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REVIEW

Microfluidic single-cell analysis of intracellular compounds

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Biological analyses traditionally probe cell ensembles in the range of 10^3–10^6 cells, thereby completely averaging over relevant individual cell responses, such as differences in cell proliferation, responses to external stimuli or disease onset. In past years, this fact has been realized and increasing interest has evolved for single-cell analytical methods, which could give exciting new insights into genomics, proteomics, transcriptomics and systems biology. Microfluidic or lab-on-a-chip devices are the method of choice for single-cell analytical tools as they allow the integration of a variety of necessary process steps involved in single-cell analysis, such as selection, navigation, positioning or lysis of single cells as well as separation and detection of cellular analytes. Along with this advantageous integration, microfluidic devices confine single cells in compartments near their intrinsic volume, thus minimizing dilution effects and increasing detection sensitivity. This review overviews the developments and achievements of microfluidic single-cell analysis of intracellular compounds in the past few years, from proof-of-principle devices to applications demonstrating a high biological relevance.

Keywords: single cell; microfluidic; separation; analysis; destructive; non-destructive

1. INTRODUCTION

What innovation potential is behind single-cell analysis? Why is there an increasing interest of researchers from a variety of disciplines to investigate and develop single-cell analytical tools? The answer to these questions is given very comprehensibly by a recent feature article from *Analytical Chemistry* (Di Carlo & Lee 2006): consider a bimodal distribution of a specific compound in a cell line, such as a high- and low-copy-number expression of a specific protein. An ensemble method would detect a mean value masking the bimodal distribution within the cell ensemble. However, analysing individual cells could resolve two subpopulations—one with high and another with low copy numbers—thus revealing the two different states of expression levels. This simple example can be amplified to more complicated and relevant questions of biological importance, such as the different states during differentiation, proliferation or disease and different responses to external stimuli and intracellular reactions. Single-cell analytical tools further offer the possibility of dynamical studies on the single-cell level as well as the access to the investigation of subcellular compartments. Biological disciplines in which such studies would be of great importance include, but are not limited to, environmental and developmental biology, systems biology or tissue engineering.

The difficulty in single-cell analysis arises from the manipulation of the tiny cellular objects that can be as small as a micrometre or even below, such as in the case of bacteria. Furthermore, single-cell analytes of interest are extremely complex as well as often only abundant in minute amounts. Confining small cells in environments such as micrometre-sized containers close to their intrinsic volume thus allows the handling and analysis of single cells with minimized dilution. Hence, the first approaches for single-cell analysis have been demonstrated with microcolumn separation techniques (Kennedy *et al.* 1989) and capillary electrophoresis (CE; Wallingford & Ewing 1988). This was followed by approaches from several research laboratories, demonstrating impressive examples of single-cell analysis in the capillary format including one- and two-dimensional separation methods. A variety of capillary-based analysis methods for single cells have recently been reviewed (Cannon *et al.* 2000; Stuart & Sweedler 2003; Woods *et al.* 2004; Liu *et al.* 2006; Arcibal *et al.* 2007).

However, conventional capillary-based techniques lack the ease of high-throughput analysis of single cells due to sophisticated cell-loading procedures.

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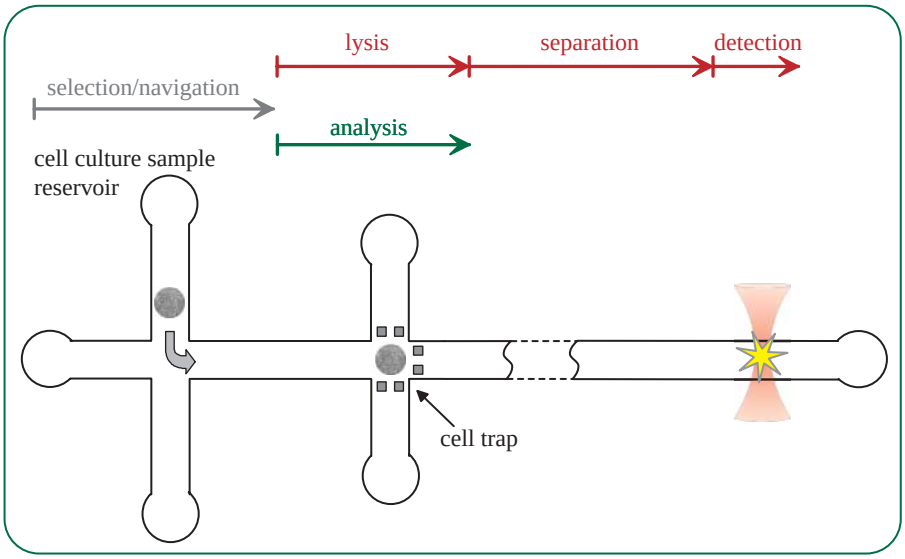

Figure 1. Generic workflow for single-cell analysis on a microfluidic platform (top view). The first step includes the selection and navigation of single cells from a cell ensemble to a lysis or an analysis position in a microfluidic channel. In this example, single-cell trapping is performed geometrically with posts defining the trapping position. *Non-destructive* analysis of intracellular compounds can be carried out at this position as indicated by the green arrow. For *destructive* single-cell analysis, additional single-cell lysis, separation and detection of cytosolic compounds are performed, as demonstrated by the red arrows. Laser-induced fluorescence is schematically indicated at the detection position. (Dimensions of channels and reservoirs are not drawn to scale.)

Microfluidic devices offer a versatile alternative to overcome this problem. These devices exhibit the possibility of integrating a variety of cellular operations on the micrometre scale, such as the positioning, trapping or lysis of single cells, as well as detection, analysis and even separation of cellular compounds. Such lab-on-a-chip devices further allow the analysis with improved performance, throughput, parallelization and automation. Apart from these advantages, one has to recognize some unique physical principles governing analytical techniques in microfluidic systems. In general, low Reynolds numbers assure laminar flow conditions and mass transport is diffusion dominated with important consequences for mixing and dispersion. A variety of physical principles can be employed to provoke fluid manipulation and pumping or the generation of novel separation principles. We refer the interested reader to recent review articles describing important physical phenomena occurring in microfluidic systems (Stone *et al.* 2004; Squires & Quake 2005) and concentrate here on the biological achievement and significance of microfluidic single-cell analysis tools.

Microfluidic devices have been an intensive field of research in the analytical and bioanalytical community since the 1990s and biologically relevant applications in the field of proteomics and genomics have been reviewed extensively (Auroux *et al.* 2002; Reyes *et al.* 2002; Lion *et al.* 2003; Vilkner *et al.* 2004). Designing and constructing a microfluidic device usually requires knowledge from several disciplines, such as microengineering (e.g. device fabrication and integration), biology (e.g. under which conditions the cells can be handled with minimum stress), chemistry (e.g. the knowledge of the surface characteristics of the microfluidic device and successful derivatization strategies) or physics (e.g. physical phenomena at small scales and detection and manipulation techniques).

In general, microfluidic devices for single-cell analysis are fabricated with standard technologies, ensuring a straightforward interplay of the various components needed for a functioning platform. The design and the necessary components of single-cell analytical platforms are specifically adapted in their size, biocompatibility, suitability for the employed detection or separation technique, cell manipulation, fluid and analyte pumping, etc. Microfabrication methods have been reviewed in textbooks in which basic introductions to microtechnology are covered in Bhushan (2004) and Geschke *et al.* (2004), whereas a more comprehensive overview is given by Madou (2002). However, two well-established methods are mostly used for microfluidic single-cell analysis devices. First, standard procedures for microfabrication in glass combining photolithography, wet etching and final bonding procedures find frequent application. Second, numerous single-cell analytical tools have been developed via moulding of poly(dimethylsiloxane) (PDMS) from a master wafer also known as soft lithography (Xia & Whitesides 1998; Sia & Whitesides 2003). These two techniques provide transparent devices that are especially favourable for fluorescence-based detection techniques. Furthermore, glass resembles many properties of fused silica, which was traditionally employed as the capillary material in CE and is already well characterized. PDMS provides an easy integration of valves and pumping units into a microfluidic device, which facilitates single-cell operations. Additionally, it is gas permeable and microfluidic assembly can be performed without sophisticated clean-room equipment, and it is thus particularly suited for use in standard (bio-)analytical laboratories.

A generic microfluidic device for single-cell analysis is schematically depicted in figure 1. A typical workflow for single-cell analysis can be outlined as follows. A cell suspension such as a several microlitre sample of

an off-chip culture is placed in a reservoir of the microfluidic device. A selection step of a particular cell of interest follows together with a navigation step of the cell downstream the microfluidic device in order to immobilize the cell at a certain trapping position in the channel. A variety of methods for selection, navigation and trapping have been employed and reviewed in the literature (Andersson & van den Berg 2004*a*,*b*; Toriello *et al.* 2005; Yi *et al.* 2006; Price & Culbertson 2007). In our example, a geometrical cell trap is illustrated, which consists of posts confining the cell at a channel crossing.

Once in the cell trap, two general methods of single-cell analysis can be distinguished. *Non-destructive* analysis represents the first case in which the single cell is directly studied according to a detectable signal from a specific cell response. With the second method by contrast, the cell is lysed at the cell trapping position, thus delivering the intracellular components to the surrounding solution. This lysis allows a subsequent separation step that is highly advantageous for resolving the high complexity of analytes that can be found in a single cell. However, subsequent separation steps may be critical due to analyte adsorption on microchannel surfaces and the low abundance of many analytes of interest. A detection step finalizes this second analysis mode, to which we refer as destructive analysis. In our example, a laser focus indicates a sensitive laser-induced fluorescence (LIF) detector.

In this review, we intend to give an overview of destructive and non-destructive single-cell separation and analysis methods demonstrated previously. In contrast to review articles already available in the field of microfluidic single-cell analysis, we explicitly factor out selection, navigation and lysis involved in single-cell analysis. Clever techniques with sophisticated designs have been reported, which are reviewed in Longo & Hasty (2006), Price & Culbertson (2007), Roman *et al.* (2007) and Sims & Allbritton (2007). Here, we explicitly concentrate on intracellular analytes investigated in single cells with destructive and non-destructive techniques in the past few years, from proof-of-principle devices to applications with a high biological relevance.

2. DESTRUCTIVE ANALYSIS OF CELLULAR CONTENTS

This section focuses on the metabolites analysed from single cells probed by post-lysis (destructive) separation and detection. Traditionally, and as motivated in §1, most destructive techniques are based on separation by CE after single-cell lysis. LIF—a well-established and highly sensitive standard technique in CE—can be easily transferred to microfluidic devices. Hence, CE-LIF techniques were used for microfluidic single-cell analysis in many examples, as described in §2.1–2.4. We further divide this section into the groups of analytes investigated.

2.1. Dyes/metabolites

Well-established biological reactions such as the cellular uptake and hydrolysis of fluorescent dyes were among the first biological systems investigated in single cells on microfluidic platforms. McClain *et al.* (2003) could show sequential single-cell analysis in a glass microfluidic device combining hydrodynamic forces and electric fields. A double T-injector was used to deliver single Jurkat T cells to a lysis intersection prior to mixing with an emulsification reagent. A combination of AC voltages at 75 Hz with an additional DC offset resulted in complete cell lysis and simultaneously allowed electrophoretic separation of the previously incorporated cytosolic fluorescent dyes, Oregon green and carboxyfluorescein, from the Jurkat T cells. With a throughput of approximately 7–13 cells min^{-1}, this study could show that in approximately 10 per cent of analysed cells hydrolysis anomalies occurred. This example showed that microfluidic single-cell analyses have the potential to proceed faster than standard CE techniques due to the requirement for often time-consuming cell manipulation steps as well as longer separation times in conventional CE.

Munce *et al.* (2004) presented another method of electrophoretic dye separation from calcein-labelled myeloid leukaemia cells. Their polymethylmethacrylate microfluidic device was fabricated by laser ablation in combination with milling for larger features on the platform. In this device, cells were directed to a lysis position via optical tweezers, electromechanical shear was used to lyse cells and calcein derivatives could be detected in the subsequent electropherograms. Although sequential due to the optical tweezers' positioning step, this microfluidic device was capable of separating four myeloid leukaemia cells in parallel and could analyse approximately 24 cells h^{-1}.

The Fang group adapted the concepts described above and investigated reactive oxygen species in single erythrocytes. Glass microdevices consisting of simple layouts with one cross point for injection or with an additional T-junction were employed by this group. Their first example probed the fluorescence of rhodamine with a LIF detection set-up upon reaction of reactive oxygen species with non-fluorescent dihydrorhodamine (Sun *et al.* 2005). A single erythrocyte was analysed in approximately 3 min and the device was capable of detecting the occurrence of reactive oxygen species upon challenging the cell with hydrogen peroxide. Furthermore, this group demonstrated electropherograms of labelled glutathione in single erythrocytes. Post-lysis, cytoplasmic glutathione was labelled with naphthalene-2,3-dicarboxaldehyde (NDA) on-chip and analysed in subsequent electrophoresis with LIF detection (Gao *et al.* 2004).

The combination of the latter two techniques allowed the detection of intracellular reactive oxygen species and glutathione simultaneously. Both the species could be separated electrophoretically and detected with a LIF set-up with a detection limit in the attomole range, and allowed the analysis of these two compounds with respect to external stimulation with H_2O_2 (Ling *et al.* 2005). A variation of this design was presented with channels of different depths in a glass substrate by Sun & Yin (2006). This modification allowed the analysis of reactive oxygen species and glutathione of single HepG2 (human carcinoma) cells, which are generally hard to separate due to their strong adherence to surfaces. Using

hydrostatic pressure differences, cells could be effectively driven towards the lysis junction and trapped at a weir by applying pinching potentials. This device achieved a throughput of 15 cells h^{-1}.

The internal concentration of β-galactosidase, a widely used reporter gene product, was probed in HL-60 cells by Ocvirk *et al.* (2004) in a microfluidic glass chip. The cells were lysed on-chip in a Y-shaped mixing junction by incubation with a lysing buffer that also contained di-β-D-galactopyranoside fluorescein, a substrate of the β-galactosidase. The resulting fluorescein was determined by LIF measurement and allowed the quantification of β-galactosidase activity. An optimized protocol allowed the detection of β-galactosidase activity within 2 min after the lysis when compared with 1 hour in a conventional flow cytometer.

Electrochemical detection in a glass microfluidic device for single-cell analysis was exploited by Xia *et al.* (2005). A three-electrode detector was butted against the end of the separation channel of the microdevice and both the elements were mounted on a single slide. In this set-up, the detection sensitivity for ascorbic acid was 5 μM and thus allowed the electrophoretically based separation and electrochemical detection for ascorbic acid from single wheat callus cells. Although only one example of an electroactive species was given, this technique should be capable of analysing a variety of other electroactive species in single cells.

The examples within this section mostly describe proof-of-principle devices for single-cell analysis with a few well-characterized dyes or metabolites. However, they represent important studies demonstrating the feasibility of single-cell micofluidic analysis and point towards the investigation of more sophisticated biological problems and analytes with single-cell microfluidics, which we describe in the following sections.

2.2. RNA

The analysis of a cell's complete set of mRNA, the transcriptome, is an important step towards understanding cellular regulation. As expression changes are often very rapid and can be the result of minute changes in environmental conditions, mRNA levels and composition can vary considerably even in very homogeneous cell populations. RNA is found in higher abundance than DNA in the cell, but mRNA represents only a small fraction. In addition, it is well known that mRNA is unstable, especially due to degradation with RNases, making it a very challenging analyte. Incorporating all the necessary steps for RNA isolation and analysis, which usually includes a reverse transcription (RT) step into a single device, can reduce the necessary reaction times and thus prevent RNA degradation. However, to date, few examples exist for the complete integration of RNA isolation and RT-polymer chain reaction (PCR) on a single microfluidic chip with single-cell resolution.

Among the few examples of single-cell RNA analysis after destructive lysis, the work of Marcus *et al.* (2006) can be found. This multilayer PDMS device incorporated cell lysis, affinity purification of mRNA as well as cDNA synthesis on a single chip, and its capabilities were demonstrated with single mouse fibroblast cells in up to four parallel reactions. The cells were lysed in a ring section and the cell contents were then pressure driven over an area stacked with paramagnetic beads derivatized with oligo(dT) sequences to capture the mRNA. The authors found that the extracted amounts of mRNA per cell (0.5–2.5 pg) were well within the range of expected values and also more efficient than a number of commercial kits. These findings indicate that within the time frame necessary for the complete reaction, no significant degradation of RNA has occurred.

Zhong *et al.* also constructed a multilayer PDMS device capable of processing 20 single cells simultaneously. The authors used the microfluidic device to extract RNA from single human embryonic stem cells and convert the mRNA to cDNA with approximately five times the efficiency of conventional bulk assays (Zhong *et al.* 2008). Based on this, the authors estimated that the microdevice would be able to detect as low as two mRNA copies per cell under analysis. Moreover, after probing the expression of a number of genes on the single-cell level by off-line quantitative PCR, the authors found the gene expression levels of individual cells to be very diverse. This is of high interest as the cells were pre-sorted to be in the G0/G1 cell cycle phase. These results clearly indicate the higher resolution of single-cell analyses when compared with traditional cell-sorting-enabled bulk analyses.

A very sophisticated method for single-cell whole-transcriptome analyses was recently described by Bontoux *et al.* (2008). The starting material was single embryonic neuronal mouse cells isolated from caudal ganglionic eminence (CGE) explants. Cell lysis and RT followed by PCR were carried out on a rotary microfluidic platform. Again, a multilayer PDMS device is employed, which is described in more detail in figure 2. Deformable, soft walls integrated in the microfluidic platform serve as valves and peristaltic pumps to actuate the liquid and perform cell trapping. Figure 2*a* shows the corresponding two layers of fluidic channels (in black) as well as actuation channels serving as pumping or valving channels (in grey). Valves can be created by one single actuation membrane, whereas a peristaltic pump is created by cyclically driving three actuation channels. Accordingly, a cell trap formed by four deformable actuators is shown in figure 2*b*. Deformable membranes realized in PDMS devices (Unger *et al.* 2000) are insensitive to almost all physico-chemical properties of the microdevice (Squires & Quake 2005); however, valves are sensitive to high pumping pressures. This principle has been applied to a variety of other analytical microfluidic devices, some of them for single-cell analysis and are included in this review (Hong *et al.* 2004; Wu *et al.* 2004; Marcus *et al.* 2006; Huang *et al.* 2007; Marcy *et al.* 2007*a*,*b*; Zhong *et al.* 2008).

The PCR products resulting in the device depicted in figure 2 were analysed on whole-genome DNA microarrays (Bontoux *et al.* 2008). The device showed overall improved RT in nanolitre volumes when compared with conventional approaches. Moreover, a comparison of the profiles created from single cells revealed that, on average, approximately 5000 genes were expressed, whereas only 2445 were common

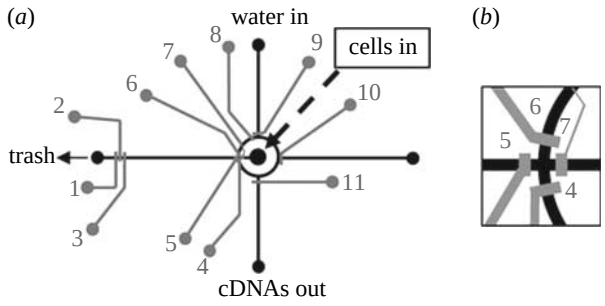

Figure 2. Schematics of a rotary device for single-cell transcriptome analysis. (*a*) A multilayer PDMS microfluidic platform incorporates both fluidic channels (black lines) and actuation channels (grey lines) that serve as peristaltic pumps, valves or cell traps. (*b*) The cell trapping position at the cross of the circular channel with the cell inlet channel. Subsequent to single-cell selection, the RT reaction of a single cell is performed after lysis in the circular channel under optimum RT temperature and continuous peristaltic pumping. cDNA product can be extracted thereafter from the device for further analysis. Adapted from Bontoux *et al.* (2008) with permission of the Royal Society of Chemistry.

among all cells. Together with the fact that the detected transcripts represent only 21 per cent of all expressed genes in the whole CGE explant, it is apparent that single-cell studies allow the identification of hitherto unobservable heterogeneity in tissues. This work also demonstrates the feasibility of combining single-cell analyses with traditional whole-throughput techniques to acquire global data with unprecedented resolution.

All these reports demonstrate that transcription analyses of single eukaryotic cells are feasible with microfabricated devices. Interestingly, the efficiency of the RT reactions was consistently higher within the microdevices when compared with microlitre-scale standard reactions. This is most likely a result of the high concentration of RNA in the small volume confinement of the microarrays. In all reports, the cell-to-cell variation of transcript abundance was found to be very high, regardless of the genes under investigation. This was mostly attributed to the heterogeneity within the sample; however, it was not unequivocally determined whether the differences might also result from experimental variation or from cell manipulations within the devices.

2.3. DNA

Interestingly, only few examples can be found for DNA separation and analysis of single cells in microfluidic devices. This is astonishing, as rapid and high-throughput analysis of DNA as well as sequencing was one of the cornerstones of microfluidics. Among the few examples, one finds the work of Prinz *et al.*, who used osmotic pressure to lyse single *Escherichia coli* cells in a T-junction by diffusive mixing with deionized water in a PDMS microdevice. The device did not include a means for the on-device separation of cells, but required loading with a cell dilution. However, the camera set-up allowed the monitoring of the lysis of single cells. After lysis, the bacterial chromosome was separated from the other cytoplasmic content by dielectrophoretic trapping (Prinz *et al.* 2002). A disadvantage of this system is that *E. coli* cells had to be pretreated with lysozyme in order to yield osmotic unstable spheroblast cells to achieve effective lysis.

Another PDMS device for DNA isolation was created by the Quake group who used a rotary mixer to effectively lyse untreated *E. coli* cells within a total volume of 5 nl (Hong *et al.* 2004). The DNA was captured on an affinity column and subsequently recovered with an elution buffer. Moreover, the basic design of this chip allowed a parallelization of this process and the authors demonstrated this with a chip that allowed three isolations in parallel.

At this point, one might actually question the virtue of isolating DNA from single cells. As the genotype within pure cell lines is usually identical, it can be argued that single-cell DNA extractions do not provide more information than regular bulk extractions. The work from Kleparnik, however, demonstrated that meaningful information can be gained from such microfluidic devices (Kleparnik & Horky 2003). They used a microfluidic device based on a commercial polycarbonate platform to semi-quantitatively measure DNA fragmentation in mouse cardiomyocytes upon doxorubicin treatment. The cells were moved to a cross section of the polycarbonate microdevice by hydrodynamic flow and lysed with NaOH for approximately 5 min. By applying an electric field, the DNA was separated within a linear polyacrylamide matrix in the electrophoresis section of this device. The migration distance of 4 mm was sufficient to distinguish fragment sizes with a resolution of 180 bp. Doxorubicin is a widely used anticancer chemotherapeutic; however, chronic use can lead to the destruction of cardiomyocytes and, ultimately, heart failure. The results in this report demonstrate a link between DNA fragmentation and doxorubicin exposure, which was not apparent using immunohistochemical staining methods. Thus, information gained from this device can lead to further insights into the cardiotoxicity of doxorubicin and might also be employed for the analysis of other DNA damaging drugs on a single-cell level.

An even more intriguing application of microfluidic devices is the analysis of complex populations of organisms with different genotypes. An example is the dissection of complex bacterial communities by the analysis of the DNA of individual cells. A commercially available PDMS microfluidic device has been applied as a high-throughput microfluidic PCR system with which the lignocellulose-decomposing microbial community of wood-feeding termite hindguts was analysed (Ottesen *et al.* 2006). The cell mixture obtained from termites was separated on the chip and individually partitioned cells were used as templates for multiplexed PCR to amplify a gene with known metabolic function, in this case a gene essential for homoacetogenesis, as well as the corresponding 16S rRNA gene sequence of the bacterium. This strategy allows the linking of certain metabolic activities to individual species via the 16S rRNA sequences as taxonomical markers. Traditional bulk strategies are usually not able to assign metabolic function to specific

taxons, unless complex labelling strategies are employed. This strategy could be used to gain valuable information from bacterial communities that are lost in pooled DNA or RNA samples.

Other approaches elaborate on this basic principle and have been applied to sequencing of whole genomes. In order to increase signal intensity, the Quake group integrated a multiple displacement amplification (MDA) step using the Φ29 bacteriophage polymerase onto a PDMS microfluidic platform. This strategy has been successfully employed to automatically isolate and amplify chromosomal DNA from uncultivated bacteria derived from complex human mouth samples. Using an elaborate sequential valve-controlled chamber system, the cells were first placed in a cell-sorting chamber, then flushed into the lysis chamber and allowed to mix via diffusion. Afterwards, the whole solution is flushed into a neutralization compartment and then finally into the main reaction chamber in which the amplification reaction occurs within a total volume of 50 nl. After an additional off-chip DNA amplification step, sufficient DNA was obtained to perform direct sequencing (Marcy *et al.* 2007*b*). In another work, the same group analysed the efficiency of MDA in nanolitre volumes on a PDMS microchip device in more detail and came to the conclusion that the quality of the amplification was higher than that in traditional microlitre volumes (Marcy *et al.* 2007*a*).

These examples demonstrate that single-cell manipulations combined with whole DNA amplification are expected to be a powerful tool for single-cell sequencing projects. The possibility of performing single-cell genomics opens up completely new avenues to dissect complex interactions as well as to identify and genetically characterize low-abundance organisms in nature.

2.4. Proteins and amino acids

As motivated in §1, expression levels as well as composition of proteins can vary extremely within single cells in one population. Moreover, no simple means exist to amplify proteins as is possible with nucleic acids by PCR, a problem that is exacerbated by the fact that the properties and quantities are very diverse. Generally, proteins are considered as low abundant if their copy number is less than 10^5. If we consider a typical eukaryotic cell with a diameter of 10 μm, one can consider that this protein number is contained in a volume of approximately 1 pl. Thus, for low-copy-number proteins, concentration limits below 100 nM have to be accomplished. Again, CE-LIF is a suitable method, as detection limits for proteins in the picomolar to nanomolar range can be accomplished for both unlabelled proteins with UV-LIF (Lee & Yeung 1992; Tseng & Chang 2000) and labelled proteins employing LIF in the visible range (Harvey *et al.* 1998; Lee *et al.* 1998). Therefore, it was only obvious that this method was also applied for the single-cell analysis of proteins on microfluidic platforms.

An electrophoretic approach to protein analysis from single cells was developed by Hellmich *et al.* (2005). The group used optical tweezers for cell selection and navigation in the microfluidic device and combined it with geometrical cell traps as the lysis position. Sf9 insect cells from *Spodoptera frugiperda* exhibiting a protein GFP-mutant were successfully positioned individually, lysed and the cell content transported electrokinetically in a separation channel where it was detected with a LIF detector on a PDMS microfluidic device. Later, two fluorescent protein species, a GFP- and a YFP-mutant, could be separated and detected from single Sf9 cells (Ros *et al.* 2006).

Omitting the necessity of protein labelling with dyes or via genetic engineering, UV-LIF detection for proteins in single cells was reported. A UV laser (wavelength 266 nm) and a UV-sensitive photomultiplier on an inverted microscope suitable for operation in the UV range could demonstrate detection sensitivity and separation efficiency from standard samples of the fluorescent amino acid tryptophan in the nanomolar range. The microfluidic devices fabricated in PDMS and the optical set-up were further improved to detect the first single-cell electropherograms from Sf9 cells with UV-LIF detection (Hellmich *et al.* 2006).

Another interesting approach is to identify specific proteins in single cells by targeted protein labelling. A non-electrophoretic microfluidic approach was constructed by Irimia *et al.*, which allowed the biochemical analysis of single cells at a picolitre scale on a PDMS device. Single human lymphoblast cells were trapped and lysed with SDS using the gas–water interface as passive valves and barriers (Irimia *et al.* 2004). With this device, the authors measured the release of a preloaded fluorescent dye from cells and were able to estimate the internal concentration. More importantly, in a second experiment, the authors used the device to determine the concentration of intracellular insoluble actin filaments labelled with fluorescent Oregon green phalloidin. While designed as a proof-of-principle experiment, it was demonstrated that quantitative analyses of protein contents in single cells are possible.

Richard Zare and his group developed a system for the on-chip lysis of Jurkat T cells in 70 pl chambers on a PDMS platform. The released amino acids were derivatized with NDA and separated by micellar electrokinetic chromatography (Wu *et al.* 2004), thus allowing the identification of individual amino acids. The Zare group further presented the first example to count proteins in single cells (Huang *et al.* 2007). They employed a PDMS microfluidic chip with integrated valves and pumps to select and lyse single cells. The lysing buffer was supplemented with fluorescent antibodies to tag target proteins. Upon electrophoretic transport and separation, the fluorescent signals from individual proteins were detected with the use of a cylindrical lens and thus protein copy numbers from single cells were counted. With this innovative approach, the heterogeneous expression of human β2-adrenergic receptors in insect cells could be quantified. In another experiment, the degradation of phycobiliprotein complexes of individual photosynthetic *Synechocystis* cells during nutrient deficiency was analysed (figure 3). An interesting finding was that remarkable variation in the number of detectable phycobiliprotein complexes within the

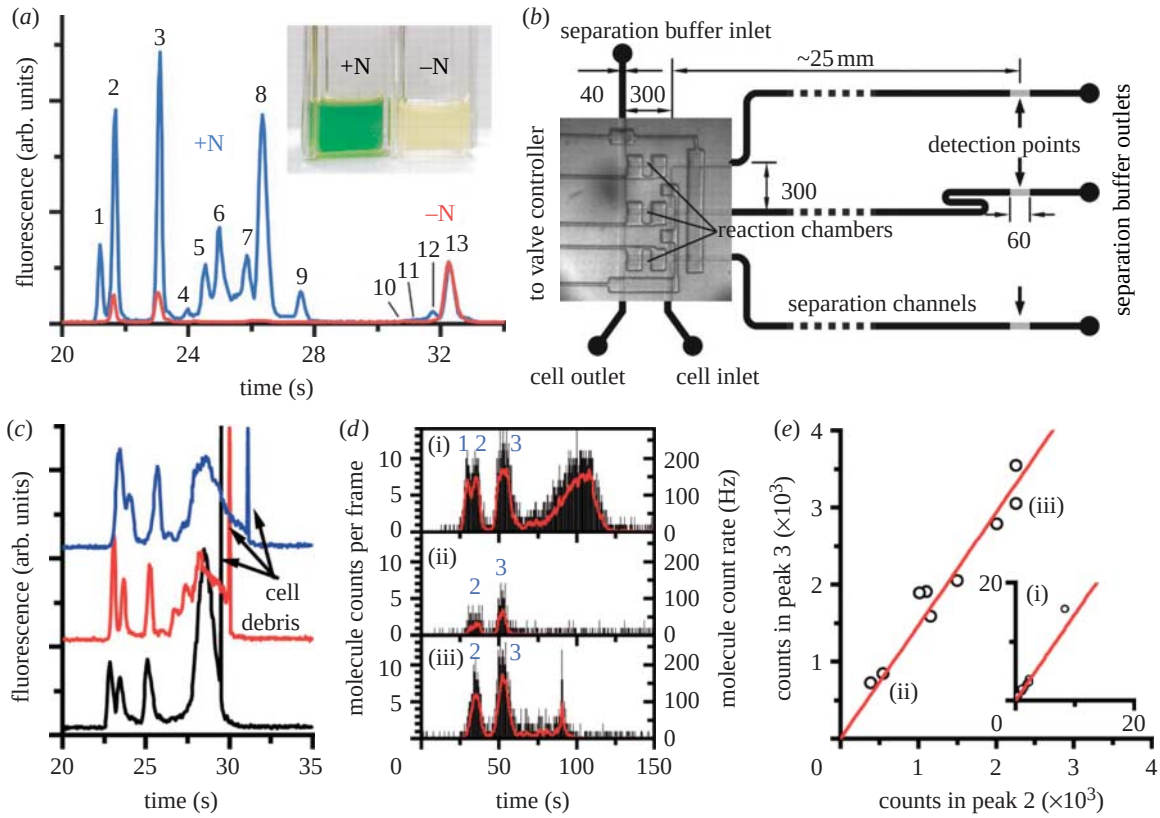

Figure 3. Microfluidic single-cell *Synechococcus* study. (*a*) Electropherograms of cell culture lysate under nitrogen-rich (+N) and nitrogen-depleted (−N) conditions. (*b*) The layout of the microfluidic device exhibits all necessary components for single-cell capture, lysis, separation and detection and allows the analysis of three cells on the same microfluidic platform. Note that pneumatic actuators control single-cell capture and lysis (photograph inset). (*c*) Examples of three single-cell electropherograms of phycobiliprotein complexes from +N cultures. (*d*) Single molecule counting results of three cells under −N conditions indicating remarkable differences in the number of detected phycobiliprotein complexes. (*e*) The high correlation between phycobiliprotein complexes of peaks 2 and 3 suggests a coordinated degradation of phycobilisome components. Adapted from Huang *et al.* (2007) with permission of AAAS.

nitrogen-depleted population suggested an inherent difference in the degradation process. Owing to the low occurrence of these cells (approx. 5% of all cells), they would not have been detected in population assays.

Interestingly, these studies do not report on protein degradation due to proteases. We attribute this to the favourable conditions in the microfluidic devices, in which single-cell analysis is carried out. The dilution is low due to the confined volume, and short lysis and separation times are achieved, and these studies were further carried out under denaturing conditions leading to minimal degradation.

3. NON-DESTRUCTIVE ANALYSIS

A possible disadvantage of destructive analyses of cells is the loss of temporal information as the same cell cannot be subjected to different subsequent stimuli. As many intracellular analytes are not naturally fluorescent or easily measurable with other means, a specific labelling of the respective compound is necessary. A common technique to monitor dynamic changes in living cells is the use of fluorescent dyes to track certain compounds or to use reporter gene fusions to analyse protein expression. Unfortunately, many fluorescent dyes are membrane impermeable, thus requiring a suitable technique to efficiently transfer compounds into single cells. In the following, we first introduce the use of electroporation as a means of compound delivery and further review examples of non-destructive single-cell analyses.

3.1. Electroporation

Electroporation is an important method in biology, delivering gene vectors and other membrane-impermeable substances to cells. Once successfully introduced, the operational genes can serve to produce a variety of cellular responses, such as the expression of a fluorescent fusion protein. While not being an analytical method *per se*, the integration of electroporation into a microfluidic device might offer a platform for automated (genetic) manipulation as well as the direct analysis of the manipulated cells. Besides actual transfer of compounds into a cell, the same set-up can also be used for rapid lysis of cells without the need for a lysis buffer. To date, most reports on electroporation in microfluidic devices concentrate more on the analysis of the electroporation reaction itself, rather than its use in integrated microdevices. However, owing to their potential for single-cell manipulations and analyses, these are included in this review.

An electroporation device for single cells was described by Huang & Rubinsky (2001). The microelectroporation device consisted of three layers of silicon chips, forming two liquid chambers. A micrometre-sized hole connects the cell sample containing a top layer with the electroporation electrode. By applying pressure differences, single cells are sucked into the hole, effectively plugging it before a voltage is applied. This device was used to analyse the effect of different electroporation conditions on the membrane integrity of human prostate adenocarcinoma cells. As only the cells trapped into a hole are affected by the electric field, similar set-ups might be useful to select and lyse or electroporate specific cells within a microdevice.

In Lee's group, PDMS chips for electroporation were developed, which combined parallelization and ease of use. In one device, HeLa cells were trapped in a small channel and pulled laterally into a trapping channel by negative pressure. This deformed the cell locally (Khine *et al.* 2005), thus creating an area of the cell with a higher resistance. This in turn allowed a local electroporation at a lower voltage. The actual electroporation was executed by a focusable electric field and the change in cell resistance during electroporation was monitored by measuring current jumps and delivery of the dye trypan blue into the cell. Using the same principles, an improved set-up was introduced later, which allowed highly parallelized electroporation assays. It also included a sophisticated feedback control system that facilitated the release of the electric field immediately after electroporation and thus improving resealing and viability of the cell (Khine *et al.* 2007).

A different strategy was employed by Ionescu-Zanetti *et al.* to enhance the efficiency of compound delivery. Before the electroporation, the molecules to be delivered are pre-concentrated electrophoretically in the PDMS device and after the electroporation, an electric field is maintained to drive the molecules into the cell (Ionescu-Zanetti *et al.* 2008). This system was used to translocate otherwise impermeable calcein and Oregon Green Dextran 514 into HeLa cells.

Valero *et al.* (2008) demonstrated successful electroporation of single myoblastic mouse cells as well as human mesenchymal stem cells on a silicon–glass microfluidic device. After successful electroporation with a vector, the device also allowed the continuous monitoring of the nuclear translocation of the introduced GFP-mutant in mesenchymal stem cells. While this experiment was intended to demonstrate the efficiency of microfluidic devices for the electroporation of rare samples such as bone-marrow-derived mesenchymal cells, it also hints at possible future developments of total integrated devices that incorporate genetic manipulation as well as further downstream analyses of single cells.

An electrode-free method of compound delivery within microfluidic devices is that of sonoporation. This method uses acoustic-driven bubble pulsations to create pores in membranes by inducing shear stress. Sonoporation of single cells in a microfluidic device was demonstrated by Gac *et al.* (2007). The cells were loaded into a PDMS chip and a single cavitation bubble was induced by a focused laser beam. The cell poration of human promyelocytic leukaemia cells has been probed by preloading the cell with calcein and monitoring the release of the dye after interaction with the bubble. While this method has not been as widely used as electroporation, it presents itself a possible alternative, integrated tool in microfluidic devices.

3.2. Impedance

The measurement of the electric properties of a cell allows the interrogation of certain global cellular properties. Especially, impedance spectroscopy over a wide frequency range allows the analysis of cell morphology, membrane capacitance and cytoplasm conductivity. As the impedance of cells is influenced by a multitude of aspects, including DNA content, protein expression and membrane composition, it is mostly suited to distinguish cells within a population according to these properties. This technique is further labelling free and it has been adapted for a number of flow cytometric approaches (e.g. Gawad *et al.* 2001, 2004; Cheung *et al.* 2005).

Recent advances aim towards more thorough impedance measurements on single trapped cells. James *et al.* (2008) used impedance spectroscopy to interrogate a single-cell response at a micrometre-sized pore. Single macrophages were trapped into pores of a silicon chip and the impedance of the cells was queried upon exposure to lipopolysaccharides (LPS) that are components of the outer membrane of Gram-negative cells and are a potent immunostimulant. An increased impedance across the capture pore holding the single cell was detected in the presence of the LPS, and this was related to a change in adhesion of the cells on the micrometre-sized pore. The robust set-up allows long-term measurements and clearly showed differences in the cellular response due to stimulation. This indirect approach of assessing cytoplasmic analytes has the potential to be coupled to lysis, separation and analysis of intracellular compounds.

A microdevice that uses micropillars as cell traps as well as microelectrodes for single-cell impedance spectroscopy was described by Jang & Wang (2007). This system was used to measure the impedance of individual HeLa cells. Another system set up by Gawad *et al.* (2007) applies impedance spectroscopy to single cells using the maximum-length sequence (MLS) measurement technique. The system was tested on human erythrocytes, and it was demonstrated that MLS measurement techniques are suitable for high-speed analyses.

In conclusion, impedance techniques can provide tools for globular differentiation even on the single-cell level; however, the application of these techniques to biological questions of more complexity remains to be demonstrated.

3.3. Fluorescence-based methods

A number of microfluidic devices have been used to monitor cellular responses based on fluorescence-tagged compounds. A natural target for non-destructive single-cell analyses is cell signalling pathways. Cellular

responses can have very specific and unique temporal patterns that might not be visible in population-averaged experiments. Owing to the importance of Ca^{2+} as second messengers, a number of groups employed fluorescence techniques to track Ca^{2+} fluxes in single cells in microfluidic devices.

Wheeler *et al.* (2003) used a PDMS microfluidic device capable of selecting single cells from bulk solutions and precisely delivering nanolitre volumes to single cells. The device exploits the fact that within a T-junction under low Reynolds number conditions, a point of stagnation develops where the fluid divides into two streams. The authors created a docking area precisely at this position to trap the cells that are delivered via a focused sheath flow. With this device, viability assays and ionomycin-mediated calcium flux measurements of single Jurkat T cells as well as receptor-mediated calcium measurements in single U937 cells could be accomplished. Fluorescence signal recording of a Fluo3 Ca^{2+}-sensitive dye could reveal ringing and sustained elevation of Ca^{2+} concentrations in Jurkat T cells. Moreover, both ringing and single spike Ca^{2+} concentrations were monitored with single U937 cells.

A V-shaped retention structure incorporated into a commercial glass chip was used by Li *et al.* to capture and monitor Ca^{2+} flux in single cardiomyocytes (Li & Li 2005). This group further refined their technique with a weir structure fabricated by a low cost and one-step procedure for glass chips (Li *et al.* 2007). Dynamic responses in external stimulation of caffeine, daunorubicin (a cardiotoxic chemotherapeutic drug) and isoliquiritigenin (a herbal anticancer candidate) were monitored successfully. Ionomycin stimulation and Ca^{2+} flux were also investigated by Zhang *et al.* (2008) in single living Chinese hamster ovary cells. Their approach combined Raman and confocal microscopy and allowed agonist-evoked Ca^{2+} flux monitoring upon ionomycin stimulation.

Other targets for non-destructive fluorescent single analyses include the measurement of enzyme activities, provided that fluorescent substrates exist for them. Such a device from the Lee group allowed a highly parallelized assay of enzyme activity (Di Carlo *et al.* 2006). The PDMS device uses a high density of regular arrays to isolate single cells by a pure hydrodynamic flow. The trapped cells can then be monitored individually under a microscope. Using this device, the authors analysed the variation of carboxylesterase activity in three different cell lines (HeLa, 293T and Jurkat), as well as the inhibition of the esterases by nordihydroguaiaretic acid.

These examples demonstrate that dynamic signalling processes can be resolved with accurate time resolution and should be further applicable to other signalling processes within single cells.

3.4. Other techniques

An electrochemical approach to gene function analysis in yeast cells expressing the β-galactosidase reporter gene was demonstrated by Yasukawa *et al.* (2008). The cells were manipulated and trapped electrophoretically within a PDMS device. In order to measure β-galactosidase activity of the cells, *p*-aminophenyl galactopyranoside (PAPG) was used as a substrate. The enzymatic hydrolysis of PAPG to *p*-aminophenol was detected by cyclic voltammetry and amperometry in the analytical chamber of the device. The device could in theory facilitate subsequent analyses as after the analyses, the cells can be moved further by electrophoretic means.

A very interesting combination of on-chip analyte concentration and off-chip MS analysis was conducted by Jo *et al.* (2007). A single *Aplysia californica* bag cell was loaded in a PDMS microchannel with a functionalized gold strip that allowed the collection of neuropeptides released from the cell upon stimulation. Using a pressure-driven buffer flow, the released peptides could be directed into different channels, allowing a separate collection of peptides released pre-, during and post-stimulation. After the collection step, the microfluidic device is peeled off and a matrix is applied to the sample. The matrix-coated surface was loaded into a MALDI-TOF instrument with which peptides were identified. This allowed the specific detection of peptides, including acidic peptide as well as α-bag cell peptide released upon stimulation of the cell.

4. CONCLUSION AND OUTLOOK

Microfluidic devices offer the integration of various processing steps for single-cell analysis such as selection, navigation, positioning or lysis, and further allow high-efficiency separation and detection in biologically relevant concentration ranges. The first examples of microfluidic single-cell analyses addressed simple biological questions such as the uptake and hydrolysis of specific dyes into single cells. These examples have impressively shown the proof of principle of microfluidic single-cell analyses. During the past few years, these approaches have been refined and researchers from different disciplines collaborated in order to investigate more complex biological questions. Their studies have demonstrated that some of the expected open questions can indeed be addressed with microfluidic single-cell analysis. Such examples include, for example, the quantitative access to low-copy-number proteins from single cells (Huang *et al.* 2007) or the whole-transcriptome analysis of single cells, revealing a hitherto unobservable heterogeneity in tissue (Bontoux *et al.* 2008).

In the future, the microfluidic analysis and detection techniques reviewed here will be further improved such that microfluidic single-cell analysis can be extended to a multitude of other biological questions. Among them, one can find important questions of cellular responses to specific external stimuli, such as drugs or nanoparticles. The detection techniques will necessarily have to resolve dynamic cellular responses in order to give deep understanding of the involved mechanisms and pathways. Furthermore, highly parallel microfluidic platforms (first examples of which already exist) have to be constructed, in order to provide statistically relevant results of subpopulation behaviour when compared with the averaged ensemble. This may yield the required accurate data that are needed for systems biology approaches. Moreover, as already

demonstrated or at least foreshadowed by some of the reports described in this review, these devices are expected to have a profound impact on a number of further biological disciplines dealing with complex cell samples. Finally, one can easily envision the incorporation of a number of techniques to cultivate, manipulate and analyse cells in parallel under defined conditions, thus enabling high-throughput analyses with single-cell resolution. We believe that microdevices are on the edge of growing into a standard technique and serving for routine biological applications, as was previously the case, for example, for DNA microarrays. In the future, a collaborative effort for creating reliable, reproducible and user-friendly microfluidic platforms will most probably provide the breakthrough for single-cell analysis, providing insight into biological problems hitherto not unravelled.

REFERENCES

Andersson, H. & van den Berg, A. 2004*a* Microfabrication and microfluidics for tissue engineering: state of the art and future opportunities. *Lab Chip* **4**, 98–103. (doi:10.1039/b314469k)

Andersson, H. & van den Berg, A. 2004*b* Microtechnologies and nanotechnologies for single-cell analysis. *Curr. Opin. Biotechnol.* **15**, 44–49. (doi:10.1016/j.copbio.2004.01.004)

Arcibal, I. G., Santillo, M. F. & Ewing, A. G. 2007 Recent advances in capillary electrophoretic analysis of individual cells. *Anal. Bioanal. Chem.* **387**, 51–57. (doi:10.1007/s00216-006-0690-0)

Auroux, P. A., Iossifidis, D., Reyes, D. R. & Manz, A. 2002 Micro total analysis systems. 2. Analytical standard operations and applications. *Anal. Chem.* **74**, 2637–2652. (doi:10.1021/ac020239t)

Bhushan, B. 2004 *Springer handbook of nanotechnology*. Berlin, Germany: Springer.

Bontoux, N., Dauphinot, L., Vitalis, T., Studer, V., Chen, Y., Rossier, J. & Potier, M. C. 2008 Integrating whole transcriptome assays on a lab-on-a-chip for single cell gene profiling. *Lab Chip* **8**, 443–450. (doi:10.1039/b716543a)

Cannon Jr, D. M., Winograd, N. & Ewing, A. G. 2000 Quantitative chemical analysis of single cells. *Annu. Rev. Biophys. Biomol. Struct.* **29**, 239–263. (doi:10.1146/annurev.biophys.29.1.239)

Cheung, K., Gawad, S. & Renaud, P. 2005 Impedance spectroscopy flow cytometry: on-chip label-free cell differentiation. *Cytometry A* **65**, 124–132.

Di Carlo, D. & Lee, L. P. 2006 Dynamic single-cell analysis for quantitative biology. *Anal. Chem.* **78**, 7918–7925.

Di Carlo, D., Aghdam, N. & Lee, L. P. 2006 Single-cell enzyme concentrations, kinetics, and inhibition analysis using high-density hydrodynamic cell isolation arrays. *Anal. Chem.* **78**, 4925–4930. (doi:10.1021/ac060541s)

Gac, S. L., Zwaan, E., van den, B. A. & Ohl, C. D. 2007 Sonoporation of suspension cells with a single cavitation bubble in a microfluidic confinement. *Lab Chip* **7**, 1666–1672. (doi:10.1039/b712897p)

Gao, J., Yin, X. F. & Fang, Z. L. 2004 Integration of single cell injection, cell lysis, separation and detection of intracellular constituents on a microfluidic chip. *Lab Chip* **4**, 47–52. (doi:10.1039/b310552k)

Gawad, S., Schild, L. & Renaud, P. 2001 Micromachined impedance spectroscopy flow cytometer for cell analysis and particle sizing. *Lab Chip* **1**, 76–82. (doi:10.1039/b103933b)

Gawad, S., Cheung, K., Seger, U., Bertsch, A. & Renaud, P. 2004 Dielectric spectroscopy in a micromachined flow cytometer: theoretical and practical considerations. *Lab Chip* **4**, 241–251. (doi:10.1039/b313761a)

Gawad, S., Sun, T., Green, N. G. & Morgan, H. 2007 Impedance spectroscopy using maximum length sequences: application to single cell analysis. *Rev. Sci. Instrum.* **78**, 054 301. (doi:10.1063/1.2737751)

Geschke, O., Klank, H. & Tellemann, P. 2004 *Microsystem engineering of lab-on-a-chip devices*. Weinheim, Germany: Wiley-VCH.

Harvey, M. D., Bandilla, D. & Banks, P. R. 1998 Subnanomolar detection limit for sodium dodecyl sulfate capillary gel electrophoresis using a fluorogenic, noncovalent dye. *Electrophoresis* **19**, 2169–2174. (doi:10.1002/elps.1150191221)

Hellmich, W., Pelargus, C., Leffhalm, K., Ros, A. & Anselmetti, D. 2005 Single cell manipulation, analytics, and label-free protein detection in microfluidic devices for systems nanobiology. *Electrophoresis* **26**, 3689–3696. (doi:10.1002/elps.200500185)

Hellmich, W., Greif, D., Pelargus, C., Anselmetti, D. & Ros, A. 2006 Improved native UV laser induced fluorescence detection for single cell analysis in poly(dimethylsiloxane) microfluidic devices. *J. Chromtogr. A* **1130**, 195–200. (doi:10.1016/j.chroma.2006.06.008)

Hong, J. W., Studer, V., Hang, G., Anderson, W. F. & Quake, S. R. 2004 A nanoliter-scale nucleic acid processor with parallel architecture. *Nat. Biotechnol.* **22**, 435–439. (doi:10.1038/nbt951)

Huang, Y. & Rubinsky, B. 2001 Microfabricated electroporation chip for single cell membrane permeabilization. *Sens. Actuators A* **89**, 242–249. (doi:10.1016/S0924-4247(00)00557-4)

Huang, B., Wu, H. K., Bhaya, D., Grossman, A., Granier, S., Kobilka, B. K. & Zare, R. N. 2007 Counting low-copy number proteins in a single cell. *Science* **315**, 81–84. (doi:10.1126/science.1133992)

Ionescu-Zanetti, C., Blatz, A. & Khine, M. 2008 Electrophoresis-assisted single-cell electroporation for efficient intracellular delivery. *Biomed. Microdevices* **10**, 113–116. (doi:10.1007/s10544-007-9115-x)

Irimia, D., Tompkins, R. G. & Toner, M. 2004 Single-cell chemical lysis in picoliter-scale closed volumes using a microfabricated device. *Anal. Chem.* **76**, 6137–6143. (doi:10.1021/ac0497508)

James, C. D., Reuel, N., Lee, E. S., Davalos, R. V., Mani, S. S., Carroll-Portillo, A., Rebeil, R., Martino, A. & Apblett, C. A. 2008 Impedimetric and optical interrogation of single cells in a microfluidic device for real-time viability and chemical response assessment. *Biosens. Bioelectron.* **23**, 845–851. (doi:10.1016/j.bios.2007.08.022)

Jang, L. S. & Wang, M. H. 2007 Microfluidic device for cell capture and impedance measurement. *Biomed. Microdevices* **9**, 737–743. (doi:10.1007/s10544-007-9084-0)

Jo, K., Heien, M. L., Thompson, L. B., Zhong, M., Nuzzo, R. G. & Sweedler, J. V. 2007 Mass spectrometric imaging of peptide release from neuronal cells within microfluidic devices. *Lab Chip* **7**, 1454–1460. (doi:10.1039/b706940e)

Kennedy, R. T., Oates, M. D., Cooper, B. R., Nickerson, B. & Jorgenson, J. W. 1989 Microcolumn separations and the analysis of single cells. *Science* **246**, 57–63. (doi:10.1126/science.2675314)

Khine, M., Lau, A., Ionescu-Zanetti, C., Seo, J. & Lee, L. P. 2005 A single cell electroporation chip. *Lab Chip* **5**, 38–43. (doi:10.1039/b408352k)

Khine, M., Ionescu-Zanetti, C., Blatz, A., Wang, L. P. & Lee, L. P. 2007 Single-cell electroporation arrays with real-time monitoring and feedback control. *Lab Chip* **7**, 457–462. (doi:10.1039/b614356c)

Kleparnik, K. & Horky, M. 2003 Detection of DNA fragmentation in a single apoptotic cardiomyocyte by electrophoresis on a microfluidic device. *Electrophoresis* **24**, 3778–3783. (doi:10.1002/elps.200305667)

Lee, T. T. & Yeung, E. S. 1992 High-sensitivity laser-induced fluorescence detection of native proteins in capillary electrophoresis. *J. Chromatogr.* **595**, 319–325. (doi:10.1016/0021-9673(92)85174-R)

Lee, I. H., Pinto, D., Arriaga, E. A., Zhang, Z. R. & Dovichi, N. J. 1998 Picomolar analysis of proteins using electrophoretically mediated microanalysis and capillary electrophoresis with laser-induced fluorescence detection. *Anal. Chem.* **70**, 4546–4548. (doi:10.1021/ac980360t)

Li, X. J. & Li, P. C. H. 2005 Microfluidic selection and retention of a single cardiac myocyte, on-chip dye loading, cell contraction by chemical stimulation, and quantitative fluorescent analysis of intracellular calcium. *Anal. Chem.* **77**, 4315–4322. (doi:10.1021/ac048240a)

Li, X., Huang, J., Tibbits, G. F. & Li, P. C. 2007 Real-time monitoring of intracellular calcium dynamic mobilization of a single cardiomyocyte in a microfluidic chip pertaining to drug discovery. *Electrophoresis* **28**, 4723–4733. (doi:10.1002/elps.200700312)

Ling, Y. Y., Yin, X. F. & Fang, Z. L. 2005 Simultaneous determination of glutathione and reactive oxygen species in individual cells by microchip electrophoresis. *Electrophoresis* **26**, 4759–4766. (doi:10.1002/elps.200500232)

Lion, N. *et al.* 2003 Microfluidic systems in proteomics. *Electrophoresis* **24**, 3533–3562. (doi:10.1002/elps.200305629)

Liu, B. F., Xu, B., Zhang, G., Du, W. & Luo, Q. 2006 Microseparation toward systems biology. *J. Chromtogr. A* **1106**, 19–28. (doi:10.1016/j.chroma.2005.09.066)

Longo, D. & Hasty, J. 2006 Dynamics of single-cell gene expression. *Mol. Syst. Biol.* **2**, 64. (doi:10.1038/msb4100110)

Madou, M. 2002 *Fundamentals of microfabrication.* New York, NY: CRC Press.

Marcus, J. S., Anderson, W. F. & Quake, S. R. 2006 Microfluidic single-cell mRNA isolation and analysis. *Anal. Chem.* **78**, 3084–3089. (doi:10.1021/ac0519460)

Marcy, Y., Ishoey, T., Lasken, R. S., Stockwell, T. B., Walenz, B. P., Halpern, A. L., Beeson, K. Y., Goldberg, S. M. & Quake, S. R. 2007*a* Nanoliter reactors improve multiple displacement amplification of genomes from single cells. *PLoS Genet.* **3**, 1702–1708. (doi:10.1371/journal.pgen.0030155)

Marcy, Y. *et al.* 2007*b* Dissecting biological "dark matter" with single-cell genetic analysis of rare and uncultivated TM7 microbes from the human mouth. *Proc. Natl Acad. Sci. USA* **104**, 11 889–11 894. (doi:10.1073/pnas.0704662104)

McClain, M. A., Culbertson, C. T., Jacobson, S. C., Allbritton, N. L., Sims, C. E. & Ramsey, J. M. 2003 Microfluidic devices for the high-throughput chemical analysis of cells. *Anal. Chem.* **75**, 5646–5655. (doi:10.1021/ac0346510)

Munce, N. R., Li, J. Z., Herman, P. R. & Lilge, L. 2004 Microfabricated system for parallel single-cell capillary electrophoresis. *Anal. Chem.* **76**, 4983–4989. (doi:10.1021/ac0496906)

Ocvirk, G., Salimi-Moosavi, H., Szarka, R. J., Arriaga, E. A., Andersson, P. E., Smith, R., Dovichi, N. J. & Harrison, D. J. 2004 β-Galactosidase assays of single-cell lysates on a microchip: a complementary method for enzymatic analysis of single cells. *Proc. IEEE* **92**, 115–125. (doi:10.1109/JPROC.2003.820551)

Ottesen, E. A., Hong, J. W., Quake, S. R. & Leadbetter, J. R. 2006 Microfluidic digital PCR enables multigene analysis of individual environmental bacteria. *Science* **314**, 1464–1467. (doi:10.1126/science.1131370)

Price, A. K. & Culbertson, C. T. 2007 Chemical analysis of single mammalian cells with microfluidics. Strategies for culturing, sorting, trapping, and lysing cells and separating their contents on chips. *Anal. Chem.* **79**, 2614–2621.

Prinz, C., Tegenfeldt, J. O., Austin, R. H., Cox, E. C. & Sturm, J. C. 2002 Bacterial chromosome extraction and isolation. *Lab Chip* **2**, 207–212. (doi:10.1039/b208010a)

Reyes, D. R., Iossifidis, D., Auroux, P. A. & Manz, A. 2002 Micro total analysis systems. 1. Introduction, theory, and technology. *Anal. Chem.* **74**, 2623–2636. (doi:10.1021/ac0202435)

Roman, G. T., Chen, Y., Viberg, P., Culbertson, A. H. & Culbertson, C. T. 2007 Single-cell manipulation and analysis using microfluidic devices. *Anal. Bioanal. Chem.* **387**, 9–12. (doi:10.1007/s00216-006-0670-4)

Ros, A., Hellmich, W., Regtmeier, J., Duong, T. T. & Anselmetti, D. 2006 Bioanalysis in structured microfluidic systems. *Electrophoresis* **27**, 2651–2658. (doi:10.1002/elps.200500923)

Sia, S. K. & Whitesides, G. M. 2003 Microfluidic devices fabricated in poly(dimethylsiloxane) for biological studies. *Electrophoresis* **24**, 3563–3576. (doi:10.1002/elps.200305584)

Sims, C. E. & Allbritton, N. L. 2007 Analysis of single mammalian cells on-chip. *Lab Chip* **7**, 423–440. (doi:10.1039/b615235j)

Squires, T. M. & Quake, S. R. 2005 Microfluidics: fluid physics at the nanoliter scale. *Rev. Mod. Phys.* **77**, 977–1026. (doi:10.1103/RevModPhys.77.977)

Stone, H. A., Stroock, A. D. & Ajdari, A. 2004 Engineering flows in small devices: microfluidics toward a lab-on-a-chip. *Annu. Rev. Fluid Mech.* **36**, 381–411. (doi:10.1146/annurev.fluid.36.050802.122124)

Stuart, J. N. & Sweedler, J. V. 2003 Single-cell analysis by capillary electrophoresis. *Anal. Bioanal. Chem.* **375**, 28–29.

Sun, Y. & Yin, X. F. 2006 Novel multi-depth microfluidic chip for single cell analysis. *J. Chromtogr. A* **1117**, 228–233. (doi:10.1016/j.chroma.2006.03.088)

Sun, Y., Yin, X. F., Ling, Y. Y. & Fang, Z. L. 2005 Determination of reactive oxygen species in single human erythrocytes using microfluidic chip electrophoresis. *Anal. Bioanal. Chem.* **382**, 1472–1476. (doi:10.1007/s00216-005-3352-8)

Toriello, N. M., Douglas, E. S. & Mathies, R. A. 2005 Microfluidic device for electric field-driven single-cell capture and activation. *Anal. Chem.* **77**, 6935–6941. (doi:10.1021/ac051032d)

Tseng, W. L. & Chang, H. T. 2000 On-line concentration and separation of proteins by capillary electrophoresis using polymer solutions. *Anal. Chem.* **72**, 4805–4811. (doi:10.1021/ac0004613)

Unger, M. A., Chou, H. P., Thorsen, T., Scherer, A. & Quake, S. R. 2000 Monolithic microfabricated valves and pumps by multilayer soft lithography. *Science* **288**, 113–116. (doi:10.1126/science.288.5463.113)

Valero, A., Post, J. N., van Nieuwkasteele, J. W., Ter Braak, P. M., Kruijer, W. & van den Berg, A. 2008 Gene transfer and protein dynamics in stem cells using single cell electroporation in a microfluidic device. *Lab Chip* **8**, 62–67. (doi:10.1039/b713420g)

Vilkner, T., Janasek, D. & Manz, A. 2004 Micro total analysis systems. Recent developments. *Anal. Chem.* **76**, 3373–3385. (doi:10.1021/ac040063q)

Wallingford, R. A. & Ewing, A. G. 1988 Capillary zone electrophoresis with electrochemical detection in 12.7 microns diameter columns. *Anal. Chem.* **60**, 1972–1975. (doi:10.1021/ac00169a027)

Wheeler, A. R., Throndset, W. R., Whelan, R. J., Leach, A. M., Zare, R. N., Liao, Y. H., Farrell, K., Manger, I. D. & Daridon, A. 2003 Microfluidic device for single-cell analysis. *Anal. Chem.* **75**, 3581–3586. (doi:10.1021/ac0340758)

Woods, L. A., Roddy, T. P. & Ewing, A. G. 2004 Capillary electrophoresis of single mammalian cells. *Electrophoresis* **25**, 1181–1187. (doi:10.1002/elps.200405842)

Wu, H. K., Wheeler, A. & Zare, R. N. 2004 Chemical cytometry on a picoliter-scale integrated microfluidic chip. *Proc. Natl Acad. Sci. USA* **101**, 12 809–12 813. (doi:10.1073/pnas.0405299101)

Xia, Y. N. & Whitesides, G. M. 1998 Soft lithography. *Angew. Chem. Int. Ed.* **37**, 551–575.

Xia, F. Q., Jin, W. R., Yin, X. F. & Fang, Z. L. 2005 Single-cell analysis by electrochemical detection with a microfluidic device. *J. Chromtogr. A* **1063**, 227–233. (doi:10.1016/j.chroma.2004.12.022)

Yasukawa, T., Nagamine, K., Horiguchi, Y., Shiku, H., Koide, M., Itayama, T., Shiraishi, F. & Matsue, T. 2008 Electrophoretic cell manipulation and electrochemical gene-function analysis based on a yeast two-hybrid system in a microfluidic device. *Anal. Chem.* **80**, 3722–3727. (doi:10.1021/ac800143t)

Yi, C., Zhang, Q., Li, C. W., Yang, J., Zhao, J. & Yang, M. 2006 Optical and electrochemical detection techniques for cell-based microfluidic systems. *Anal. Bioanal. Chem.* **384**, 1259–1268. (doi:10.1007/s00216-005-0252-x)

Zhang, X. L., Yin, H. B., Cooper, J. M. & Haswell, S. J. 2008 Characterization of cellular chemical dynamics using combined microfluidic and Raman techniques. *Anal. Bioanal. Chem.* **390**, 833–840. (doi:10.1007/s00216-007-1564-9)

Zhong, J. F., Chen, Y., Marcus, J. S., Scherer, A., Quake, S. R., Taylor, C. R. & Weiner, L. P. 2008 A microfluidic processor for gene expression profiling of single human embryonic stem cells. *Lab Chip* **8**, 68–74. (doi:10.1039/b712116d)