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.
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; Vilkin 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 Blaushan (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 2004a,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 calcine-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 calcine 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$_2$O$_2$ (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 microfluidic 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 2a 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 2b. 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. 2007a,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
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 spheroplast 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 termites 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 organisms.
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. 2007b). 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. 2007a).

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 derive 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. 2007b). 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. 2007a).

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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 the only 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 as 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 Fluoro 3 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, danno-ubin (a cardiotoxic chemotherapeutic drug) and isoloquinigriligenin (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 neuro-peptides 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- 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
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J. R. Soc. Interface (2008)


