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Microfluidics for single cell analysis

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Substantial evidence shows that the heterogeneity of individual cells within a genetically identical population can be critical to their chance of survival. Methods that use average responses from a population often mask the difference from individual cells. To fully understand cell-to-cell variability, a complete analysis of an individual cell, from its live state to cell lysates, is essential. Highly sensitive detection of multiple components and high throughput analysis of a large number of individual cells remain the key challenges to realise this aim. In this context, microfluidics and lab-on-a-chip technology have emerged as the most promising avenue to address these challenges.In this review, we will focus on the recent development in microfluidics that are aimed at total single cell analysis on chip, that is, from an individual live cell to its gene and proteins. We also discuss the opportunities that microfluidic based single cell analysis can bring into the drug discovery process.

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Introduction

Cellular analysis underpins many fields including life science, diagnostics, the pharmaceutical industry and renewable energy [1,2]. Conventional cell-based assays measure the average response from a population of cells, assuming that an average response is representative of a typical cell within a population. However, this simplification can result in a misleading interpretation. For example, an average of 50% protein expression in a cell population can represent either a 100% response in half the cells or a 50% response in all.

A plethora of evidence has shown cellular heterogeneity commonly exists within an isogenic or clonal population [3]. This heterogeneity can arise through genetic drift, differences in cell development or cell cycle status, differences in cell age due to proliferation and passaging as well as non-genetic heterogeneity arising from the inherent stochasticity of cellular processes. Whether in isolation or caused through a combination of the above events, cellular heterogeneity can dramatically influence cellular decision making and cell fate [4], however, this can be masked by the average response from a population. One approach to solve this dilemma is to analyse a population at individual cell level. However, multiple individual cells are required to obtain statistically meaningful data, and therefore high throughput analysis is critical.

A number of single cell analysis methods have been established. Some conventional examples are listed in Table 1. Microscopic imaging of a cell is the most obvious approach and has been well established for wide range of applications, such as physiological studies, measurements of gene and protein expression. However, assays on single cells are difficult to perform. The patch-clamp technique enables highly sensitive measurement of changes in ion channels, however it requires high skills to perform and has limitations when detecting complex changes. Furthermore, both of these methods are low throughput. Conventional high throughput tools for single cell analysis include well established methods such as Flow Cytometry (and Imaging Flow Cytometry, Fluorescence Activated Cell Sorting) that can detect, sort and collect cells with desired properties. However, as data are only collected at a single time point, these techniques still do not permit dynamic monitoring of cell response.

Microfluidics has emerged as a powerful enabling technology for investigating the inherent complexity of cellular systems [5]. Typical microfluidic channels have dimension of tens to hundreds of microns that are comparable to the size of a single cell (~10 μm in size and roughly ~1 pL in volume) [5]. At this length scale, the physical behaviour of fluids is fundamentally different from that seen in large channels: Laminar flow forms when two fluid streams come together and mixing of molecules across their interface only occurs through diffusion. This phenomenon permits the accurate realisation of complex molecular trajectories and has been effectively exploited in many circumstances, such as in gradient formation [6,7] and in localised stimuli delivery to subcellular compartments [8].

In addition to the conventional methods already described, new microtechnology based tools for single cell analysis have also emerged in the last decade; for example, optical/magnetic tweezing and the use of

Table 1

Comparison of exampled approaches to single cell analysis

Approaches	Main applications	Key advantage	Key disadvantage
Microscopic imaging	• Morphological studies • Gene and protein expressions • Intracellular communications	Generic and well established	Difficult to perform assays on single cells
Patch clamp	• Ion channel studies	Very sensitive	Limited applications
Flow cytometry	• Gene and protein expression • Population studies	Very high throughput	Requires labeled cells in suspension
Tweezing (e.g. optical, magnetic)	• Manipulation • Single cell mechanics	Low force range (pN)	Requires complex optical systems
Patterned substrates	• Cell–cell communication studies • Controlled cell proliferation and differentiation, guidance	Simple and versatile	Requires fabrication capability
Microfluidics	• All of the above, that is, a wide range of applications from cell manipulation to total single cell analysis	Enabling technology for integrated total single cell analysis	Has not yet gained popular acceptance

patterned substrates (Table 1). However, these can only address challenges in one or a few aspects of single cell analysis. However, from an individual live cell to its gene and proteins. By contrast, microfluidics has been rapidly developed into a powerful approach capable of integrating multiple functions for micrototal analysis of biological systems (μTAS) and single cell analysis [2,9•].

A rapid increasing body of new discoveries have been enabled by microfluidics that would not be possible otherwise. As discussed in this review, whole-genome molecular haplotyping of single human cells has recently been achieved using microfluidic technology [10••]. It is arguable that microfluidics will be a central part of the next generation sequencing focused towards personal genomics. Capabilities in other single cell 'omic' studies, including metabolomics, transcriptomics and proteomics [11•] have also been significantly accelerated partly owing to the rapid development of microfluidics. This advancement will enable information on the subtle variability that exists in biological systems to be revealed, and will have significant implications for understanding the progression of a disease as well as potential of transforming system biology.

As an enabling technology, microfluidics owes its power to handing small volumes of liquid (nanoliter to picoliter), streamlining multiple procedures on a single chip, with scope for parallelization. Recently developed droplet-microfluidics has also emerged as a new forerunner for single cell encapsulation and analysis with massive parallelization. High throughput screening of rare cells to a drug library has been achieved, providing addition information on cell heterogeneity response [12••]. The use of microdroplet confinement has enabled new insights into the nature of quorum sensing, suggesting it is a ''cell-autonomous mechanism for diffusion or efficiency sensing'' [9•].

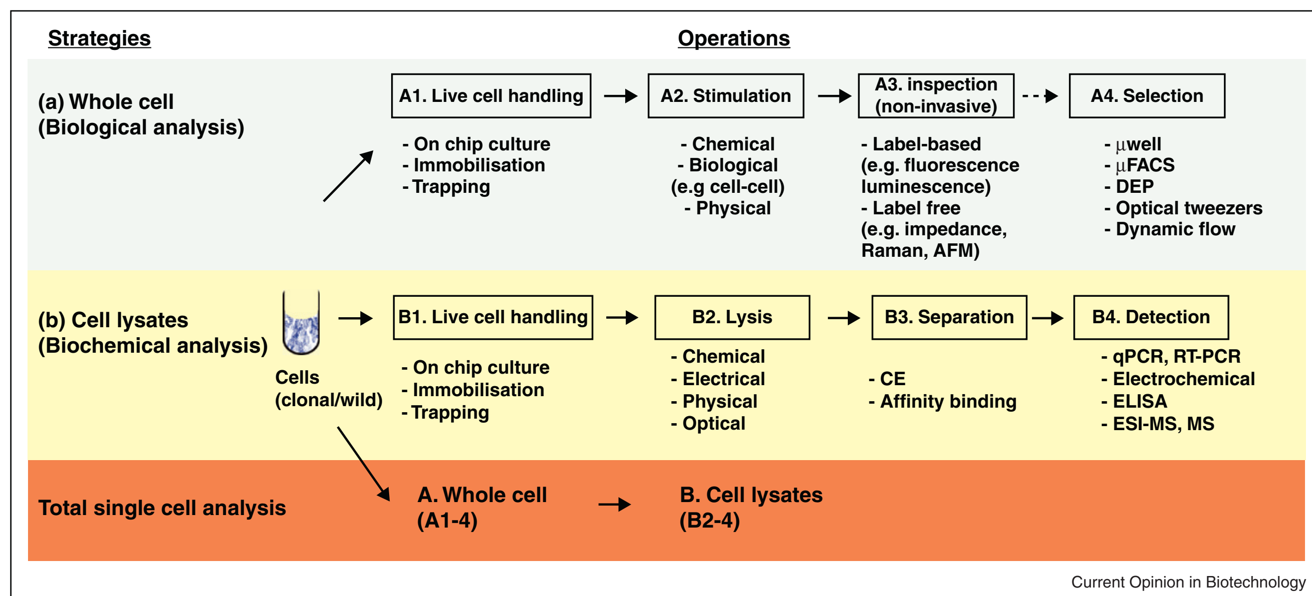
In this review, we will discuss various microfluidic applications for single cell analysis. We focus on recent developments aimed at total single cell analysis on chip, that is, from an individual live cell to its gene and proteins. We recognise the rapidly expanding number of applications for single cell analysis and do not attempt to cover all these topics. Instead, we will focus on the opportunities that microfluidic based single cell analysis can bring into the drug discovery process.

Single cell analysis on chip: overview

Most cellular processes are not isolated single events, but are interconnected and hierarchically organised from molecules to a whole cell. As such, multiparameter analysis, for example, coupling epigenetic to gene expression or protein expression to physiological measurement at the level of single cells would enable a better understanding of the whole process. To achieve this, a complete analysis of an individual cell, from its live state to cell lyses, is essential. We define such a process as ''total analysis of single cells'' and list it as one of the three strategies that could be employed for cell analysis on chip (Figure 1). The three strategies are roughly categorised by the subject of interest, that is, whole cells or cell lysates or both, and therefore the associated on chip operations vary. In the last decade numerous technologies have been developed for each operation making it impracticable to list them all [1,2,13,14]. However, a few major approaches are illustrated for each operation.

Although it appears that ''total analysis of single cells'' is just a sequential combination of the other two strategies (i.e. using whole cell and cell lysates respectively), it is far from trivial. The total amount of analyte in a single cell is

Figure 1



Overview of microfluidic approaches for single cell analysis via three different strategies. The total single cell analysis requires the sequential combination of strategies A and B. The approaches listed for each operation are not exhaustive. More detail can be found in several excellent reviews [1,2,13,14]. *Abbreviations*: CE, Capillary Electrophoresis; ESIMS, Electrospray Ionization Mass Spectrometry; MS, Mass Spectrometry; DEP, Dielectrophoresis; ELISA, Enzyme linked Immunosorbent Assays; qPCR, quantitative Polymerase Chain Reaction; RT-PCR, Reverse Transcription Polymerase Chain Reaction; µFACS, Micro Fluorescence Activated Cell Sorting.

very low (N.B. a single somatic cell weighs ~500 pg) [15]. Furthermore, the majority of the targets of interest are of low copy number and among abundant interferences. These complexities and limitations make the detection of an analytes in a single living cell highly challenging. Whether the combination of these operations is even capable of appropriately sensitive detection is perhaps the first thing to consider. For example, mass spectrometry is the major tool for proteomics. However, integration of mass spectrometry based proteomics and on chip single cell analysis has yet to be achieved. The challenges for single cell analysis on chip are substantial but also provide great opportunities to advance technologies in the field of microfluidics and its associated applications (see below).

Single cell analysis on chip: challenges and prospects

A detailed understanding of the complex heterogeneity in cell populations and its impact on cell behaviour and biological responses can only be achieved using highly sensitive methods with resolution at the single cell and ideally subcellular level (e.g. down to the level of single molecules). In addition, since the processes occur with varying time scales (e.g. from seconds to hours), dynamic control of conditions is essential. However, to achieve total single cell analysis, the development of high throughput systems capable of handling and analysing individual cells is essential. In this section, we highlight the recent advances towards this goal.

Spatiotemporal single cell manipulation

Several single cell immobilisation methods have been developed, including microwells and traps. Traps can function via a range of means including physical geometry, hydrodynamics, magnetic force, dielectrophoresis and optical or acoustic tweezers [16,17••,18] (Figure 2). In the early stages of trap development, enhancement of trapping efficiency has been a major focus – single cell trapping efficiency has now reached 97% with optimised trap architectures [18]. Recent development has seen a tendency towards cell–cell interaction and long term measurement of cell activities [18,20,21,22••,23]. Of special interest is recent work that tracks the lineages of hundreds of single cells in parallel for detailed study of the time scales of heterogeneity in a population [22••] (Figure 2). Via a similar method, the mechanisms governing hematopoietic stem cell fate decision (e.g. self-renewal and differentiation processes) were revealed [23].

Recently, hydrodynamic cell trapping systems have shown great promise for high throughput handing and manipulation of single cells that would otherwise be impractical [17••,19••,24•] (Figure 2). Various designs of microwell, weir and microjail arrays have been used to passively trap hundreds of single cells. Controlled pairing and fusing of different cell types for fusion-mediated reprogramming has also been achieved [19••] (Figure 2). Successful long term observation of the response of normal and disordered single cells to drugs

demonstrates the potential of microfluidics in personalised diagnostics [24•].

Single living cell detection

Although cell response can be measured quantitatively in many ways, non-invasive methods (and treatments) are required for living cell detection. At present fluorescence techniques remain the most common methods owing to their established nature and abundance of commercially available probes. Since cell-to-cell variability is temporally and spatially dependent [4], high content quantification of all components involved would be ideal. A single time point fluorescence measurement, such as flow cytometry, is well suited for high throughput single component quantification. Spatiotemporal measurement of protein translocation, interactions and modification rely on imaging technologies such as Fluorescence Resonance Energy Transfer (FRET) and Quantitative Time-Lapse Fluorescence Microscopy [13,25]. However, the large data rich files generated by these techniques compromise throughput.

Recently, an intriguing approach has been developed for both high content and high throughput detection [26]. It used a photomultiplier to combine one-dimensional imaging with microfluidic flow cytometry and demonstrated its usefulness in high content screening with a speed of several thousands cells per second. This work, together with the work from Chung *et al.* [27] illustrates that integration between microfluidics, automation and conventional microscopy could expand the ability of a biological lab for high resolution and large-scale quantitative experiments.

Development of label free techniques continues. A powerful method capable of measuring growth rate at the single cell level was developed using a Suspended Microchannel Resonator technique coupled with microfluidic control [28•]. It is envisaged that this method will contribute to our understanding of many cellular processes that affect cell growth.

Cell lysate analysis

Substantial developments have been made to integrate major analytical methods currently used for genomics and proteomic for single cell analysis [11•]. A few notable examples that could contribute to future developments are discussed below.

Gene

Gene expression analysis of single cells has been demonstrated by various groups [29,30••,31]. For example, Toriello *et al.* have developed integrated microfluidic devices that couple single cell selection and capture, enzymatic reaction and quantitative detection all on a single platform [30••] (Figure 3). By quantifying mRNA from two distinct populations at the single cell level, they illustrated that stochastic variations in gene expression and silencing within single cells is masked by bulk measurements [30••]. Quake and colleagues have pioneered large-scale gene expression analysis from single cells. The technology they developed has been exploited in a wide range of applications, such as a recent work on the whole-genome molecular haplotyping of single human metaphase cell [10••] (Figure 3). Indeed, developments in this field advance rapidly, and many technologies have started to enter commercial markets. Examples of companies offering the technology include Fluidigm Corporation, Oxford Nanopore Technology, and Genechip Affymetrix. These technologies are massively expanding the throughput for gene expression measurements as well our understanding of the differential gene expression profiles that underlie cell behaviours. For example, Petriv *et al.* recently used the Fluidigm dynamic array system to perform 80,000 RT-qPCR assays to map the expression of micro RNA's in the hematopoietic hierarchy and showed using single cells the major reprogramming events that occurs upon cell differentiation [32].

Epigenetics

Changes in gene expression within a cell population can be caused by mechanisms other than the underlying DNA sequence. Processes such as DNA methylation or histone deacetylation can activate or silence genes without genomic alteration. These epigenetic processes are a major factor in non-Mendelian disease such as Alzheimer's and Parkinson's disease [33] and can influence the way cells behave in response to drugs [34]. Unlike gene expression studies the application of single cell approaches to epigenetic analysis has so far been limited. However, a recent study by Kantlehner *et al.* [35] demonstrated an approach for DNA methylation profiling within the regulatory CpG islands of single cells for genes which are aberrantly methylated in several types of cancer. Their single cell approach was aided by the fact they could reduce sample volumes down from the standard 0.2–1.5 mL PCR volumes to 5 μL which increased their experimental success rate. This type of analysis would therefore lend itself to microfluidic approaches where the reactions volumes could be reduced even further and it is probably only a matter of time before these studies are reported in the literature.

Protein

In comparison to gene expression analysis, measuring proteins from a single cell represents another level of challenge [11•,14]. This is due to the extremely low concentrations of proteins (sometimes less than 1000 copies per cell) and that, unlike DNA or RNA, proteins cannot be directly amplified. Currently, single cell proteomics is still in its infancy. Two main development focuses are prominent, namely, sensitivity enhancement and improved integration and automation.

Figure 2

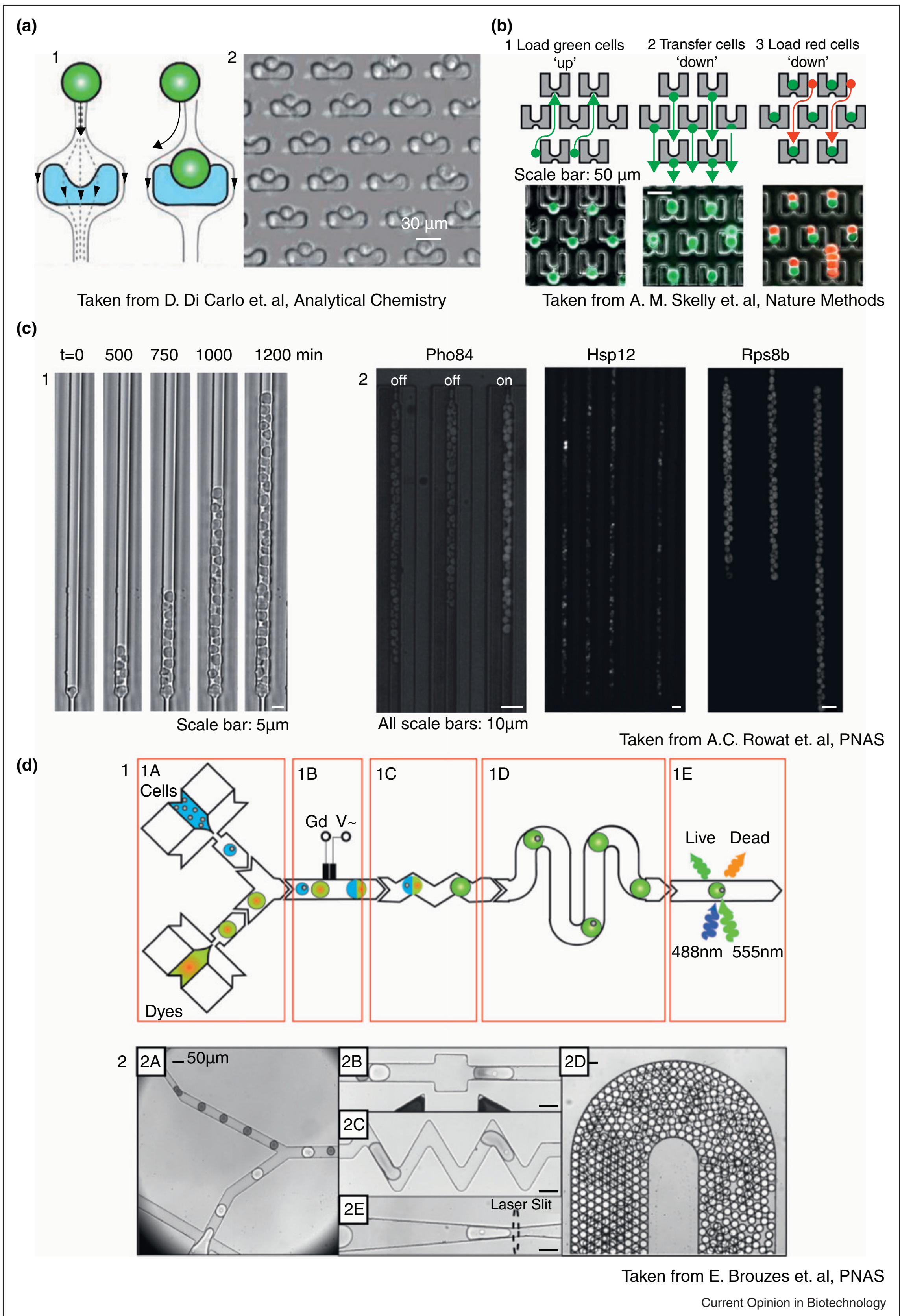


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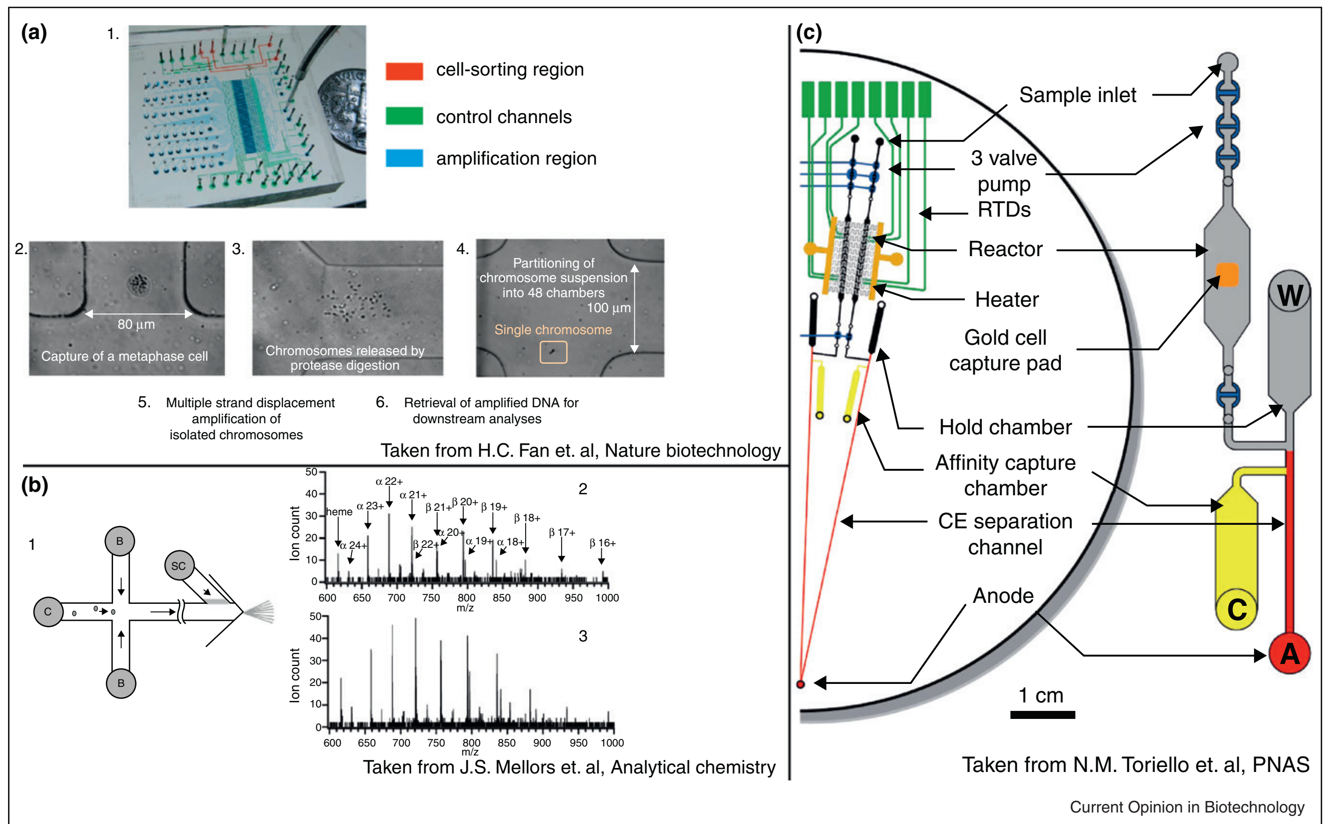
Figure 3



Individual cell analysis from a live cell to gene or protein expressions. **(A)** An integrated microfluidic approach for whole-genome molecular haplotyping of single cells. (1) An overview image of the device. (2–6) The stages that a cell progresses through during analysis. Reproduced by permission of Nature [10••]. **(B)** integration of microfluidic device with ESI-MS for on line single cell analysis. (1) A diagram showing the basic operation. (2) Mass spectra generated by a normal single cell lysis and (3) by a cell with higher haemoglobin content. Reproduced by permission of the American Chemical Society [38••]. **(C)** An integrated device for gene expression analysis from a single cell. The processes of single cell capture, mRNA transcription and amplification were all integrated on chip. Reproduced by permission of the National Academy of Sciences [30••].

Innovative combinations of microfluidic devices and optical microscopy have proven to be an efficient way to cope with the detection of low number of proteins [36•,37]. The whole process is essential to this, namely single cell manipulation, separation and detection all in one device. This not only minimises loss of protein and reduces contamination but also enables a correlation between protein expression and the phenotypic stage of a singe cell at a specific time point (i.e. when it is lysed). A good example is shown in the work from Huang *et al.* where individual β_2 adrenergic receptors from a single cell were successfully counted in a microfluidic channel using cylindrical optics [36•]. With the aim towards single cell proteomics, Salehi-Reyhani *et al.* have developed an integrated microfluidic antibody capture chip and demonstrated detection of the human tumour suppressor protein p53 from a single cell [37].

Despite many advances in fluorescence based technologies, problems in spectra overlapping and uncertainties from labeling procedures ultimately restrict the total number of simultaneous measurements. A promising alternative is to combine single cell analysis with mass spectrometry. Currently, full integration has been challenged by a lack of an effective interface between the mass spectrometer and miniaturised sample preparation

(Figure 2 Legend) Single cell manipulation on chip. **(A)** Cell trapping by hydrodynamic focusing principles. (1) A schematic and (2) A phase contrast image of an array of high quantity single-cell isolates. Reproduced by permission of the American Chemical Society [17••]. **(B)** Single cell manipulation enabling precise control of cell-to-cell contact for cell fusion. (1) to (3) show schematics and corresponding red/green micrographs of a three-step cell-loading protocol. Reproduced by permission of Nature [19••]. **(C)** Restrained and monitored growth of individual cells allowing interrogation of the lineage of single cells. (1) Bright field image showing single progenitor cells constrained to grow in a line. (2) Variations of three different protein expressions in lineages of cells were revealed. Reproduced by permission of the National Academy of Sciences [22••]. **(D)** Droplet microfluidic for high throughput single cell manipulation and analysis. A series of schematics (1) and corresponding micrographs (2) showing 5 optimised modules employed for analysis of cell viability. Reproduced by permission of the National Academy of Sciences [12••].

platform [38••,39] (Figure 3). However, this is starting to be addressed. For example, Mao *et al.* [40], have recently reported the development of a microfluidic system which allows high throughput nanoelectrospray into the mass spectrometer providing sensitivity comparable to the larger volume standard capillary emitters. While this is not a fully integrated system it does open the possibility for handling the volumes obtained from single cells and presenting them to the mass spectrometer. Achieving this complete integration between the two platforms will be an important step forward towards single cell proteomics.

Metabolites

The cellular metabolome can provide a highly sensitive measure of a cellular phenotype with quantification of intracellular metabolite concentrations forming an integral part of systems biology and pre-clinical drug toxicity analysis [41]. Similar to protein analysis, single cell metabolomics is challenging due to the small quantities of analytes which are present in single cells, with a typical 1 pL cell volume containing metabolite concentrations in the low femtomole range [15]. In common with single cell protein analysis, direct amplification of the analytes cannot be performed. To date, most metabolite profiling studies on single cells have used approaches based on molecular sensors such as FRET [42] which limits the analysis to specific metabolites and prevents 'omics' level detection. More recently however, mass spectrometry based approaches have reached the limits of detection required for single cell metabolomics making the technology suitable for use in combination with microfluidic systems [38••,43]. For example, Zenobi's group developed a microfluidic device which can position single cells onto a specialised slide for metabolome analysis of ADP, ATP, GTP, and UDP-Glucose by matrix assisted laser desorption ionization (MALDI) mass spectrometry. Meanwhile, Ramsey's group coupled a microfluidic system which incorporated elements for single cell lysis, a solution electrophoresis channel, where cellular constituents can be separated, and an electrosmotic pump to direct the eluted cell components to the mass spectrometer. These microfluidic based mass spectrometry systems, while still at the experimental stage, open up the possibility for multiple simultaneous quantitative detection of multiple metabolites at the single cell level and could uncover the subtle metabolite concentration differences that are currently hidden due to stochastic variability.

Cells in droplets

In the last few years droplet microfluidics has emerged as a promising avenue for single cell encapsulation, dynamic living cell assay, and single cell immunoanalysis [12••,44,45] (Figure 2). In these cases, microfluidic devices enable high throughput generation of femtoliter sized and picoliter sized aqueous droplets in an immiscible carrier, such as oil. These droplets are effectively nanolabs that accommodate single cells and host all subsequent reactions. High throughput cytotoxicity screening of drugs [12••] (Figure 2), in-droplet cell lysis and intracellular content analysis have all been illustrated [46]. An advantage of in-droplet analysis is that the droplet confines the lysate, preventing its dilution through diffusion. Currently, most of these analyses are based on fluorescence and therefore suffer from the limitations described above. Recently, online Mass spectrometry of individual microdroplets has been demonstrated [47•]; this could greatly enhance the sensitivity of droplet-based single cell analysis.

Microfluidic single cell analysis in drug discovery

Cell based assays form fundamental practices at various stages of the drug discovery process. To reduce the cost of discovery, high throughput screening using robotics and multi-well plates is the principal tool for pharmaceutical industry. In general, the average response from a population in a well (~tens or hundreds of cells) is used as the readout. However, the increasing evidence of heterogeneous responses from individual cells invites the introduction of new strategies capable of revealing information at both the individual and population level. We envisage that the development of microfluidic single cell analysis is one of the most attractive approaches towards this goal, and illustrate its potential with a few recent examples below.

Concentration profiling

The concentration and time course of a drug (and multiple drugs) interacting with a cell are essential in evaluating its efficacy [48]. Laminar flow within microfluidic devices provides a unique advantage to create purpose designed concentration profiles (e.g. gradients) for various applications, such as the study of chemotaxis [7]. Dependent on the cell type studied, the drug concentration can be delivered by either flowing over an adhered cell layer on a substrate or by encapsulation together with cells in a microdroplet [12••]. In the case of adhered cells, different gradient generators have been reported for creating concentration profiles for the pharmacological screening of voltage-gated human hERG K^+ channels [49] and toxicity tests [50]. Such systems enable the rapid acquisition of a large amount of high content data that is essential to draw statistically meaningful conclusions. It should also be noted that shear stress can induce similar response to the chemical stimuli for adherent cells [51] and the high flow rate used for gradient generation might damage shear sensitive cells [50]. The droplet-based cytotoxicity screen is of specific interest for large scale single cell based screening [12••] as it offers high throughput, precise delivery and powerful manipulation permitting a multitude of possibilities.

Miniaturisation for future discoveries

In recognition of the challenges that current target-driven drug discovery faces, there is an ongoing paradigm shift

towards pathway-driven drug discovery [52]. Multi-parameter phenotype profiling of a compound is essential to understand the biological pathway but faces many challenges [52]. In this context, microfluidic technology will be one of the enabling technologies to address these challenges, as exemplified in the many new discoveries that would not be possible by conventional methods [53,54]. For example, recently, Bao *et al.* has developed a microfluidic system that enables precise timing control with integration of multiple simultaneous experiments. With this system, tracking responses of individual cells across multiple stimulations was achieved, leading to the new finding that the variability in G-protein-Coupled Signalling is associated with long-lived cell state difference rather than from stochasticity [54].

Conclusions

The impact of the rapid expansion of high throughput single cell analysis is evident in its great potential for numerous applications, including drug discovery, diagnostics, cancer research, regenerative medicine, system and synthetic biology, and many others. The implementation of microfluidic technologies in single cell analysis is one of the most promising approaches that not only offers information rich, high throughput screening but also enables the creation of innovative conditions that are impractical or impossible by conventional means. The possibilities for distinguishing the difference between individual cells and the benefits from miniaturisation (e.g. confinement) have led to many discoveries in both traditional biopharmaceutical communities and in emerging fields such as synthetic biology [9•,52]. Recent research has only just started to show the full potential of microfluidics in this field.

Major challenges still remain. Full integration of microfluidic (and the miniaturised platforms) for single cell manipulation with established analytic methods, such as mass spectrometry and NMR, traditionally used for high sensitivity single molecule detection is still some way off. Developing effective strategies for extracting system level information the very large data sets generated by total single cell analysis of population is essential. New breakthroughs in these areas will enable microfluidics approaches to transition from an interesting research topic to routinely used tool for drug screening and diagnostics.

Acknowledgements

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- •• of outstanding interest

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