Microtechnologies and nanotechnologies for single-cell analysis

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Many efforts are currently underway to try and mimic the properties of single cells with the aim of designing chips that are as efficient as cells. However, cells are nature's nanotechnology engineering at the scale of atoms and molecules, and it might be better to envision a microchip that utilizes a single cell as an experimentation platform. A novel, so-called laboratory-in-a-cell concept has been described, where advantage is taken of micro- and nanotechnological tools to enable precise control of the biochemical cellular environment; these tools also offer the possibility to analyse the composition of single cells. Methods for single-cell handling and analysis are being developed and will be required for this concept to progress further.

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Abbreviations

CE	capillary electrophoresis
DEP	dielectrophoresis
LIC	lab-in-a-cell
LOC	lab-on-a-chip
µTAS	micro total analysis system

Introduction

The understanding of many biological processes would greatly benefit from the ability to analyse the content of single cells. Today, there are only a few conventional systems that enable direct intrinsic studies of single cells, including capillary electrophoresis (CE) and flow cytometry [1]. These systems, however, are based upon conventional technologies and instrumentation, they give only limited information about the cell content and do not present a general method for single cell analysis. Recent, rapid developments in micro- and nanofabrication technologies, have already led to the successful so-called 'laboratory-on-a-chip' (LOC) concept and these developments offer great opportunities for the analysis of single cells.

The concept of the LOC or 'micro total analysis systems' (µTAS) has received increasing interest over the past ten years, as illustrated by several review articles [2•,3•,4–6]. Initially, there were two approaches in this field. The first aimed to combine microsensors with fluidic components (e.g. pumps, flow sensors) into systems (e.g. for ammonia or phosphate sensing) [7,8]. The second approach, which had a much greater impact, focused on the miniaturization of analytical chemical methods. Separations were of particular interest and following the first demonstration with amino acids [9] much emphasis was placed on genetic (DNA) analysis [10–13]. As genetic analysis is now more or less routine, research focus has moved to the use of µTAS for protein analysis [3•].

In addition, over the past few years the analysis of even more complex biological systems, such as living cells, with the use of microfabricated structures has attracted increased attention. A recent review discussed the use of microtechnologies as a very useful tool for cell manipulation and analysis [14•], and illustrated that they have advanced to a level that allows control of mechanical, electrical and biochemical parameters down to the nanometer scale. Most of the cited work derives from the past five years, with a clear trend towards single-cell analysis, as illustrated by the development of chip-based devices for single cell ion channel studies [15,16•,17]. Although no reports of direct chemical analysis of single cells in microfluidic devices are known, Waters *et al.* [18] used microstructures to lyse single cells, followed by PCR and CE for the analysis of cell DNA. Nevertheless, the availability of cell analysis even without the need for amplification only seems to be a matter of time, as new approaches to single-cell analysis by CE have been recently proposed by Zabzdyr *et al.* [19], albeit in a conventional system.

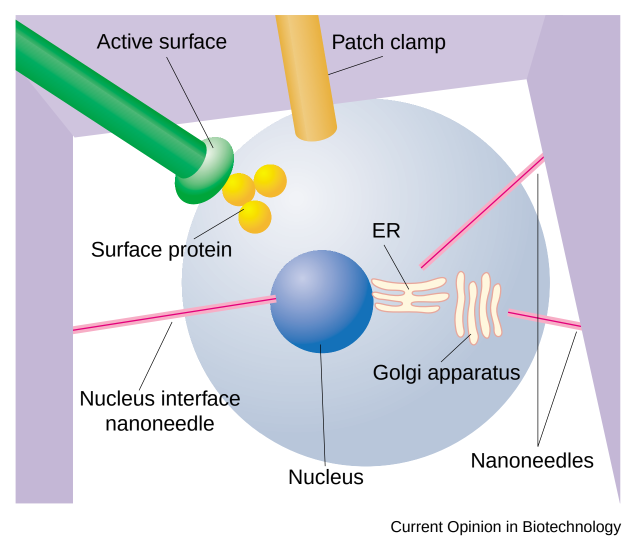
It is clear that with recent technological developments many life-science researchers have obtained very powerful tools for detailed cellular studies [20•]. The novel concept of a laboratory-in-a-cell (LIC) described in this article intends to combine the best of both worlds: to use the biological 'unit', a cell, as a laboratory to perform complex biochemical operations, and to employ advanced micro- and nanotechnological tools to access and analyse this laboratory and to interface it with the outside world. This idea of combining efficient and specific functioning, generated by 'natural' structures, with man-made micro- or nanofabricated devices has similarities with other developments; for example, the use of the natural ionophores (e.g. the antibiotic valinomycin) to create highly selective ion sensors [21], the entrapment of enzymes such as glucose oxidase for obtaining a microglucose sensor [22], the utilization of ion channel proteins (e.g. α-hemolysin) incorporated in lipid bilayers for single molecule DNA sequencing [23], and the concept of cell-based sensors [24]. The LIC concept, however, aims to exploit the incredible complexity and effectiveness of individual cellular processes in a much broader scope, as we will illustrate below.

The lab-in-a-cell concept

In biological systems, such as single cells, the parallel handling of small numbers of molecules is inherent. A differentiated eukaryotic cell can perform some 10^3–10^4 different chemical operations simultaneously, depending on the protein content, using 10^6 molecules of ATP per second [25•]. All this is performed in a volume in the order of 1 pL. How is this chemical multiprocessing capability in extremely small volumes possible? Part of the solution to this question is found in five main features: compartmentalization (i.e. the use of specialized reaction containers such as organelles with volumes of 10^{-15}–10^{-21} L, and with controlled input and output properties); molecular recognition (highly specific interactions between reacting molecules or binding interactions in a sorting/counting step); a combination of small scale and complex function; targeted transport and controlled mixing of components (e.g. by the use of vesicles as cargo carriers between organelles) [25•]; and the preservation of internal laboratory conditions. Many efforts are currently underway to try and mimic these properties of single cells and to design chips that are as efficient as cells. We believe it might be better to take advantage of the optimized natural 'laboratory' represented by a cell and to vision a chip where a single cell constitutes the core — a laboratory-in-a-cell.

Imagine a life-science laboratory represented by a single cell on a chip. You would need several different components on the chip to put your 'laboratory' on a specific position on the chip, to study what is going on inside the 'laboratory' and to deliver and/or withdraw data from it (see Figure 1). Hence, for the new LIC concept three functions have to be provided: cell manipulation and immobilization; cell characterization (electrical, mechanical, biochemical or optical); external and internal connection and communication. In the literature the following has been presented for single-cell analysis on a chip.

Figure 1



Conceptual drawing of the laboratory-in-a-cell concept where nano- and microtechnological tools enable precise control and analysis of single cells.

Single-cell manipulation and immobilization

Dielectrophoresis

Among the many manipulation techniques, the electric field-based approach is well suited for miniaturization because of the relative ease of microscale generation and structuring of an electric field on microchips. Dielectrophoresis (DEP) has been successfully applied on microchip scales to manipulate and separate a variety of biological cells including bacteria, yeast and mammalian cells. Voldman and colleagues [26] developed a microfabricated device for use in parallel luminescent single-cell assays that can sort populations of cells upon the basis of dynamic functional responses to stimuli. This device is composed of a regular array of noncontact single-cell traps. These traps use DEP to confine cells and hold them against disrupting fluid flows. By situating an array of these traps in a microchannel it was shown that cells could be loaded, optically observed and sorted on the basis of their dynamic fluorescent response to a stimulus.

Electrophoresis

As most cells have a net negative charge at neutral pH, electrical fields can be used to move individual cells in microfluidic systems. When applying an electrical field across a thin membrane containing a micro- or nanopore, cells will be automatically transported towards the pore and will stick there. This enables the localization of single cells on top of the pore, where they can be analysed using patch-clamping techniques [14•,15,16•].

Optical trap

A system for the random separation of a single microorganism, such as a living cell or a microbe, in a microfluidic device that integrates a laser trapping force and DEP has been presented [27]. Once the target cell was trapped at the focal point of the laser trap, excess cells were excluded by controlling the electrical field. Another system combining optical trapping and DEP has been employed to manipulate cells and beads and enabled pN force measurements on single cells [28]. The beads were caught in the optical trap and brought into contact for defined times with cells held in the DEP. The interaction between biotinylated live cells and strepavidin-coated beads was studied. In both systems the optical tweezers are off-chip and the DEP integrated on-chip.

Characterization of single cells

Electrical characterization

Chip-based patch clamping has the objective to replace traditional patch electrodes with a planar array of recording interfaces miniaturized on the surface of either a silicon, polymer or glass substrate. One chip-based device for patch clamping was presented by Schmidt *et al.* [15] and consists of planar insulating diaphragms on silicon. In this work it was shown that stable gigaohm seals over micrometer-sized holes can be obtained in the time frame of seconds by the electrophoretic self-positioning of charged lipid membranes. Recording chips can be produced in large numbers with defined geometry and material properties by standard silicon technology. Multiple recording sites can be integrated on one single chip because of the small lateral size of the diaphragms.

Three-dimensional silicon oxide micronozzles integrated into a fluidic device for patch clamping were developed by Lehnert *et al.* [16•]. A cell can be positioned on the nozzle by suction through the hollow nozzle that extends to the back of the chip. A microanalysis system for multipurpose electrophysiological analyses has been presented by Han *et al.* [17]. This system has the capability to perform whole-cell patch clamping, impedance spectroscopy and general extracellular stimulation/recording using integrated, multi-electrode configurations.

The loss of physical integrity in the plasma membrane is one of the major indications of cell death. Cell viability is thus usually determined through examination of membrane integrity with colorometic of fluorescent dyes. Huang *et al.* [29•] developed a new technology that employs a microfabricated device for high-resolution, real-time evaluation of membrane electrical properties of single cells. The chip allows a single cell to be probed with low electrical potentials without introducing membrane damage and permits the corresponding electrical currents flow through that cell to be measured. Electrical resistances of dead (membrane impaired) cells and live cells were found to be significantly different. This suggests that evaluating membrane resistances of individual cells can provide an instant and quantitative measure to determine cell membrane integrity and cell viability of single cells.

Mechanical characterization

Cells in viable tissues respond to mechanical stimuli under physiological and pathophysiological conditions through alterations in the activity of ion channels and the concentrations of signaling molecules, which ultimately lead to modifications of the cytoskeleton and extracellular structures. To begin to understand the complex biological mechanisms that organize cellular response to mechanical forces, many different *in vitro* devices have been developed to apply static and dynamic mechanical stimulus to cell cultures [30]. Micromechanical systems (MEMS) technology offers the ability to shrink the entire force transducer down to a size comparable to that of a single cell. A fully submersible force transducer system has been implemented using MEMS designed for use with single living heart cells [31]. The scale of the device works well for the study of many cell types with dimensions in the range 25–250 μm. The cell force transducer was successfully operated with cardiac myocytes in a saline bath and survived multiple solution exchanges under steady state and oscillatory conditions [31].

Optical characterization

Yasuda and colleagues [32] has presented a system for the continuous observation of isolated single cells, which enables genetically identical cells to be compared using an on-chip microculture chip and optical tweezers. The microchambers are connected by a channel through which cells are transported by the optical tweezers from a cultivation microchamber to an analysis chamber, or from the analysis chamber to a waste chamber. Differential analysis of isolated direct descendants of single cells showed that this system could be used to compare genetically identical cells, helping to explain heterogeneous phenomena.

(Bio)chemical sensing

(Sub)micron-size biochemical sensors and electrodes can be used for the analysis of intracellular parameters (e.g. pH, conductivity) and to detect the presence of cell metabolites (e.g. calcium). The electrochemical signature of peroxynitrite oxidation, an important biologically active species, has been studied using microelectrodes at the single-cell level [33]. A method for preparing platinum electrodes with nanometer dimensions has been reported [34], demonstrating the ability to voltammetrically detect zeptomole quantities of an electroactive species. Recently, an attempt was made to make a micro-ion sensor array to determine intracellular ion concentrations [35].

External and internal communication

Electroporation

Numerous high-resolution techniques exist to detect, image and analyse the biochemical contents of single cells and organelles, but few methods exist to control or selectively manipulate the biochemical nature of these compartments. The plasma lipid membrane surrounding cells is impermeable to most compounds of biological and medical interest (e.g. dyes, drugs, DNA, RNA, proteins, peptides and amino acids). Thus, to introduce or withdraw such compounds from the cell the bilayer membrane has to be broken. Electroporation is a non-contact method for transient permeabilization of cells. The first system for single-cell electroporation was presented by Huang and Rubinsky [36–38]. The chip is a three-layer device that consists of two translucent poly-silicon electrodes and a silicon nitride membrane, which together form two fluid chambers. The two chambers are interconnected through a single micron-size hole in the insulating silicon nitride membrane. In a typical process the two chambers are filled with conductive ionic solutions. One chamber contains cells: individual cells can be captured in the hole and, thus, incorporated in the electrical circuit between the two electrodes of the chip. Experiments show that the chip has the capability to manipulate and induce electroporation on specific individual cells. As indicated in a recent review on single-cell electroporation [39], this methodology makes it possible to investigate cell-to-cell variations in a population and to manipulate and investigate the intracellular chemistry of a cell. Further miniaturization of the electrodes to the nanoscale will allow the selective manipulation of single organelles within a cell. Another possibility is to combine electroporation with analytical techniques such as CE separation and mass spectroscopy to perform single-cell proteomic studies.

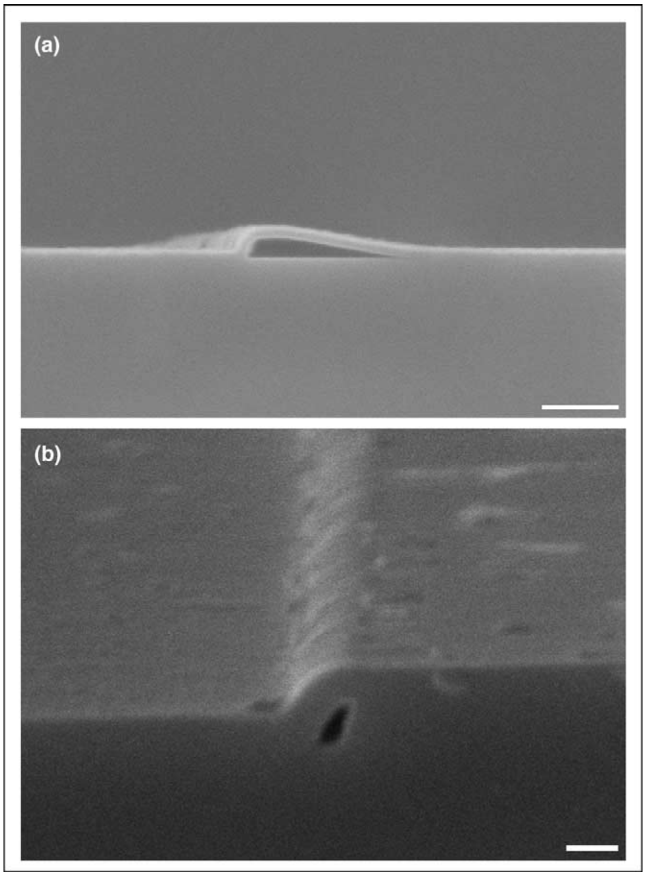
Nanoneedles

Two new methods for the fabrication of nanochannels by conventional micromachining with submicrometer dimensions have been reported [40•]. The first method is based on sacrificial etching of a nanowire, which was formed on the side wall of a step generating 40 nm wide and 90 nm high channels (Figure 2a). The second method is based on the adhesion of the capping layer to the substrate after removal of a sacrificial strip separating the two, generating 50 nm high and 400 nm wide channels (see Figure 2b). The fabricated nanochannels are localized and can be connected to microchannels and reservoirs. These could also be redesigned into nanoneedles and integrated in microfluidic channels where, for example, they could be used to penetrate the lipid bilayer of single cells injecting or withdrawing samples.

Nanotubes

Novel, complex two-dimensional microscopic networks of phospholipid bilayer nanotubes and containers in which the connectivity, container size, nanotube length and angle between the nanotube extensions can be controlled has been presented by Orwar and colleagues [41] (see Figure 3). Containers within these networks can be chemically differentiated and material successfully routed between two containers connected by a common nanotube. These networks will enable model systems to be devised for studying confined biochemical reactions, intracellular transport phenomena and chemical computations. The method is based on the propensity of liposomes to undergo complex shape transitions as a result of mechanical excitation.

Figure 2

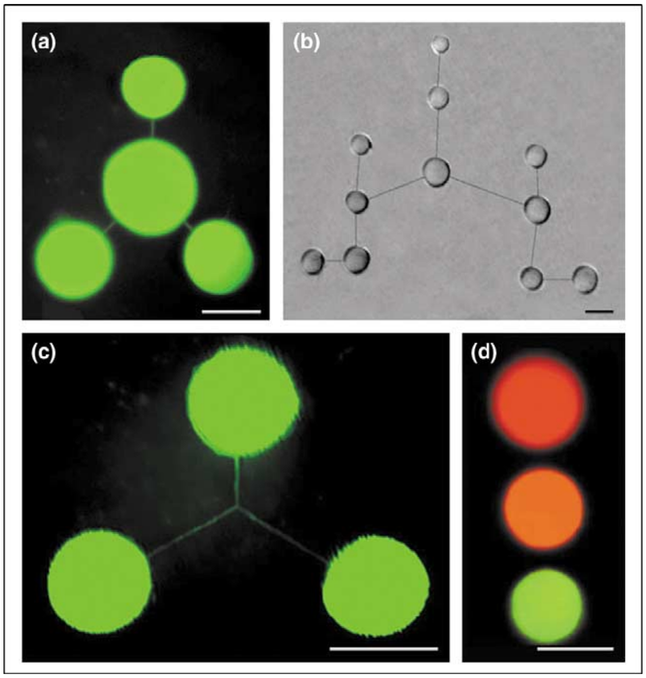


Fabricated two-dimensional confined nanochannels produced by two different methods. **(a)** SEM photograph of a nanochannel (90 nm high and 40 nm wide) made using the sacrificial etching of a nanowire. **(b)** SEM photography of a nanochannel (50 nm high and 400 nm wide). The method used here is based on the adhesion of the capping layer to the substrate after removal of a sacrificial strip separating the two. (Figure reproduced from [40•] with permission.)

Liquid control

To withdraw or deliver subpicoliters to and from the cells a very precise pump is required. A nanofluidic bubble pump using surface tension directed gas injection has been presented by Tas *et al.* [42]. Gas bubble injection is a simple and generic method for the displacement of picoliter volumes. Reproducible dispensing of approximately 40 pL was demonstrated [41]. The combination of nanochannels with electrochemical actuation would allow control of flow rates down to the subpicoliter per second range, which would enable dosing of, for example, drugs into a single cell (NR Tas, personal communication). This subpicoliter dosing is particularly interesting if combined with nanopipettes, allowing for the localized injection of chemicals into different regions in the cell.

Figure 3



Microscopic liposome networks. **(a)** Fluorescence image showing a network of four stained multilamellar liposomes connected by nanotubes. **(b)** Bright-field image of an 11-liposome network. As the nanotubes are not visible under bright-field optics, lines have been drawn between liposomes. **(c)** Fluorescence image of a symmetric three-way nanotube junction created by pulling the middle liposome in a V-shaped three-liposome network away from the other two liposomes. **(d)** A three-liposome network with photochemically differentiated containers created by division of a single DiI liposome (where DiI is 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) also containing fluorescein. Bottom, liposome depleted of DiI by laser; top, liposome depleted of fluorescein by laser; middle, unaltered liposome. (Figure reproduced from [41] with permission.)

Outlook and conclusions

As mentioned in the introduction, populations of cells have been used as the 'workhorse' for producing biological compounds or as sensing elements since the beginning of 20th century. However, it has not been possible to access and make use of the processes of individual cells owing to the lack of tools able to operate and interact with them. New micro- and nanotechnological tools have now opened new opportunities for realizing novel methods (biochemical, electrical, mechanical and optical) to characterize single cells.

LIC systems are likely to have many applications. These could include detailed studies of intracellular processes and mechanisms (e.g. detecting ion-channel responses as a function of external stimuli), the use of single cells as nanoreactors for combinatorial chemistry, the use of single cells as platform for drug testing (which would reduce the need of animal testing), and the development of single cell based sensors, to name but a few. Hopefully, this article will promote awareness among biologists that single cells can be considered as experimental platforms, as well as providing the stimulus for micro- and nanoengineers to further develop and refine the required instrumentation.

There are many efforts today trying to mimic the properties of single cells with the aim to design chips that are as efficient as cells. However, cells are nature's nanotechnology engineering at the scale of atoms and molecules and it will be very difficult (impossible) to create LOCs that are as efficient as cells. Therefore, it might be better to envision chip solutions where a single cell constitutes the core, the workhorse, and the chip is the interface that enables manipulation, characterization and communication.

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