create defined microenvironments and to facilitate their adjustment to the particular demands of cells. Total microenvironmental control not only considers chemical control, such as that of transmitter concentrations and nutrient supply as well as a precisely defined chemical composition surrounding the isolated single cell, but also enables physical control, such as temperature control and defined mechanical impact

such as pressure changes or shear stress. The resulting data potentially can be used to standardize cellular responses and will help to identify the reasons behind population variability. Particularly promising is the combination of the data with mathematical balancing of the smallest catalytic complex involved in a bioprocess. This would provide the boundary conditions necessary to solve the nonlinear equation system of models describing the biochemical dynamics of omics in single cells and enable the accurate modeling and simulation of a whole-population response. Online metabolite analysis and integrated parameter identification for automated predictive model setup



would be the ultimate tools to identify process modules as targets for the rational improvement of microbial catalytic performance.

CHALLENGES FOR SINGLE-CELL ANALYSIS

Many exciting technologies for SCA have been developed in recent years and offer unprecedented opportunities. Numerous approaches allow the analysis of single cells with a level of detail that is close to that of routine bulk measurements with populations (10). SCA has already proved itself as a valuable and indispensable technology in medicine and biotechnology; however, its full potential has yet to be reached, and key challenges lie ahead.

Breakthroughs in micro- and nanotechnology have been the strongest driving forces toward the status quo in SCA. Numerous sophisticated microfluidic devices have been designed to meet the dimensions of single cells and to enable precise cell handling and manipulation, including the specific sorting, separation, and cultivation of individual cells (43, 58, 63). Despite the widespread application of various cell retention and manipulation methods, the influence of each technology on cellular physiology has not yet been clarified. Therefore, the design and engineering principles of LOC devices for cell cultivation and manipulation must be reconsidered and optimized for undisturbed analysis of biological functions. Only such a technology can provide data for the exact identification of stress modules on the basis of the real-time responses of cellular functions to different chemical and physical perturbations.

For the real-time analysis of live single cells, the most prominent analytical technique uses fluorescent reporter systems to visualize and quantify the target processes and involved biomolecules (123). Here, multiplexing of fluorescent probes will be the key to the simultaneous multiparameter determination of transcriptional activity, enzyme activity, and enzyme quantity as well as metabolite concentrations in living cells. Another approach for noninvasive analysis of single cells aims at the secretome (126). The secretome comprises all biomolecules that are secreted, including intermediates or products from metabolic pathways as well as extracellular peptides and proteins. The secretome has been investigated rarely, but this is likely to change, especially with regard to the analysis and evaluation of production strains with technological and/or industrial relevance. Biological SCA also encompasses the separation and subcultivation of analyzed single cells for physiological investigation of the resulting population. Thus far, most cultivation and separation technologies do not include the separation of daughter cells, although studies on subcultivation of single cells deliver valuable information about phenotypic stability. An exception is FACS analysis,

Figure 3

(*a*) Schematic representation of the flow injection flow cytometry system (147), which combines fully automated sample preparation, direct coupling of bioreactor and real-time flow cytometry, and computerized online data analysis and interpretation. The flow chart shows the three sections of the flow injection flow cytometry system with a defoaming, an injector, and a dilution section. The programmable automation controller (PAC) regulates the pumps (P1–5), the injector (I1), and the valves (V1, V2) via digital and analog in- and outputs (DO, AO, DI, AI). The PAC also monitors the conductivity for calibration (GI1, GI2) and is connected to a computer (PC) via a network router. The PC runs a LabView algorithm to control the flow cytometer and collects analysis data using a Matlab algorithm. The sample from the bioreactor is defoamed, injected with an injection volume (V_{Loop}) into the mixing chamber with the mixing volume (V_M), and subsequently analyzed with the flow cytometer. (*b*) Histogram of cell-fluorescence distribution in *Pichia pastoris* measured with the flow injection flow cytometry system during fed-batch cultivation. A, the increase of fluorescence caused by the leaky AOX promoter system; B, induction of the expression of fluorescence-tagged protein by a methanol pulse; C, a strong increase in fluorescence intensity in the exponential mixed-feed mode; and D, end of fermentation.

which includes sorting and separation mechanisms for subsequent subcultivation of cells that are of particular interest (151). However, a future task will definitely be the parallelization of biological SCA with a focus on high-throughput approaches.

Sample processing strategies preserve target intracellular biomolecules for subsequent analysis and therefore constitute an integral part of chemical SCA. Cell cultivation, cell lysis, quenching, lysate processing, and sample separation are ideally combined in one integrated unit with direct coupling to the appropriate analytical instrument. The strategy for cell lysis and lysate quenching thus must be carefully chosen on the basis of the analyte classes and their vulnerability to thermal or chemical decomposition (152). We consider the realization of a mild and sufficiently fast cell lysis with the full preservation of all liberated biomolecules for subsequent omics analysis as one of the main engineering challenges in the chemical analysis of single cells.

Furthermore, the reduction of the sample volume correlating to the minute amounts of analyte molecules from a single cell requires specific adaptation of microfluidic devices. Beyond volume reduction, a crucial point for integrated design of microfluidic LOC devices is the direct and loss-free coupling of the cultivation and sample processing unit to the analytics. Reliable and reusable microfluidic world-to-chip connectivity and sealing concepts remain major obstacles that only a few research groups have addressed so far (153). Although MS-based methods exhibit sufficient specificity and selectivity in the low attomole range for the analysis of whole classes of metabolites and proteins from single cells, handling and transfer of sample volumes in the low picoliter range still require the development of high-quality interfaces between microfluidic chips and mass spectrometers. The importance of microfluidic LOC systems for sample handling and processing has already been demonstrated in other omics disciplines. Significant improvements in WGA approaches have been realized with microfluidic nanoreactor chips, principally because these chips provide reaction volumes free of contaminant DNA (91). In addition to the hardware developments, sophisticated software solutions for automated acquisition and mathematical interpretation of the massive amounts of data obtained from high-throughput SCA must be developed as well (148). Finally, we are convinced that these challenges will be overcome in the next few years and that SCA based on case-by-case solutions will become a broadly applicable key technology for the elucidation of the many features and functions of life.

SUMMARY POINTS

1. Flow cytometry and cell sorting approaches have been implemented on LOC devices to create combined noninvasive μFACS sorting systems.
2. Advances in microfluidic single-cell cultivation allow highly parallelized, noninvasive, and hence spatiotemporal analysis.
3. Microfluidic single-cell cultivation with total microenvironmental control has been demonstrated.
4. Detection techniques for biological SCA on the genomic, transcriptomic, proteomic, and metabolomic levels with spatiotemporal resolution are available.
5. Several microfluidic lysis methods exist that can be used for specific applications depending on the cell type and target analytes.
6. Analytical instruments recently exceeded the single-cell threshold for sensitivity and specificity, which has enabled chemical SCA.

7. Cellular heterogeneity was proven to affect the performance of bioprocesses.
8. Thus far, only a few strategies have been developed to exploit the potential of SCA for the improvement of biocatalytic processes.

FUTURE ISSUES

1. Innovative multifunctional μTAS require the sophisticated integration of currently available LOC technologies for cell sorting as well as biological and chemical SCA in defined microenvironments.
2. Integration of microfluidic sample processing technologies and direct coupling to analytical instruments for further sensitivity improvement is needed.
3. Automation of μTAS for high-throughput SCA is required.
4. Identification of process modules as targets for the rational improvement of microbial catalytic performance with SCA is needed.

DISCLOSURE STATEMENT

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