Novel Micro-scale Analytical Devices for On-line Bioprocess Monitoring: A Review

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Review
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This review examines the potential of novel micro-scale microfluidic analytical devices – lab-on-a-chip (LOC), micro total analysis systems (μ-TAS) – for on-line monitoring and control of industrial bioprocesses. First, motivation for the current study is presented and potential benefits from the use of micro-scale analytical devices in bioprocess control and monitoring are outlined. This is followed by a review of the state of the art in the relevant application domain (cell analysis) for novel microfluidic analytical devices. Finally, the conclusion provides a summarizing comparison of the main features of the reported micro-scale analytical devices evaluating their potential applicability for on-line bioprocess monitoring, with the most promising concepts identified.

Key words:
Bioprocess monitoring, lab-on-a-chip, micro total analysis systems, single-cell analysis

Introduction

A fundamental prerequisite for successful bioproduction is the choice of microorganism and the examination of the optimal conduction for obtaining the desired product. Keeping the production process within the conditions given by the protocol is one of the most difficult and challenging tasks. Even a small concentration change of an important nutrient can cause a radical change in cellular metabolism resulting in obtaining undesired product concentrations or composition. Considering these facts, bioprocess monitoring presents one of the limiting factors of a given bioprocess performance. Additional requirements arise due to regulatory affairs, such as the Process Analytical Technology (PAT) initiative issued by the FDA. Its main goal is to improve the understanding and thus control of the manufacturing process as such – quality cannot be tested in products alone, it has to be inherently built-in or achieved by design. PAT is seen as a system for designing, analyzing, and controlling manufacturing through timely measurements (*i.e.* during processing) of critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality.[1,2]

In this context, the physiological state control approach represents a concept capable of addressing the above-mentioned issues. It is more than 20 years since this ambitious concept was first proposed by Konstantinov and Yoshida.[3] This advanced approach to bioprocess automation was based on the description of the physiological state of the cultivated microorganism by a set of on-line measurable process variables providing relevant information about the organism's state. Using appropriate algorithms for on-line recognition of the current physiological state of the cell culture, the aim of process control then was to keep this status within a desired range, achieving thus optimal performance of the production bioprocess through maximizing the performance of the microorganism used as the biocatalyst.

Even though the potential benefits to industry were obvious, this advanced concept has hitherto found only limited practical application due to the limitations of conventional measuring devices, usually only capable of monitoring environmental conditions, providing thus only indirect and incomplete information on the state of the cell culture.

Following important technological advances in analytical tools in the 1990's, the original concept of physiological state control was revisited by Schuster[4] – information on the actual state of the cell culture is to be gained directly from the analysis of the microbial cells, using measurements of internal variables inside the cells. This approach can be further differentiated according to the specific cellular component to be monitored on the intracellular level as follows:

- analysis of whole cells
  - population-wide analysis
  - single-cell analysis
- groups of cellular components
- specific compounds which serve as markers for a certain physiological status.

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At the time of Schuster's report, however, the choice of available tools capable of on-line measuring of relevant intracellular variables (proteins, RNA, metabolites or intracellular storage material) was quite limited.

What is the situation like today, 10 years later? There is a clear trend towards single-cell analysis, as most microbial cultures are by no means a mass of identical cells. However, the choice of commercial systems that enable direct intrinsic studies of single cells is still limited – mainly conventional flow cytometry. This applies not only to the application area of productive bioprocesses, but also to the whole field of life sciences as such.

And it is here, in this context, where the latest developments in micro- and nanofabrication technologies, which have already led to the successful lab-on-a-chip concept, offer promising opportunities for the analysis of single cells.[5,6]

The commonly cited advantages of micro-scale analytical devices (LOC, μ-TAS) include increased sample throughput; low sample, reagent and power consumption; short reaction time; portability for in situ use; low cost; versatility in design, and potentials for parallel operation and for integration with other miniaturized devices; and last but not least, the capability to automate complex laboratory processes in a single device.[7–9]

So far, the research interest in the area of single-cell analysis by micro-scale analytical devices has focused mainly on genetic analysis (on-chip genomics), with a recent shift towards using μ-TAS systems for protein analysis (on-chip proteomics). In addition, in the past few years, the interest in analysis of even more complex biological systems such as entire living cells with the use of microfluidic systems has attracted increased attention. Andersson *et al.* have summarized in their review the specific reasons making microfluidic devices and systems particularly interesting for cellomics:[6]

– increased interest in biochemical experimentation/analysis of living single cells *e.g.* for studying effects of drugs, external stimuli on cell behaviour etc.

– possibility of easy integration of all kinds of analytical standard operations into on microfluidic system

– several methods for manipulating large numbers of cells simultaneously can be used in microfluidic systems

– the cells size fits very well with that of commonly used fluidic devices (10–100 μm)

– micromechanical devices are very well capable of manipulating single objects with cellular dimensions.

Today, it is beyond any doubt that the application of micro-scale systems has really entered the life science field and has started to serve as a driving force for discovery in cell biology, neurobiology, pharmacology and tissue engineering.[6] It is therefore reasonable to expect that similar development will also take place within the application area of bioprocess control and monitoring.

The following sections will take a closer look at reported applications of these novel technologies in single-cell as well as population-wide analysis to evaluate their potential for monitoring of productive bioprocesses. Particular attention will be paid in this respect to features critical for eventual on-line implementation (sample preparation, high throughput capability/measurement rate, etc.).

Micro-scale analytical devices for cell analysis

Transcriptomics

The gene expression activities of cells can be determined primarily by their messenger RNA (mRNA) – transcriptome, and finally by their protein pattern – proteome (Fig. 1). However, cellular RNA consists mainly of ribosomal RNA (rRNA) and transfer RNA (tRNA). Only 4 % of the total RNA pool in bacteria like *E. coli* represents mRNA. The mRNA level of a specific gene can be used as a measure of its expression status.[10]

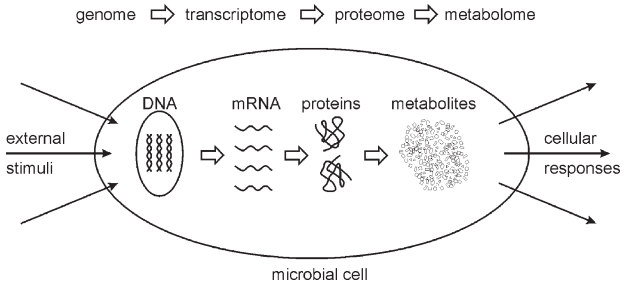


Fig. 1 – *Scheme of a general cellular response to extracellular stimuli*

Based on the knowledge of the base composition of a distinct bacterial genome, an expression profiling of all genes of an organism can be performed by means of optical DNA-chips (also known as DNA-arrays). This technique allows the analysis of the mRNA levels of all genes of a microbial genome at one time. However, even though the DNA-chip technology is a powerful tool in gene expression analysis, it is not very useful for frequent expression monitoring of a selected subset of genes – characteristic for biotechnological diagnostic purposes. The DNA-chip based analysis is too

expensive and slow for such a routine application. The optical DNA-chips usually require a rewriting of the isolated mRNA molecules into cDNA by means of reverse transcriptase. This step is necessary in order to convert the unlabeled mRNAs into labeled nucleotide sequences that can be detected, for example, by fluorescence. The time needed for the mRNA analysis by these approaches, from cell disruption and RNA isolation to the final data analysis, is up to 24 hours.[11,12] The hybridisation is the most time-consuming step of the optical DNA-chip analysis. Alternative faster DNA/RNA detection techniques are characterised by one common feature. In comparison to optical DNA-chips, they do not allow a parallel analysis of thousands of samples at one time.[10,13]

Therefore, even though the optical DNA-chip analysis is irreplaceable for comprehensive off-line bioprocess analysis, because only a genome wide transcriptional profiling enables a global overview on cellular activities, it is the expression analysis of marker genes sets (focused expression analysis) which represents a more appropriate approach for process monitoring applications. For this application, the micro-scale (microfluidic) approach based on the lab-on-a-chip concept appears to be particularly promising.[13]

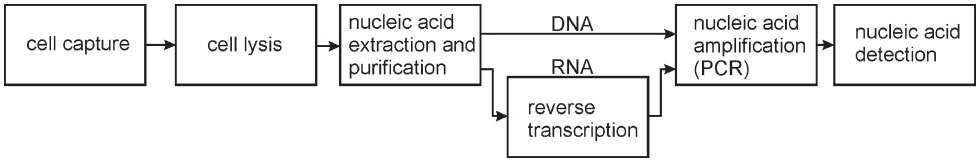
Although the field of nucleic acid analysis by microfluidic devices has undergone extensive development in the past decade, particularly with the integration of sample amplification by the polymerase chain reaction (PCR) and detection steps on single chip in microfluidic format, there are still only a few fully integrated microfluidic devices truly realizing the "sample-to-answer" concept in quantitative terms among the reported applications – see the recent reviews.[14–17] The on-chip integration of the sample preparation steps appears to be the most challenging obstacle for construction of a fully integrated microfluidic analytical device capable of a quantitative DNA/RNA analysis. Specifically, since the subsequent sample amplification step (PCR) requires pure nucleic acid samples, a successful on-chip integration of sample preparation steps, *i.e.* cell capture, lysis, DNA/RNA extraction, purification and preconcentration is essential (Fig. 2, Table 1).[15,16]

Most advanced in this respect are the experimental devices developed by Motorola Labs[18] and Samsung Bio Lab,[19] respectively. The team of P. Grodzinski at Motorola Labs developed a self-contained fully integrated microfluidic device ("biochip") that included sample preparation, PCR amplification and DNA micro-array with electrochemical detection. The device is intended primarily for medical applications (pathogen bacteria detection in whole blood samples). The on-chip analysis starts with the preparation process of a whole blood sample, which includes magnetic bead based target cell capture, cell preconcentration and purification, and cell lysis, followed by PCR amplification and electrochemical DNA microarray-based detection. Functionality of the device has been demonstrated in *E. coli* K12 cells detection in rabbit blood sample by recognizing a specific gene fragment. The whole analysis from loading the blood sample and different reagents into the storage chambers to obtaining the hybridization results took 3.5 hours. The durations for the different operations were as follows: sample preparation 50 min, PCR amplification 90 min, pumping and valving 10 min, and hybridization 60 min.[18]

A different, fully integrated microfluidic pathogen detection system has been developed by Samsung Bio Lab that is capable of rapid and efficient DNA extraction and subsequent real-time detection by combining laser irradiation and magnetic beads. The device integrates cell lysis, sample extraction, amplification and detection by real-time PCR in a single chamber of a microchip achieving a remarkably fast analysis (total time < 32 min). Again, as with the previous system from Motorola, its functionality has been demonstrated in bacterial pathogen detection in biological samples by identification of a specific segments of bacterial genome (*E. coli* and Gram-positive bacteria).[19]

As noted above, both fully integrated microfluidic systems for DNA analysis are intended primarily for medical applications. If these concepts were to be used also for mRNA analysis, the steps of mRNA sample extraction, purification and conversion to cDNA by reverse transcription would have to be integrated on the microfluidic chip as well. The feasibility of this approach has been recently demonstrated by Bontoux *et al.*[20]

However, for industrial bioprocess monitoring applications a rather more robust concept, avoiding the very sensitive step of the PCR-based sample amplification, would be highly desirable. Such a



F i g . 2 – *Flow chart of unit operations related to transcriptomic analysis*

Table 1 – *On-chip transcriptomic analysis – working principles of unit operations*

Unit operation	Working principle
Cell capture	In the first step, cells of interest that are to be analyzed, must be isolated from the fluid sample. Still, in many applications this operation is performed off-chip, reported on-chip solutions can be roughly divided into two categories: techniques requiring cell labeling and label-free solutions. The former include fluorescence- and magnetic-based approaches where the cells of interest (typically bacterial cells) are attached to specific fluorescently or magnetically labeled antibodies. Label-free approaches are often based on various types of microfabricated mechanical filters (*e.g.* used for blood cells capture), other reported solutions use electrical (separation by dielectrophoresis) or optical principles (*e.g.* optical traps based on highly focused laser beams).
Cell lysis	In order to provide the transcriptomic material (mRNA) for the subsequent analytical steps, the captured cells are lysed by disrupting the cell membrane. The methods of choice for cell lysis on the microfluidic platform include electrical (typically lysis via a high voltage electrical pulse), thermal, chemical, and mechanical approaches. From these, the former two appear to be the most appropriate for microfluidic transcriptomic analysis as all the necessary technology can be relatively easily implemented on-chip and there is no need for additional chemical reagents that might potentially interfere with the subsequent sensitive analytical procedures.
Nucleic acid extraction and purification	As most of the used analytical methods require relatively pure nucleic acid (NA) samples, free from potentially contaminant cellular debris, the transcriptomic material of interest has to be extracted and purified. On the microfluidic platform, this is usually implemented via various separation techniques based on solid-phase extraction (SPE). Reported solutions range from NA extraction via specific binding to magnetic or silica microparticles to various types of microfluidic filters or microstructured surfaces for NA capture and immobilization, generally followed by washing off the unbound material. Among the more frequently used separation techniques are those based on magnetic microparticles (typically magnetic beads combined with specific probes for complementary binding with targeted NA molecules), the resulting NA-bead conjugates and can then be processed in a variety of ways using advanced magnetic particle processors, this approach is even capable of continuous nucleic acid extraction.
Reverse transcription	Transcriptome analysis requires that prior to the subsequent steps (amplification and detection), the extracted transcriptomic material (mRNA) be reverse transcribed into complementary DNAs (cDNA). The process of reverse transcription from mRNA to cDNA mediated by the reverse transcriptase enzymes is often implemented in conjunction with the amplification step by polymerase chain reaction (PCR) in the form of a technique known as reverse transcription polymerase chain reaction (RT-PCR). This critical operation is very sensitive to sample impurities; its on-chip implementation must therefore be closely integrated with the sample preparation steps (cell lysis, NA extraction and purification).
Nucleic acid amplification	The process of NA amplification in the form of a standalone PCR (for DNAs) or as PCR in conjunction with reverse transcription (for RNAs) is a key operation in NA analysis. Generally, the enzyme-based process of PCR amplification comprises a series of repeated thermocycles, each featuring several steps characterized by different temperatures (typically 3 steps – denaturation, annealing, extension) during which the initial NA amount (NA template) is enzymatically replicated to yield up to millions of copies. In comparison to a conventional macro-scale setup, the microfluidic implementation of PCR is more advantageous in terms of thermodynamics due to small device dimensions. Reported microfluidic implementations of PCR vary in their modes of operation (batch-wise operating well-based PCR *vs.* continuous flow-through PCR) and detection (standard mode where detection follows PCR completion *vs.* real-time PCR featuring simultaneous amplification and quantification of the targeted DNAs due to the use of specific fluorogenic probes.
Nucleic acid detection	Apart from microfluidic systems where NA amplification and detection are combined into one step (real-time PCR), the operation of NA amplification is followed by the detection of the replicated NAs. The on-chip NA detection methods of choice fall into two basic categories: systems based on electrophoretical separation (capillary gel electrophoresis – CGE) and hybridization-based (DNA-arrays). Compared to hybridization-based detection systems, those based on CGE generally provide shorter sample-to-answer times but the amount of information obtained is limited as the target NA detection is based only on size separation. In this respect, the hybridization-based approaches where the NA detection is sequence-based (the amplified NAs are hybridized to oligonucleotide probes immobilized to a solid array) can provide more detailed information about the NA sample with a higher throughput. On the other hand, the NA detection by DNA-array is slower than CGE due to the time-consuming hybridization step. Even though the DNA-array technology is traditionally coupled to optical detection (hybridized pairs are identified on the array using laser excited fluorescence detection), it is the electrochemical detection (electrochemical signals linked to hybridization are detected on the array), which appears to be more suitable for on-chip integration as optical systems are generally more difficult to miniaturize onto microfluidic platform.

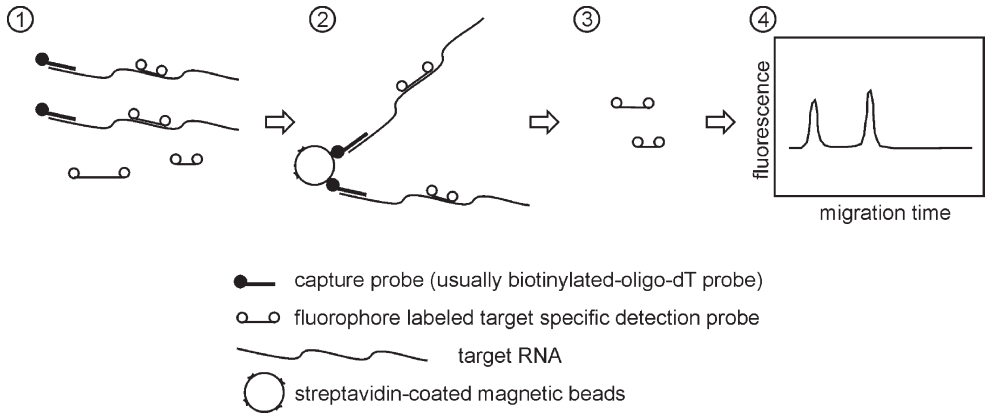
concept has been developed recently by the VTT Technical Research Centre (Finland) and is currently being commercialized by VTT spin-off company PlexPress Oy. Their TRAC technique (transcript analysis with the aid of affinity capture) is intended for focused gene expression monitoring through multiplexed and quantitative analysis of RNA (tens of genes can be monitored simultaneously). It is based on solution hybridization between RNA and multiple fluorophore labeled target specific probes of distinct sizes. After hybridization, the RNA-probe complexes are captured, washed and finally the hybridized probes are eluted and analyzed by capillary electrophoresis (CE). The probe signal intensities correspond to the amount of RNA in the sample, while the probe size indicates the specific gene expressed (Fig. 3).[13,21]

Macro-scale functionality of this concept for gene expression monitoring in industrial microbial cultures (filamentous fungus *Trichoderma reesei*) using oligonucleotide probe pools has been successfully demonstrated. Rapid transcriptional analysis of selected mRNAs has been achieved with automated sample treatment in 96-well format using a magnetic bead particle processor and crude cell lysates. For 96 samples the total analysis time was approximately 2–3 hours. For a lower number of samples with an optimised protocol this time can be shortened to 1–2 hours. Even more reduced analysis times are expected in integrated microfluidic format of this concept that is currently being developed as the so-called TRAC chip. This implementation should also be capable of on-line measurement.[13,21]

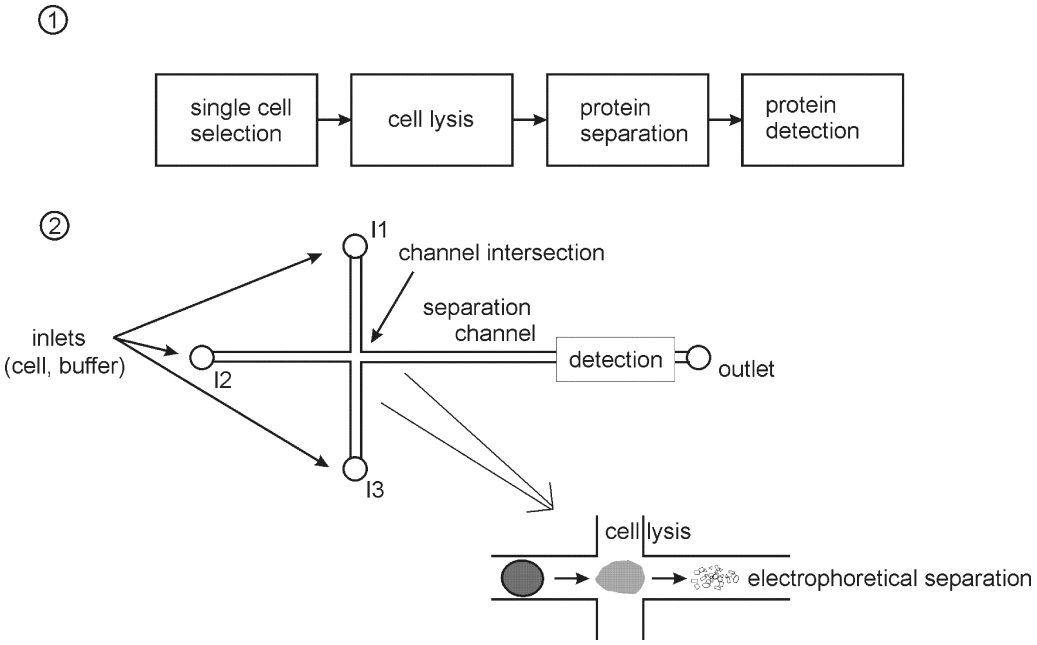
Proteomics and metabolomics

Even though transcriptomics can provide important information about the physiological state of cultivated cells, it is proteomics that makes the picture complete by being able to characterise what cannot be investigated by mRNA analysis – protein targeting, protein turnover or post-translational modifications. After all, it is the proteins that carry out the actions necessary for cellular function with changes in their concentrations having direct phenotypic consequences. Therefore, the proteomic approach is irreplaceable in directly determining protein concentrations as its estimation by analyzing the quantity of mRNA in cells does not provide precise values since the half-life and translation efficacy of mRNA is variable. Furthermore, it must be noted that under dynamic conditions, mRNA and protein levels are often markedly different. The combination of both approaches would therefore allow a much more comprehensive direct view on the physiological state of the cells in one culture.[10,22,23]

Unfortunately, proteomics is a more complex field than genomics or transcriptomics because of the considerably higher number of potential analytes – it is estimated that organisms express an order of magnitude more proteins than genes. Further, and most important, expressed proteins represent molecules of vastly different physicochemical properties as they are typically modified in a variety of ways by post-translational modifications such as phosphorylation and glycosylation. In addition, unlike nucleic acids where minute sample quantities can be amplified by PCR, it is currently impossible to amplify proteins purified from biological samples. Therefore, in case of low abundant proteins in a single cell, the applied detection method must be sensitive enough to detect amounts in the order of nanomoles (< 100 nmol). Correspondingly, single cell protein analysis in microfluidic format must be based on advanced techniques that can guarantee efficient and sensitive separation and detection (Fig. 4, Table 2).[22,24–28]



F i g . 3 – *Principle of the TRAC transcriptomic analysis: 1 – solution hybridization between target RNAs and specific fluorophore labeled detection probes of distinct sizes and specific capture probes (usually biotinylated-oligo-dT probes), 2 – hybridized targets are immobilized to magnetic beads, 3 – after washing off the unbound substances, the detection probes are eluted from the beads, 4 – identification and quantification of the detection probes by capillary electrophoresis*



F i g . 4 – *Single-cell proteomic analysis: 1 – flow chart of unit operations related to proteomic analysis, 2 – schematic diagram of a microfluidic chip for single-cell proteomic analysis*

T a b l e 2 – *On-chip proteomic analysis – working principles of unit operations*

Unit operation	Working principle
Single cell selection	The initial step of single-cell proteomic analysis – the selection of individual cells to be analyzed from a cell population and its transport to a position where the analytical steps are carried out, is of critical importance for the overall performance of the microfluidic analytical system, as it must guarantee that only one cell at a time will be analyzed. There are basically two approaches to this task: a) batch-wise operation involving two-tier-selection process where a specific cell of interest is selected for analysis from a cell ensemble previously isolated from a liquid sample in a manner similar to the cell capture for microfluidic transcriptomic analysis; b) continuous single-cell proteomic analysis (chemical flow cytometry) where all the cells of interest are analyzed one by one as they flow through the microfluidic analytical device. From the technological point of view, the reported techniques for single-cell manipulation applied in on-chip cell selection include the use of mechanical traps (geometrical trapping) or microfluidic pumps and valves, hydrodynamic focusing, incorporation into fluid droplets, dielectrophoretic, optical or optoelectronic trapping.[29]
Cell lysis	As in the case of transcriptomic analysis, the selected cell must first be lysed by disrupting the cell membrane to release the intracellular protein content for the subsequent analytical steps. Likewise, the methods of choice for cell lysis include electrical, thermal, chemical, and mechanical-based techniques. From these, the electrical (typically lysis via a high voltage electrical pulse) and chemical (usually by exposing the cell to a detergent agent) are the most frequently used as easy-to-implement techniques in the microfluidic format. Unlike the case of transcriptomic analysis where the PCR sensivity limits the use of chemical agents, the subsequent proteomic analytical procedures can be effectively coupled on chip with chemical lysis.
Protein separation	Following the lysis step, the cell lysate is typically transferred to a microfluidic separation channel where the cellular protein content is separated and individual proteins subsequently detected. For microfluidic protein separation, the dominant method is capillary electrophoresis (CE) due to its favourable properties (capability of processing extremely small samples, high separation speed and efficiency, ability to be coupled to ultrasensitive detection methods).
Protein detection	In the final step, the individual separated proteins are detected on chip. The most commonly used method for single-cell protein detection has traditionally been laser-induced fluorescence (LIF) due to its high sensitivity– in the visible range of the spectrum for natively fluorescent samples or in the ultraviolet range for fluorescently labeled compounds; or electrochemical detection (ECD) – for electroactive compounds. However, LIF or ECD-based detection techniques are limited in the amount of information they can provide about the protein analytes in terms of their chemical structure. For this reason, the recent trend has been to implement mass spectrometry (MS) as the detection method for on-chip single-cell analysis. MS is not only capable of providing for label-free identification and structural analysis of individual proteins but also other cellular compounds, such as metabolites. Both variants of MS traditionally used for macro-scale analysis of liquid and solid biological samples (electrospray ionization – ESI-MS and matrix assisted laser desorption ionization – MALDI-MS) have been successfully coupled to microfluidic proteome/metabolome analytical devices in experimental proof-of-principle studies.[26,30,39,40]

For these reasons, the most frequently used technique for single cell protein analysis is currently capillary electrophoresis (CE) because of its extremely small samples, high separation speed and efficiency, biocompatible environments and last but not least its ability to be coupled to ultrasensitive detection methods such as laser-induced fluorescence (LIF) – for natively fluorescent or fluorescently labeled compounds, or electrochemical detection (ECD) – for electroactive compounds.[31,32]

During the past five years, as first proof of principle attempts, several studies of experimental microfluidic devices based either on CE-LIF or CE-ECD for single analyte analysis have been reported:

– qualitative analysis of fluorescent dyes in Jurkat cells at average analysis rates of 7–12 cells/min,[31] respectively in AML (acute myloid leukemia) cells at analysis rates of approx. 24 cells/hour,[33] both featuring CE-LIF integrated on chip with cell handling and lysis

– quantitative and qualitative analysis of a specific small peptide (fluorescently labeled) in human erythrocyte cells at analysis rates of 15 samples/h by chip-based CE-LIF[34]

– quantitative and qualitative analysis of a vitamin C (ascorbic acid) in single wheat callus cells by chip-based CE-ECD.[35]

In the latter two applications, an experimental microfluidic device developed at the Institute of Microanalytical Systems (Zhejiang University, China) integrating single cell injection, controlled cell lysis, CE separation and LIF detection of a specific intracellular constituent of a lysed single cell has been used. The same device has also been successfully used for high-throughput single cell analysis by chip-based CE-ECD of a specific enzyme activity, alternatively with or without cytolysis at average detection rates above 1 cell/min (model system: peroxidase inside human neutrophils). The latter alternative is however limited to the assaying of cytosolic enzymes in cells that do not contain cell walls.[32]

However, probably the first microfluidic system capable of a single cell protein analysis has been developed recently by the research group of D. Anselmetti at the Bielefeld University, Germany. Their microfluidic chip platform combines single cell trapping, steering, deposition, lysis, and subsequent electrophoretical protein separation, and LIF-detection in the visible range (VIS) and in the ultraviolet (UV) spectral range. As a proof of principle application Sf9 insect cells (*Spodoptera frugiperda*) were analysed, first analyzing a single GFP-construct protein obtaining its electropherogram in the VIS.[28,36] Because LIF detection in the VIS requires adequate fluorescent labeling for the cell component of interest, further research focused on LIF detection in the UV spectral range that allows a direct label-free detection of proteins due to the favourable fluorescence properties of the aromatic amino acids tryptophan, tyrosine and phenylalanine.[25] With the device, the first single cell electropherograms (~10 distinct peaks) from Sf9 cells were recorded with native LIF detection of proteins in the UV-range.[27,37] The microfluidic device was further improved by using novel PDMS-Quartz-Window-chips in order to reduce the background fluorescence, thus considerably improving detection sensitivity – the so obtained electropherograms exhibited 5× more and 4× higher peaks as compared to the previously used chips (total width of the electropherogram = total migration time approx. 900 s).[25,38]

There is also growing interest in the research of other detection methods to be coupled to a microfluidic device for single cell protein analysis. Mass spectrometry is intended as a detection method used in a project of fully integrated glass microsystem for protein analysis combining preconcentration, labeling, separation, dilution/destaining, and detection steps with monolithically integrated electrospray needle for interfacing microfluidics with the macro-scale mass spectrometry detection part. The ultimate goal of this project is to have an integrated device where sampled cells could be presorted, selected cells then lysed, and their protein content subsequently concentrated, labeled and analyzed in a single run/operation within minutes.[26]

Mass spectrometry (MS) also appears to be the detection method of choice for chip-based single cell metabolomics. An interdisciplinary project has been completed recently at ETH Zurich aimed at developing a highly sensitive tool for the identification and quantification of cellular metabolites at the single-cell level and at a temporal resolution that would be appropriate to understand cellular processes at biologically relevant time scale. The system couples a microfluidic cell processing and sample preparation step to a MALDI-TOF mass spectrometer via an innovative fluidics/MS interface. Specifically, the microfluidic part of the system includes the following key features:[39,40]

– temperature control (metabolism stoppage through active cooling – Peltier element)

– integrated peristaltic pump

– cell sizing through differential impedance measurement

– cell lysis through electrical field pulses and cell fragment separation.

Chip-based cytometry

Conventional flow cytometry is a well-established and widely used method based on the mea-

surement of physical and biochemical properties of single cells suspended in a fluid stream passing through the measurement device. Most modern flow cytometers are commercially available full-sized laboratory bench-top instruments, which are relatively expensive for wider use as an analytical tool in microbiological laboratories, let alone routine production process monitoring. Furthermore, most of these instruments require highly trained personnel to carry out even the most routine analyses. In this respect, many attempts have been made to design flow cytometers that use microfabrication technologies. Consequently, chip-based cytometry was proposed and various miniaturized flow cytometers – microfluidic flow cytometers, have been described and are expected to be utilized in a wide range of applications. Due to their reduced sizes and lower costs, microfluidic flow cytometers are expected to compete successfully with conventional macro-scale flow cytometry.[41]

In the past decade, microfluidic flow cytometer systems have undergone rapid evolution driven by two main factors:

1. the increased need for higher quality and larger quantity of cellular analysis data, and

2. the enhanced sophistication and accessibility of microfabrication technologies.

The emerging needs coupled with the new technical capabilities have led to a variety of new developments in microfluidics for flow cytometric analysis of cells and particles. In addition to providing higher speeds, smaller sizes and lower costs, the use of microfabricated structures and microscale flow physics serves as an enabling technology to analyze and understand complex cellular processes at single-cell resolution in new ways that make use of the following advantages of microscale cellular analysis:[43]

– The typical size of microfabricated fluidic systems is comparable to that of cells and allows more precise control over multiple variables in complex cellular microenvironments.

– The small dimensions of microfluidic systems increase the surface-to-volume ratio, enhance the rate of mass transfer, and allow use of significantly less reagent volumes and shorter reaction times in biochemical cell assays.

– Single cells or functional microscale objects can be mechanically or electrically manipulated in a non-invasive manner.

– Microfabrication processes offer the possibility of massively parallel configurations with very high throughputs using arrays of microfluidic channels. Integration of multiple microfluidic systems having different analytical functions is also possible.

Depending on the underlying detection technology used, reported microfluidic flow cytometer systems can be broadly divided into two different groups:

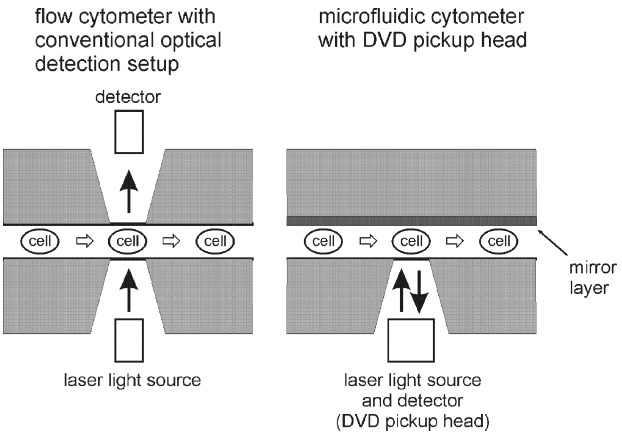
1. optical detection

2. impedometric detection.

Microfluidic flow cytometers with optical detection

The general measurement principle in optical-based flow cytometry is based on laser excitation of either natively fluorescent or fluorescently stained biomaterial and the subsequent detection of the corresponding fluorescent response, scattered and transmitted light. Following the commercial success of macro-scale conventional flow cytometers with optical detection, several successful proof-of-principle studies of microfluidic flow cytometers integrated with optical detection systems have been reported during the past decade.[41] However, in the majority of cases these systems still represent rather a combination of macro- and micro-scale technologies since not all necessary optical parts can be miniaturized to microchip dimensions, thus not being considerably different from conventional optical flow cytometers. Moreover, pretreatment steps, such as, cell staining or modification using markers or antibodies (which are usually performed out of chip) are also required. Whole systems with peripherals tend therefore to be still relatively large. In addition, the detection instruments used are expensive and require an expert for alignment (between the laser light source and the detector) or troubleshooting.

In this context, a more promising concept has been presented by Kostner and Vellekoop,[42] where the optical detection part of the microfluidic flow cytometer is based on a standard, low-cost DVD pickup head combined with a mirror layer (highly reflective titanium surface) (Fig. 5). This concept

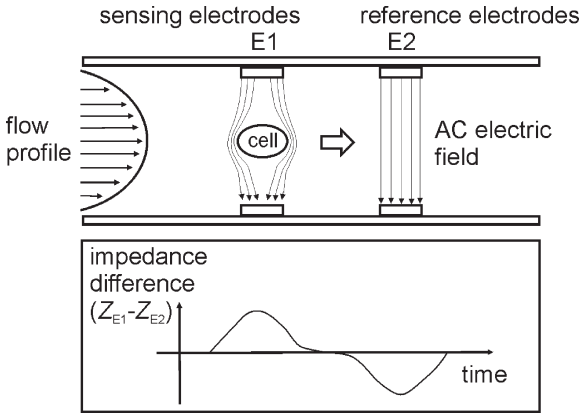


F i g . 5 – *Optical microfluidic flow cytometry: comparison of conventional optical detection setup with a mirror setup using DVD pickup head-based setup*

offers several advantages – due to the integration of the laser light source (laser diode) and the detector (photodiodes) in the DVD pickup unit, the problem of alignment is not critical and, in addition, the cost of the traditionally expensive optical part is no longer an issue because a widely available mass product is used. The functionality of this concept has been proved in a flow cytometric analysis of animal red blood and yeast cells.

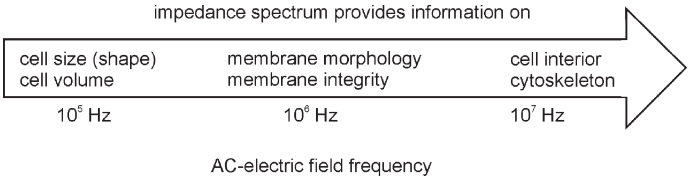
Microfluidic flow cytometers with impedometric detection

Compared to optical-based microfluidic-FC systems, microfluidic flow cytometers with electrical detection are more attractive in terms of miniaturization, simplicity, and cost effectiveness. Specifically, flow cytometers with impedometric detection do not require time-consuming sample preparation, expensive reagents or equipment, and eliminate the need for staining of live cells, which can cause cell damage.



F i g . 6 – *Principle of impedometric detection in microfluidic flow cytometry*

Generally, dielectric or impedance spectroscopy is suitable for the characterization of living biological cells. Impedance measurements over a wide frequency range give information on cell size, membrane capacitance, cytoplasm conductivity, and cytoplasm permittivity as a function of frequency. At low frequencies, the polarized cell membrane poses a barrier to the flow of electrical current and thus the impedance signal amplitude is related to the cell size. At intermediate frequencies, the polarization of the cell membrane is less intense and the signal reflects the properties of the membrane itself. Finally, at high frequencies, the polarization of the cell membrane is only minimal and the measured signal is linked to the properties of the cell interior and intracellular structures. All this information can be used to distinguish cell populations without the need for fluorescent, magnetic, or other cell markers (Fig. 7).[44–46]



F i g . 7 – *Impedance measurement for cell characterization: AC electric field frequencies and the detection of cell properties*

Case study of a microfabricated impedance spectroscopy cytometer developed at EPF Lausanne in cooperation with Leister Microsystems has been presented capable of cell discrimination based on differences in cell size, membrane capacitance and cytoplasm conductivity.[47]

The reported microfabricated impedance spectroscopy flow cytometer permits rapid dielectric characterization of a cell population. Impedance measurements over a wide frequency range provided information on cell size, membrane capacitance, and cytoplasm conductivity as a function of frequency. The information about amplitude, phase and opacity, *i.e.* the ratio of the high frequency to a low frequency impedance amplitude, were used for discrimination between different cell populations without the use of cell markers. Polystyrene beads, red blood cells (RBCs), ghosts, and RBCs fixed in glutaraldehyde were passed through the system and measured individually by using two simultaneously applied discrete frequencies. The cells were characterized at 1000 per minute in the frequency range of 350 kHz to 20 MHz. Cell size was easily measured with submicron accuracy. Polystyrene beads and RBCs were differentiated using opacity. RBCs and ghosts were differentiated using phase information, whereas RBCs and fixed RBCs were differentiated using opacity. RBCs fixed using increasing concentrations of glutaraldehyde showed increasing opacity. This increased opacity was linked to decreased cytoplasm conductivity and decreased membrane capacitance, both resulting from protein cross-linking.[48]

The microfabricated flow cytometer has also been successfully used for the detection of *Babesia bovis* infected red blood cells. The cellular modifications caused by the intracellular parasite result in a shift in impedance which can be measured dielectrically – changes of the signal amplitudes at a frequency of 8.7 MHz are clearly observable for samples containing infected cells. These changes at high frequency indicate a variation of the dielectric properties of the cytoplasm. Unlike other diagnostic tests, this method does not depend on extensive sample pre-treatment or expensive chemicals and equipment.[45]

The above system is currently being further developed by Axetris – the Microsystems Division of Leister, into a commercial microfabricated flow cytometer.[46] Its main features include: non-invasive, label-free and near online single-cell analysis (short sample preparation), high throughput (cell counting – 10^5 cells/min, cell characterisation – 10^2–10^3 cells/min).[49] In addition to the above mentioned applications, the system has been shown to be capable of detecting the presence of fatty inclusions in bacterial cells (unpublished results).

Further research in this area aims at developing a microfabricated flow cytometer capable of broadband single cell impedance spectroscopy. Correspondingly, an advanced type of microfabricated impedance-spectroscopy flow cytometer capable of measuring single cells (RBC) at high speed at a number of discrete frequencies (frequency range 100 kHz – 10 MHz, 2000 cells per frequency point) has been developed in cooperation between EPFL and University of Southampton.[50] Also in collaboration of these two research teams, another microfluidic impedometric FC has been developed that is based on maximum length sequence (MLS) method allowing the measurement of time-dependent impedance of a sample with high temporal resolution over a large bandwidth. Specifically, the applicability of this technique has been demonstrated by characterizing the impedance spectrum of red blood cells (RBCs) – the impedance being measured within 1 ms at 512 discrete frequencies, evenly distributed in the range from 976.56 Hz to 500 kHz. Such a microfabricated broadband single cell impedance spectroscopy flow cytometer should enable complete dielectric characterization of a single cell, including the membrane, cytoplasm and nucleus.[51]

Commercially available systems

The majority of the currently commercially available flow cytometers marketed as chip- or microcytometers are in fact bench-top systems on the fringe between macro- and micro-scale (all are off-line systems).

From these, probably the most widely used device is the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) – a microchip-based analysis system for simple flow cytometry primarily of eukaryotic cells, representing a relatively inexpensive system when compared to conventional macro-scale FC systems, operational without the need for substantial user training or experience. This instrument is capable of two-color fluorescence detection. The blue LED has a maximum emission at 470 nm. The maximum emission of the red laser diode is at 630 nm. The detection windows are at 525 nm for the green and 680 nm for the far-red channel. The system allows simple flow cytometric studies of cells in a disposable microfluidic chip. "On-chip" staining procedures are supported, thus eliminating time-consuming washing steps. Cells and staining-reagents are loaded directly onto the microfluidic chip and analysis is started after a short incubation time. These procedures require only a fraction of the staining reagents generally needed for flow cytometry and only 30 000 cells per sample, resulting in savings in terms of the amount of time, cells, and reagents needed.[52]

Even though the Agilent 2100 Bioanalyzer is primarily intended for the analysis of eukaryotic cells, a successful application for bacterial studies has also been reported – rapid quantification of the *E. coli* bacteria both in culture and in environmental samples. Results achieved by this technique were similar to those obtained by direct microscopic count. The time required for this on-chip flow cytometry was only 30 minutes per 6 samples.[53]

Other similar systems in this category include:

– C-Box (Digital Bio Technology, Korea) –

– A microfluidics-based bench-top system flow cytometry system incorporating a disposable plastic microchip

– Guava EasyCyte (Guava Technologies, USA) –

– A bench-top microcapillary-based sheath fluid-free flow cytometry system

– Microcytometer System (Micronics, Inc., USA) –

A portable flow cytometer for rapid blood analysis (sample to result time < 5 min).

Conclusion

In biotechnology and life sciences, single-cell analysis is currently probably the most important application field for micro-scale chip-based analytical devices. Due to their design, these devices can offer several advantages for this type of analysis over conventional macro-scale systems. However, practically all of the reported applications have been confined to off-line use. In this respect, there still are significant limitations, principally related to low measurement rate and complicated sample preparation/treatment. A comparison of reported devices from this viewpoint is presented in Table 3.

The comparison table reveals that, despite the recent remarkable progress, still only a few of the reported micro-scale chip-based analytical devices for cell analysis possess features necessary for an eventual application in on-line bioprocess monitoring and control. From this perspective, the most promising candidate appears to be the microfluidic

Table 3 – *Summarized comparison of reported micro-scale chip-based analytical devices for cell analysis*

Sensor category	Specific application	Sample treatment		Measurement/ throughput rate	On-line capability	Commercial availability
		Cell lysis	Sample staining/ labeling			
Transcriptomics	Focused gene expression monitoring[13,21]	yes	no	total analysis time: 0.5–3 h	no (planned for TRAC chip)	no
Proteomics/ Metabolomics	Single cell analysis (protein fingerprinting)[25,27,36–38]	yes	both alternatives possible	1 cell/min – 4 cells/h	no	no
Cytometry (optical detection)	Cell quantification, characterisation (flow cytometric analysis of cell fluorescence parameters)[52,53]	no	yes, in most cases	~10^3 cells/min	no	yes (*e.g.* Agilent's 2100 Bioanalyzer)
Cytometry (impedometric detection)	Cell quantification, characterisation (cell size, membrane capacitance, cytoplasm conductivity)[44–49]	no	no	~10^2 – 10^5 cells/min	at-line (on-line operation as future option)	prototype stage (commercial product expected soon)

flow cytometry with impedometric detection, combining a sufficiently high measurement rate with a relatively simple sample treatment. With regard to microbial biopolymer production processes, this technology might be particularly useful for in vivo at-line or even on-line monitoring of the accumulation of intracellular storage material consisting of biopolymer granules in microbial cells.

Potentially, in the future, devices implementing focused gene expression monitoring techniques like the TRAC chip could also provide a valuable tool for on-line monitoring of expression activities of selected genes in microbial cultures producing industrially relevant products. For this purpose, the specific genes related to the production of the desired product (*e.g.* biopolymer) in the particular microbial strain must be identified first.

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