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Review

Micro- and Nanoscale Technologies for Delivery into Adherent Cells

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Several recent micro- and nanotechnologies have provided novel methods for biological studies of adherent cells because the small features of these new biotools provide unique capabilities for accessing cells without the need for suspension or lysis. These novel approaches have enabled gentle but effective delivery of molecules into specific adhered target cells, with unprecedented spatial resolution. We review here recent progress in the development of these technologies with an emphasis on *in vitro* delivery into adherent cells utilizing mechanical penetration or electroporation. We discuss the major advantages and limitations of these approaches and propose possible strategies for improvements. Finally, we discuss the impact of these technologies on biological research concerning cell-specific temporal studies, for example non-destructive sampling and analysis of intracellular molecules.

Need for Techniques To Study Adherent Cells

A mechanistic understanding of cell biology is often limited by both the complexity of the processes and the limitations of commonly available research tools that lack temporal or spatial resolution. The lack of tools capable of providing cell-specific, non-destructive biomolecular delivery and analysis is a particular barrier to advancing fundamental discoveries of cell heterogeneity, single-cell behavior within a complex environment, and the mechanisms that govern disease states, responses to drugs or other stimuli, and differentiation of stem cells. To gain new mechanistic understanding, advances in methods for precise intracellular delivery and non-destructive biochemical analyses of non-secretory molecules (e.g., mRNA and proteins) are greatly needed such that individual cells can be experimentally controlled and repeatedly analyzed over time and/or within a particular location of the cell. For example, developing neurons must undergo a series of sequential changes in gene expression to achieve a mature phenotype; hence, understanding the process will require the ability to accurately monitor the sequence of intracellular events, within individual cells, in a non-destructive manner. In addition, neuronal maturation is influenced by interactions with surrounding cells and with the extracellular matrix, and it is therefore necessary to be able to simultaneously monitor events occurring in multiple cells that are interacting with each other and with the matrix. While the requirements are challenging, these experimental capabilities would provide unprecedented insight into the determinants both of the timing of cellular processes and of their phenotype, the principles of cell heterogeneity, and the role of cell–cell communication in homogeneous cell populations and co-cultures.

Because most cells adhere to a substrate or to other cells during their growth or differentiation [1], it is advantageous for new technologies to be capable of accessing adhered cells to avoid the

Trends

Miniaturization of biotools: micro-/nanoscale biotools for cell transfection and analysis are being developed to achieve cell-specific experimental capabilities and localized cell–tool interfaces. This allows minimal perturbation to cells and unprecedented spatial resolution, which is essential for fundamental cell studies.

Biotools for adherent cells: despite the risk for altering phenotype or stressing cells, conventional biotechnologies often require suspending and replating cells during *in vitro* studies. Novel micro/nanotechnologies are being developed to transfect and analyze adhered cells, which is particularly advantageous for longitudinal studies of individual cells or for investigating cell mechanisms.

Combination of micro-/nanotechnologies with conventional biotechnologies: various strategies use micro-/nanotechnologies with conventional analytical tools such as fluorescence array readers and atomic force microscopes. This assembly approach promises revolutionary advances in biology and medicine.

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need to disrupt cell processes by suspension and replating. Several technologies for studying adhered cells are currently being developed and, given the need for individual cell access and non-destructive probing, micro- and nanotechnologies are a natural choice because they interact with cells at the appropriate length scale, reduce the working volume of expensive reagents, require less time and space for replicates, allow for automation and integration of sequential analyses, enable portability, and reduce waste [2,3]. We present here an overview of recently developed micro- and nanotools, with a focus on trends in intracellular delivery for *in vitro* studies of adhered cells, and highlight major advantages/disadvantages of these technologies with respect to features such as individual cell selectivity, spatial resolution, non-destructive cell analysis, and potential for high throughput or automation. Finally, we discuss the exciting promise for these technologies to cause a paradigm shift in biological research by providing methods to study cells over time at the individual cell level.

Technologies for *In Vitro* Studies of Adherent Cells

Traditionally, molecules have been delivered into adhered cells by viral or chemical methods, micropipette injection, or electroporation, which are often significantly toxic and produce heterogeneous delivery results. These deleterious outcomes limit their usefulness for cell biology and biotechnology applications where high cell viability, dosage precision, and selectivity within a population are desired. By contrast, micro- and nanotechnologies offer unprecedented levels of spatiotemporal control and cell stress minimization, which enables high-efficiency and high-viability delivery of biomolecules and in some cases non-destructive live-cell analyses that could be transformative for exploring time-dependent phenotypes, heterogeneity, and differentiation mechanisms. Several recent micro- and nanotechnologies have demonstrated promising potential as alternative methods for molecular delivery into adhered cells utilizing working principles that include mechanical penetration and localized electroporation. Because studying a specific adhered cell during its natural state of growth requires accessing the cell individually, these technologies currently present a trade-off between experimental throughput and cell specificity or spatial resolution, as summarized in Table 1. Nevertheless, further development of these technologies promises to increase their ability to study, analyze, and control adhered cells.

Mechanical Penetration

Arguably the simplest mechanism to deliver molecules into cells is by microinjection, which is performed by mechanically piercing the cell membrane using a needle-like structure with a sharp tip, for example a glass micropipette, which is positioned manually using a micromanipulator (Figure 1). Despite its instrumental simplicity, microinjection has several disadvantages associated with the size and shape of traditional micropipettes. The typical tip diameter is approximately 1 μm, comparable to the diameter of small cells (5–15 μm), and it has a tapered geometry, which limits this technique to use on larger cells. As the tip is inserted deep into a cell, the size of the pierced area on a cell membrane increases, which can be a significant perturbation to the cell. In addition, inserting the micropipette is difficult when a target cell is small (spherical diameter of <15 μm) or flat (thickness of <5 μm), where the micropipette tip may contact the substrate before the piercing is complete owing to the compliant nature of the cell. The solution to overcome these disadvantages is to decrease the size of the tip such that it is much smaller than the size of the cell, which also minimizes the applied force needed to pierce into a cell membrane.

1D nanoprobes, cantilevers with nanoscale tips, and nanopipettes have been developed to increase spatial resolution and minimize cell perturbation during membrane piercing (Figure 1). This allows intracellular delivery and live-cell probing without lysing or permanently damaging the cell [4–10]. The small size of the nanoprobe allows extremely precise spatial positioning and long incubation times within the cell. The most common 1D nanostructures for nanoinjection are nanotubes and nanowires with dimensions of 1–750 nm in diameter and 0.5–20 μm in length

Table 1. Micro- and NanoTechnologies for Cell Transfection and Analysis of Adherent Cells[a]

Mechanism		Fabricated Device	Cells	Use Demonstrated	Comments	Reference
Non-Fluidic Nanoprobes	1D probes	Boron nitride nanotube with Au coating (d = 60–70 nm)	HeLa	Delivery of fluorescent QD	Single cell; position controlled by a manipulator	[4]
		Silica optical fiber with metal coating (d = 250–300 nm)	Rat liver epithelial cell	Detection of benzopyrene tetrol	Single cell; manipulator	[17]
		Carbon nanotube (200 nm) or glass nanofiber (100–500 nm) with Au nanoparticles (25–50 nm)	HeLa	Detection of trace of intracellular molecules using SERS	Single cell; manipulator	[18,95]
	Cantilever	Si-nanoneedle (200–800 nm) prepared using FIB	MSC, HEK293	Delivery of DNA	Single cell; AFM; force measurement	[16]
		Carbon nanotube (1–20 nm)	HeLa	Delivery of QD	Single cell; AFM	[13]
Fluidic Nanoprobes	1D probes	Nanotube endoscope (carbon nanotube, 50–200 nm)	HeLa, HOS	Delivery of fluorescent particles; aspiration of Ca^{2+}-labeled cytosol	Single cell; manipulator; SERS and electrochemical measurement	[5,23]
		Glass nanopipette (~100 nm tip diameter)	DRG neurons	Delivery of capsicin to the surface of neurons	Single cell; position feedback control using SICM	[5]
		Quartz capillary pipette (100 nm tip diameter)	Human fibroblast, HeLa	Aspiration of RNA samples	Single cell; position feedback control using SICM	[6]
		Carbon nanopipette (200–400 nm tip diameter)	U2OS	Delivery of tRNA	Single cell; detection for penetration of cell and nuclear membrane; manipulator	[24,25]
	Cantilever	Nanofountain probe (tip opening: d = ~0.3–0.7 μm)	HeLa, HT1080	Delivery of dextran, bovine serum albumin, RNA-/DNA-based MB, DNA plasmids, and nanoparticles	Single cell; localized electroporation or injection; AFM or manipulator; force measurement	[7,29,53,70]
		FluidFM (tip opening: d = ~300 nm)	Myoblast, neuroblastoma, HeLa	Delivery of lucifer yellow CH, DNA, FITC	Single cell; AFM; force measurements; cell manipulation	[28,30,96]

Table 1. (continued)

Mechanism		Fabricated Device	Cells	Use Demonstrated	Comments	Reference
Arrays of 1D-Vertical Structures	Non-fluidics	Nanowire (d = ~200 nm)	HeLa, fibroblast, NPC, neurons	Delivery of siRNA, peptide, DNA, protein	Population of cells; NW-induced delivery	[34]
	Fluidics	Hollow nanoneedle array (500 nm)	NIH3T3, HEK293	Delivery of dextran, DNA	Population of cell; saponin, cell membrane permeation promoter, was used	[97]
		Nanostraw (d = 100–750 nm)	HeLa, CHO	Delivery of ions, PI, plasmid DNA	Population of cells; nanostraw-induced delivery or localized electroporation	[39,40]
Lab-On-a-Chip Platforms	Microwell	Cell arraying-assisted electroporation chip (microwell: d = 100 μm)	HeLa	Delivery of PI, DNA	Population of cells; EP for cell seeding; electroporation of each well	[76]
		Microwell array (microwell: d = 500 μm)	HEK 293T, primary mouse macrophages	Delivery of PI, siRNA, DNA	Population of cells	[77]
	Perforated-substrate	Localized electroporation device (pore size: d = 600 nm to 2 μm)	HeLa, HT1080, neurons	Delivery of PI, DNA plasmids	Population of cells; long-term cell culture; on-chip differentiation; localized electroporation	[3]
		Nanofiber-based sandwich electroporation (pore size: d = 0.2–3 μm and microwell size: d = 100 μm)	Mouse embryonic stem cells	Delivery of DNA	Population of cells	[78]

[a]Abbreviations: AFM, atomic force microscopy; DRG, dorsal root ganglion; EP, electrophoretic force; FIB, focused ion beam; FITC, fluorescein isothiocyanate; MB, molecular beacon; NW, nanowire; PI, propidium iodide; QD, quantum dot; SERS, surface enhanced Raman spectroscopy; SICM, scanning ion channel microscopy.

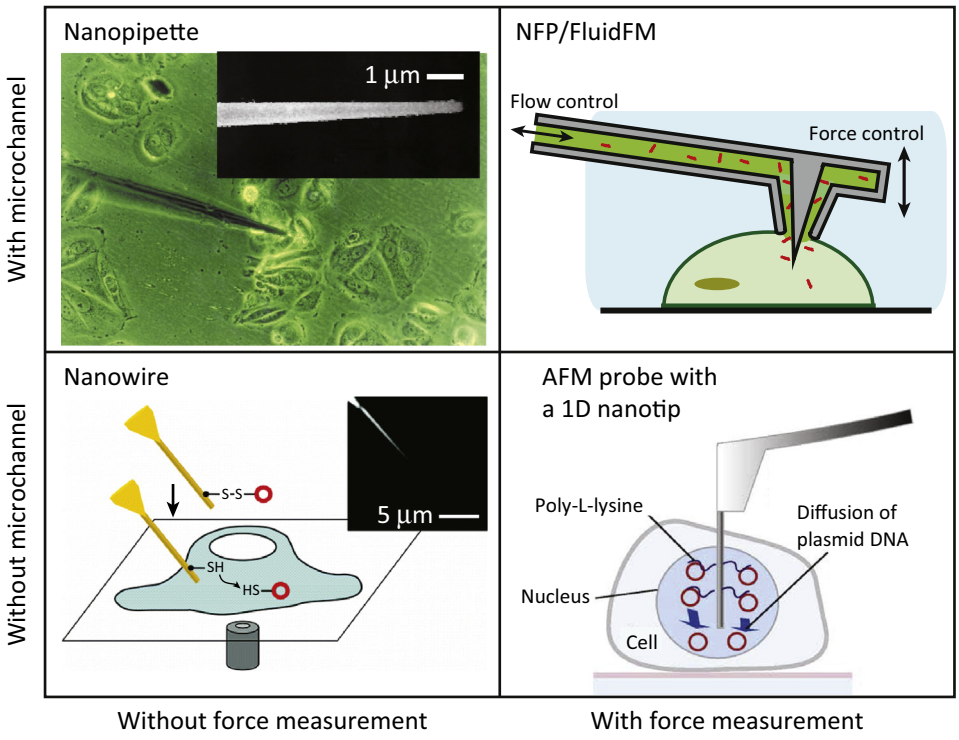

Figure 1. Mechanical Penetration. For delivery of molecules into cells, the membrane of a target cell can be mechanically pierced using a needle-like structure with a sharp tip including (counterclockwise) nanopipettes [17], 1D nanowires [4], or cantilevers with either 1D nanoscale tips [16] or embedded microchannels [7,28]. Figures reproduced with permission.

assembled on a linear wire or cantilever support [11,12]. Common nanoprobes are fabricated using carbon nanotubes [13], boron nitride nanotubes [4], and silicon nanowires [14]. The dimensions and choice of material must be balanced to provide robustness such that the nanostructure possesses sufficient bending stiffness to effectively pierce a cell membrane (and, if applicable, withstand capillary forces during immersion into aqueous solutions), but at the same time have enough strength to prevent mechanical failure during injection [15]. Although fabrication of the nanostructures has become easier with advances in micro and nanofabrication processes, assembly with macroscopic support structures remains challenging.

As in conventional microinjection, the position of the nanostructure tip is controlled using a micromanipulator while manually monitoring it relative to the target cell via an optical microscope (Figure 1, left column). However, the limited resolution of optical microscopes makes monitoring the tip a challenge when the tip diameter is <30 nm [11], which is one reason that integration of the nanostructures with a cantilevered support has become increasingly popular for use within an atomic force microscope (AFM) (Figure 1, right column) [9,13–15]. In addition to cell injection, this AFM-based approach offers the capability for mechanical quantification of cell–tip interaction, as discussed in Box 1.

From Non-Fluidic to Fluidic

Nanoinjection with a non-fluidic nanostructure (Figure 1, bottom row) requires chemical functionalization of the tip such that molecules can be released into the cell or biomarkers can be captured by binding to the functionalized surface [4,17,18]. Thus, this approach is limited to small-dosage delivery of molecules that can be coated to the nanostructure surfaces. Moreover,

Box 1. Force Control Measurement

Atomic force microscopes (AFMs) allow quantification of the force signature associated with the interaction between a probe tip and a cell during mechanical penetration of the cell membrane [13–16]. This quantitative force measurement provides key mechanical information, including the onset of cell–tip contact, penetration of the cell membrane, and dissociation – all signatures that can, in principle, provide a method for computer-aided operation for repeatable and less user-dependent protocols. Pioneering single-cell injection using an AFM was first performed with conventional AFM tips [19,98], whereas recent work has focused on using cantilevers with 1D nanostructure tips [13–16] fabricated using either a bottom-up or top-down approach, in other words the assembly of an AFM cantilever with a nanostructure within a scanning electron microscope [13,15] or fabrication of a nanoprobe tip via etching techniques [14,16], respectively. Force-controlled nanoinjection allows reduced mechanical force on the nanostructure tip (e.g., <200 pN force or <200 nm cell deformation [15], compared to 1–3 nN or >1 μm using conventional pyramid-shaped AFM tips [19]), significantly increasing the success of nanoinjection and the likelihood of cell recovery after injection. In addition, this technique allows for direct insertion of molecules into the nucleus because of the nanoscale tip size and spatial resolution, resulting in higher efficiency (about 74% GFP expression) than other conventional approaches including lipofection (~50%) and microinjection (~10%) [16]. With increasing application of AFM to biological studies, conventional AFM systems are now frequently integrated with an inverted fluorescence microscope for simultaneous optical monitoring of cells, providing real-time imaging of tip insertion into a cell.

it lacks precise control for releasing the molecules into the cell. In addition, because non-fluidic nanoinjection often relies on passive desorption of molecules, this technology requires a relatively long dwell-time, in other words the tip typically remains inside each target cell for several minutes [16,19] and, as a result, intrinsically limits its throughput. To provide active control of fluid delivery and increase the number of cells that can be treated per experiment, fluidic nanopipettes and nanoprobes are increasingly being used (Figure 1, top row).

While technology to reduce the size of glass nanopipette tips has improved such that diameters of <100 nm are routinely used [6,20,21], the fragility of the tip often results in mechanical failure during insertion into cells, and this has prompted innovations such as assembly of a carbon nanotube within a glass nanopipette, by either magnetic assembly [22], flow-through [23], or chemical vapor deposition [24], to take advantage of the superior mechanical flexibility and strength of the carbon nanotube [22–25]. Additional utility is provided in this approach by correlating the change in electrical impedance and interfacial capacitance between a cell and a carbon nanopipette during nanoinjection to independently detect penetration of the cell and nuclear membranes, a crucial capability to achieve automated injection [24,25]. Another approach to increase the mechanical stability of glass nanopipettes is to reinforce them with a thin-film coating, which can also provide useful electrical properties [26]. In practice, however, difficulties in fabricating continuous nanotube structures and clogging of the nanochannels have been reported as major challenges [27].

An important advance that combines microscale fluidics with nanoscale tips, and that eliminates the time-consuming fabrication of individual probes, is the development of cantilever-based technologies that are batch-fabricated from silicon wafers to include built-in microchannels, microreservoirs, and protruding tips with a nanoscale opening [7,28]. These versatile probes can be positioned by a manipulator or an AFM such that single-cell molecular delivery into the cytoplasm or the nucleus can be achieved [7,29,30]. Because these microfluidic devices generally rely on pressure-driven injection utilizing an external pump or electrophoretic transport, precise control of injected volume (0.5 fl to 3 pl) has been achieved with much reduced dwell-time inside a cell (<1 s [30]) compared to non-fluidic approaches. While most fluidic nanoprobe technologies consist of one probe and one reservoir, it is worth noting that the wafer-based fabrication approach enables the design and use of multiple cantilevers or reservoirs on the same chip, as demonstrated by the nanofountain probe (NFP) technology [31] which was fabricated with 12 cantilevers connected via embedded microchannels to two microreservoirs on a single chip [32]. This provides unique possibilities for on-chip multiplexing for delivery of different molecules, or for delivery followed by analysis on the same chip.

It is important to note that, similarly to microinjection, tip size and shape play a key role in nanoinjection. For example, penetration of cell and nuclear membranes using a fluidic AFM cantilever with a conventional pyramid-shaped tip requires about 30 and 60 nN, respectively [30], while the corresponding forces using an AFM probe with 1D nanowire tip are about 0.5 and 1 nN [33]. Because fluidic AFM probes use considerably large forces compared to some of the traditional AFM probes (1–3 nN [19]), perturbation to the cell membrane could be significant and its biological implications should be appropriately investigated in the future.

Arrays of 1D Nanostructures

Microfabricated substrates containing arrays of 1D nanostructures such as nanowires [34–38] and nanostraws [39,40] have been employed for delivery to and/or analysis of a population of adhered cells [12,41]. When cells are cultured on top of the microfabricated substrates, the arrays of 1D nanostructures interact with the cells, although the exact mechanism of penetration of the cell membrane is currently being elucidated [42,43]. By selecting the proper aspect ratio of the nanostructures (length to diameter), probe insertion deep into the cytosol can be obtained. The arrays are generally fabricated by either lithography-based techniques (top-down) or nanowire synthesis from a substrate using deposition techniques (bottom-up). The detailed review on these well-established micro- and nanofabrication techniques can be found in [44]. In general, use of arrays of 1D nanostructures offers higher throughput compared to nanoinjection approaches, and a simpler experimental protocol. The remaining challenges are fabricating uniform arrays of 1D nanostructures and controlling the nature of the cell-nanostructure interface, for example the number of nanowires contacting or penetrating each cell. In addition, unlike nanoinjection, it has been reported that arrays of 1D nanostructures may not penetrate through nuclear membranes, which prevents delivery of molecules directly into the nucleus [45].

Despite successful biological studies using various 1D nanostructures [34–37,39,40], the governing mechanism(s) of interaction between the nanostructure and cell membrane are currently being investigated [34,39,42,43]. For example, spontaneous endocytosis was proposed as the internalization mechanism when using arrays of nanowires (200 nm) or nanotubes (100 nm) [34,39], but high-resolution transmission electron microscope images of the interface between cortical neurons and SiO_2 nanowires (50–300 nm) indicate that cells surround the nanowires rather than penetrate the cell membranes [46]. Theoretical modeling using a mechanical continuum model of elastic cell-membrane penetration predicts that gravitational force alone is not sufficient to trigger penetration of nanowires with diameters of approximately 50 nm [47], but the authors speculate that membrane piercing may be favorable with an additional external force, for example cell adhesion. This assertion is supported by another *in situ* experimental characterization that found only approximately 7% of 100 nm diameter nanostraws penetrate cells and that the penetration is adhesion-dependent [43]. The influence of 1D nanostructures on cell phenotype is somewhat controversial because deleterious effects to the cells such as slow growth and abnormal division, development of irregular contours, lipid scrambling, and DNA damage have been observed in some cases [45,48,49]. Thus, further studies are needed toward fundamental understanding of cell–nanostructure interactions and their effects on cells.

Current and Future Trends in Mechanical Penetration

Low cell viability and low throughput have limited the expansion of conventional injection methods to biological applications beyond *in vitro* fertilization, but these limitations are being overcome by novel fabrication approaches and use of nanomaterials in the manufacturing of nanoprobes and nanopipettes. Likewise, advances in instrumentation for increased force control provide a significantly less invasive means to penetrate cells and, as a result, cell viability after mechanical penetration has improved from <50% to >90%. Indeed, these advances have allowed insertion of nanostructures into cells for over 1 h while maintaining cell viability [14], which presents unique opportunities beyond delivery of molecules toward temporal live-cell

analysis *in vitro*, potentially without repeated penetration of the cell membrane. As an example, selective capture and analysis of biomarkers is possible by coating 1D nanowires with molecules such as antibodies [17] or with gold nanoparticles to monitor DNA and proteins using surface-enhanced Raman spectroscopy [18]. However, experimental throughput is still limited owing to time-consuming positioning of the tips and, as a result, the practical applications remain in the realm of single-cell studies. This is an acceptable limitation for some applications because the unique capability of using mechanical penetration to obtain *in vitro* force measurements can provide insight into the role of mechanical signals that influence cell migration, cell growth, stem cell differentiation, and the regulation of disease states [50].

Electroporation

Since its introduction in 1982 [51], bulk electroporation has become increasingly popular because it is reproducible, versatile (almost no limitation on cell type and size), and easy to use compared to alternative approaches [52]. Electroporation causes transient nanopores to form in the cell membrane when the cell is subjected to a sufficiently large electric field, and through which molecules can be delivered inside cells [3,29,53–56]. Because the electric field is typically created by applying an input voltage between two electrodes, the appropriate placement of the electrodes is crucial for reproducibility and consistent yield. The mechanism of cell electroporation involves three steps: membrane charging, pore nucleation, and pore evolution. During membrane charging, an electrically non-conductive cell membrane behaves as a capacitor between conductive cell culture media and cytoplasm [57,58]. Formation of nanopores (with estimated diameters of 2–50 nm [58]) is triggered when the electric potential difference across the cell membrane reaches 0.2–1 V [59]. Because the whole membrane of individual cells is subjected to an applied external electric field, bulk electroporation tends to create large numbers of small pores over a large fraction of a cell membrane. The key parameters that govern density, location, and size of nanopores in the cell membrane are amplitude, duration, frequency, and shape of the electrical input signals [60]. Transport of molecules into cells through the nanopores involves several mechanisms, including diffusion, convection, electrophoresis, electro-osmosis, endocytosis, and micropinocytosis [57,61,62]. After the electric pulse, the nanopores shrink and reseal in the order of seconds. When an excessively high input voltage is applied, electroporation becomes irreversible. For example, the application of several hundreds to thousands of V/cm to cells results in cell lysis [63].

Bulk electroporation systems suffer from the need for high input voltage, non-uniformity of the electric field, formation of bubbles, and variations in local pH and temperature [52,64,65]. These challenges can be overcome by miniaturization to reduce the required input voltage, create a more uniform electric field, and rapidly dissipate heat because of the large surface-to-volume ratio [52]. Applying this miniaturization toward adhered cells has resulted in localized electroporation methods that have recently been developed in configurations suitable for single cells using fluidic nanoprobes (Figure 2, right column) and for multiple cells using a lab-on-a-chip approach (Figure 2, left column) [3,29,66].

Key Attributes of Localized Electroporation

In localized electroporation, the applied electric field is focused to a small area (typically ranging from tens to hundreds of nanometers in diameter [3,29,40]) on the cell membrane that is electrically sealed by a micro- or nanochannel in contact with the cell membrane (Figure 2). Owing to the focused electric field, this technique results in formation of relatively larger pores in a small area while allowing use of applied voltages that are orders of magnitude less than typical bulk electroporation. As a result, delivery efficiency and cell viability exceed 90% for most cell types and biomolecules. The system typically consists of metallic electrodes, a pump for fluidic control, and either a fluidic cantilever/pipette with nanoscale tip or a microporous substrate integrated within a microfluidic device.

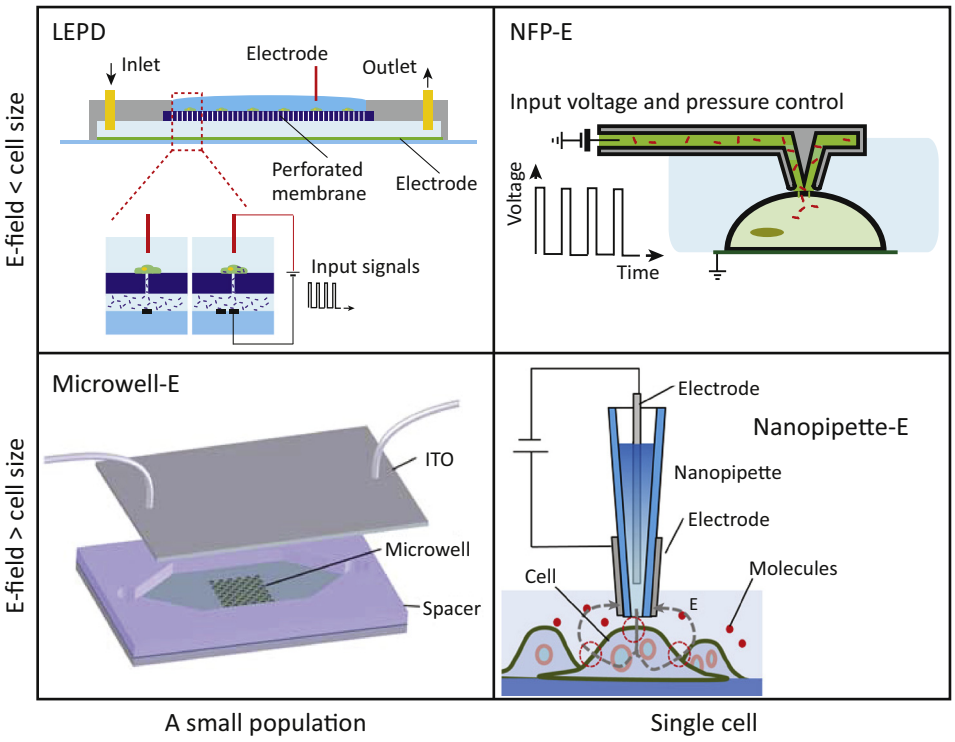

Figure 2. Localized Electroporation. When a cell is subjected to a sufficiently large electric field, transient nanopores are formed in the cell membrane through which molecules can be delivered into the cell. Electroporation has been used in both lab-on-a-chip (left column) and nanoprobe (right column) configurations including (counterclockwise) localized electroporation device (LEPD), and microwell-, nanopipette-, and nanofountain probe-electroporation (Microwell-E, Nanopipette-E, and NFP-E), respectively. Figures reproduced, with permission, from [3,29,72,76]. Abbreviations: ITO, indium-tin oxide; NFP-E, nanofountain probe electroporation.

Localized electroporation shares the same governing equation for the electric field as bulk electroporation, but accurate prediction of the focused electric field requires accounting for the effects of cell and nanochannel shape, size, and interface. Because the effective electric field applied to target cells depends on the microdevice architecture and dimensions, numerical analyses of localized electroporation are often utilized to optimize and quantify the local electric field needed for poration [3,29,67,68]. Recently, experimental studies indicate that a highly focused electric field can alter the mechanism of molecular transport, particularly for large molecules such as DNA plasmids and quantum dots. For example, use of a nanochannel creates a large electric field (70 MV/m for 200 V) only in the area of the cell membrane adjacent to the channel. This extremely large electric field, which is, interestingly, orders of magnitude larger than typical inputs for electrical cell lysis techniques, likely results in the formation of large pores and a strong electrophoretic force that can transport plasmids directly into the cytoplasm [69]. Further studies are greatly needed to fully quantify the pore size and also to understand the dynamic evolution of membrane pores and corresponding molecular transport mechanism(s) when cells are subjected to highly focused and intense electric fields.

Single-Cell Selective Localized Electroporation

Techniques for single-cell localized electroporation have been developed using either glass nanopipettes or microfluidic cantilevers (Figure 2, right column) that are integrated with an electrode to apply the electrical signal through the fluidic channel. They are positioned in contact with the membrane of an adhered cell using a micromanipulator [29,70–72]. This experimental

Box 2. Localized Electroporation with Probe–Cell-Membrane Proximity Detection

The nanofountain probe electroporation (NFP-E) system: By packaging batch-fabricated nanofountain probes with a fluidic circuit containing a wire electrode allows electrical resistance measurement, local membrane nanoporation, and electrophoretic transport of charged biomolecules. Measurement of electrical resistance enables accurate probe–cell-membrane proximity detection, which is ideal for automation and throughput. In this configuration the molecules to be delivered are loaded into the low-volume NFP chip, and cells are cultured in standard Petri dishes or in arrays of microwells or stamped matrix proteins. The arrayed cell protocol provides a multiplexing capability to conduct many experiments of interest in parallel. The NFP-E has proved to be a versatile technique for molecular delivery to many cell types including immortalized cell lines, stem cells, and immune cells [29,70]. Successful delivery of proteins, DNA and RNA hairpin molecules, and plasmids have been achieved with high rates of efficiency and viability (>90% for small molecules) into the cytoplasm or nucleus of various cells, indicating the broad applicability of the NFP-E system as a robust and versatile biotool [29].

setup and capability share many similarities to microfluidic nanoinjection, including high spatial resolution, dosage control, and versatility for delivering different types and sizes of molecules into adherent cells. However, there are two important differences that provide distinct advantages: (i) localized electroporation requires only gentle contact of the nanostructure tip with the cell membrane rather than mechanical penetration, which reduces the stress exerted on the cell and diminishes the influence of size and shape of the cell; and (ii) electrical feedback-controlled positioning of the nanostructure tip can be used to detect contact with the cell membrane, which eliminates the need to rely on a skilled operator and provides a method for automation that significantly increases throughput and delivery efficiency [71]. The nanofountain probe electroporation (NFP-E) system, a key example that demonstrates these advantages, is highlighted in Box 2.

Lab-on-a-Chip Platforms for Populations of Cells

Lab-on-a-chip platforms offer electroporation of a population of adhered cells by combining microwell arrays and perforated substrates into the chip and coupling it with electrical and fluidic controls (Figure 2, left column). Pneumatic valves are often integrated with these small-scale devices to allow automated, multiplexed, and high-throughput microfluidic control [73]. Because cells are cultured directly on these devices, biocompatibility of each component must be appropriately considered, particularly for studies of sensitive cells and long-term cell behavior. Detailed reviews of materials and fabrication for microfluidic systems can be found elsewhere [74,75].

Microwell-based microfluidic electroporation devices (see Microwell-E in Figure 2) typically consist of an array of microwells, with diameters in the range of 100–500 μm, assembled with microelectrodes (patterned metallic films on Si or glass substrates) [76,77]. Input voltages of <30 V are typically used given the small distance between electrodes, and this is often referred to as microelectroporation. Despite the small working distance, the entire cell membrane is exposed to the electric field, which may compromise cell viability, for example by up to 93% using optimal input signals [77], compared to localized electroporation methods. Owing to the array structure, these devices are often designed to be compatible with conventional microarray readers for high throughput analysis. Recently, a cell arraying-assisted electroporation (CAE) chip [76] was developed where cells can be effectively positioned into each microwell using dielectrophoretic and hydraulic forces. For selective electroporation, an array of microwells were registered with an array of independent microelectrodes such that a small population of cells in a particular microwell can be selectively electroporated.

In an effort to achieve gentler electroporation by focusing the electric field on a small region of the cell membrane, microfluidic devices using a perforated substrate (with pore diameters 0.2–2 μm) were developed [3,66]. These perforated-substrate electroporation systems consist of a porous substrate containing nanochannels (see LEPD in Figure 2), which form the substrate for cell culture on the device. Integrated microchannels are used to load and circulate cell media for long-term cell culture on the device and/or transport of a solution with molecules to be delivered. After cells are plated into the cell culture chamber, the cells adhere to the perforated substrate

and electrically seal the nanochannels such that an applied electric field is focused within the area of the cell membrane–nanochannel junction [3]. This unique feature offers effective electroporation of adhered cells in their natural *in vitro* state while maintaining high cell viability (~97%), even when transfecting sensitive cells such as embryonic stem cells and neurons [3,78]. However, similarly to an array of 1D nanostructures, cells are randomly plated on a perforated membrane or patterned microelectrodes, and therefore achieving cell selectivity and consistent cell–nanochannel interfaces remains a challenge.

Current and Future Trends in Localized Electroporation

Because of miniaturization and device architecture, localized electroporation requires much lower input voltage compared to its bulk counterpart and, as a result, achieves significantly improved cell viability. As discussed above, localized electroporation offers a versatile technique as it can be implemented in either nanoprobes or lab-on-a-chip configurations for different biological applications, in other words in the scope of selected single cells or a population of cells, respectively. Localized electroporation technologies for single cells structurally share many similarities with microfluidic-based mechanical penetration approaches; however, localized electroporation presents unique features as a result of the built-in electric circuits. For example, when comparing fluidic probes used for electroporation and mechanical penetration, the former present much greater potential in overcoming the throughput limitation by incorporating electrical feedback signals in addition to optical images. In addition to throughput, microfluidic-based mechanical penetration approaches are frequently complemented by localized electroporation to overcome their limitation on delivery efficiency. For example, 250 nm diameter nanostraws were combined with a platform for electroporation, resulting in increased plasmid delivery efficiency from 10% to 81% into CHO cells [39,40]. Exploiting high delivery efficiency, cell viability, and moderate experimental throughput, localized electroporation technologies show promising potential for unprecedented temporal studies of gene expression and cell phenotype on adhered cells that can provide data on a scale useful for systems-biology analyses [79,80].

Concluding Remarks and Future Perspectives

Vibrant, ongoing innovation and the development of probes and microfluidic systems continue to offer new capabilities for biological research and, as they are adopted into mainstream use, they will cause a paradigm shift in biological studies of adherent cells by achieving precise transfection/sampling while maintaining the natural state of growth or differentiation of the cells. They also offer the potential for automation, leading to hands-free cellular manipulation and analysis systems (see Outstanding Questions). In this regard, we envisage that integration of micro/nanodevices with different functions, such as cell sorting and long-term culture, transfection, sampling, and biomolecule detection with single-cell specificity and high throughput (Figure 3), would provide powerful biotools for advancing applications in therapeutics, diagnostics, and drug discovery, particularly for cellular engineering involving somatic cell reprogramming, stem cell differentiation, and gene editing. As an example, these integrated microfabricated biotools could greatly advance fundamental understanding of stochastic cell signaling pathways – the link between inputs and outputs through interconnected molecular interactions – for example during stem cell differentiation. It is known that stem cells display stochastic behavior within pathways during differentiation owing to crosstalk between multiple pathways, localization of reactions, and the low concentration of molecules involved in signaling [81–83]. One way to explore such complex stochastic behavior is to integrate the unique capabilities of microdevices (i.e., the cell access and analysis module in Figure 3) to include long-term cell culture [3], non-destructive cell access [29,40,66,70], and highly-sensitive sensing capabilities [84] with temporal and spatial resolution. Several other microfluidic-based methods, such as isotachophoresis [85], dielectrophoresis [86], and bio-barcodes [87], could also be integrated for the detection and analysis of biosamples by modular assembly.

Outstanding Questions

How can high-throughput transfection and analysis be achieved in the context of single-cell studies? Current micro-/nanotechnology-based biotools generally suffer from limited throughput which must be addressed by adopting automation and/or multiplexing strategies for comprehensive and statistically-relevant studies of biological complexity such as cell heterogeneity, cell differentiation, and disease mechanisms.

How can micro-/nanotechnology-based biotools be used to provide unprecedented capabilities for novel biological studies and medical applications? We foresee that micro-/nanotechnology-based biotools, which offer precise cell transfection and non-destructive biomolecular analysis, will enable new approaches to address challenges in probing biological processes of cell reprogramming and stem cell differentiation, and eventually the generation of personalized diagnostics and cell therapeutics.

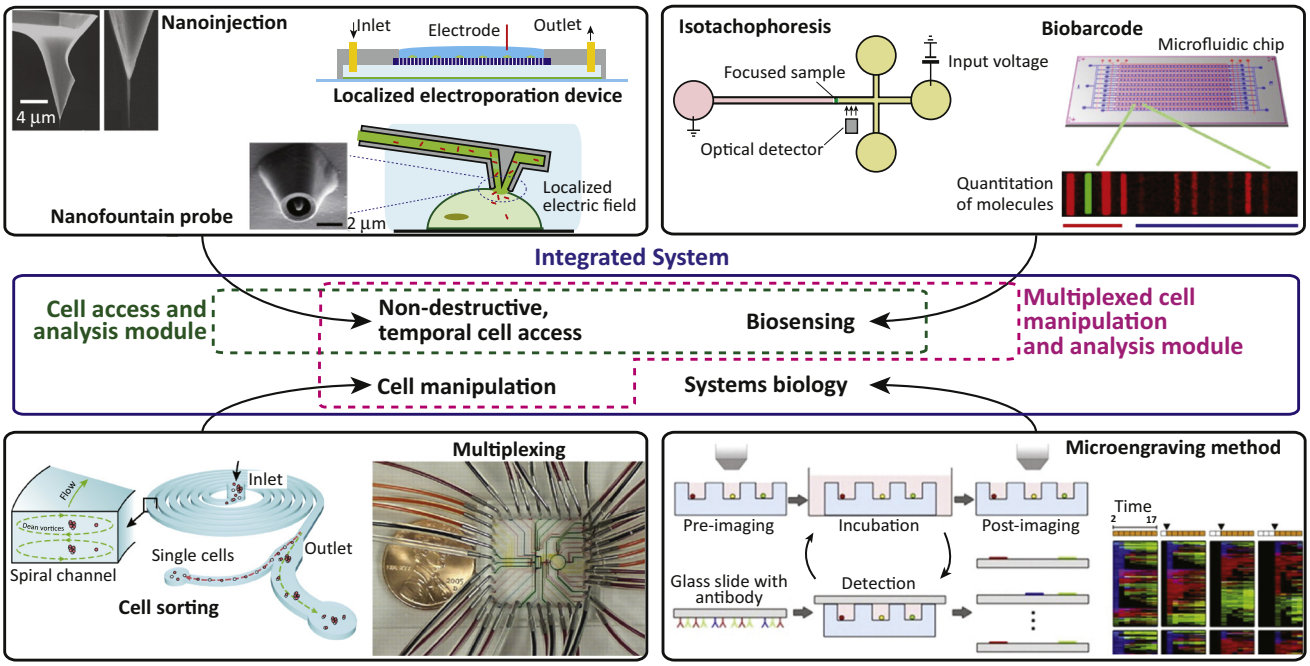

Figure 3. Example of an Envisaged Integrated Micro-/Nanofluidic Platform for Transfection, Sampling, Biomolecular Detection, Sorting, and On-Chip Cell Culture [3,7,15,85,88,92–94]. Through temporal analysis, intracellular processes leading to mechanistic understanding through systems biology is possible [79,80]. Figures reproduced, with permission, from [15,80,85,88,93,94].

To understand complex intracellular input–output relationships and to develop mathematical descriptions of cellular behavior, it is essential to have tools for multiplexed cell manipulation and analyses, for example by adding cell sorting [88] and multiplexing [89] schemes to the cell access and analysis module (Figure 3), that can be performed with throughput that is statistically significant and practical with respect to research time per datapoint (1000 cells have been suggested as a reasonable goal for statistical relevance using single-cell technologies [2,90]). In addition, systems-biology analyses of the temporal data are necessary to systematically process large sets of data from multiplexed cell analysis to elucidate the cellular behavior or mechanisms being studied [79,80]. These integrated systems will have a significant impact on fundamental biological studies and lead to advances in our ability to understand cell phenotypes and develop predictive analyses for engineering higher-level systems such as tissues and therapeutics [91].

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