Designing a nano-interface in a microfluidic chip to probe living cells: Challenges and perspectives

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Nanotechnology-based materials are beginning to emerge as promising platforms for biomedical analysis, but measurement and control at the cell–chip interface remain challenging. This idea served as the basis for discussion in a focus group at the recent National Academies Keck *Futures Initiative*. In this Perspective, we first outline recent advances and limitations in measuring nanoscale mechanical, biochemical, and electrical interactions at the interface between biomaterials and living cells. Second, we present emerging experimental and conceptual platforms for probing living cells with nanotechnology-based tools in a microfluidic chip. Finally, we explore future directions and critical needs for engineering the cell–chip interface to create an integrated system capable of high-resolution analysis and control of cellular physiology.

A major emphasis in biomedical engineering in the last decade has been placed in areas related to tissue engineering, medical diagnostics, and detection of trace amounts of biological agents. Microarray and high-throughput screening technologies are now commonly used for measuring gene and protein expression profiles and for assessing biological activity of potential drug targets (1–3). These tools identify molecular agents in a complex biological milieu and describe events at the length scale of tissues and organs. Although technology development is advancing rapidly, detection of small numbers of molecules or subtle functional defects occurring in a small subset of cells in a population is not yet feasible. Recent advances in the development of fluorescence indicators (4, 5) and microelectronics (6–8) have revealed significant heterogeneity in biological behavior at the level of single cells, presenting the possibility that a few cells within a population can guide the coherent behavior of multicellular communities toward a given tissue phenotype. This information could be used to improve patient health by controlling physiological mechanisms involved in wound healing and disease prevention or by engineering biotechnology-based pharmaceuticals. In addition, recognizing dysfunction in a few cells would provide high sensitivity for detecting cancer, quantifying biological toxins, or investigating the effectiveness of pharmaceutical leads. To realize these goals, however, an ability to probe cell function at the length scale of protein complexes involved in cellular function must be achieved.

This Perspective describes current trends and deficiencies in the understanding and control of cell–biomaterial interactions using platform technologies based on nanoprobes and microfluidic chips. The discussion is broken into three main sections. The first hypothesizes that traditional experimental approaches that characterize an average behavior in millions of cells are unable to capture single-cell phenomena that may indicate disease pathology or the presence of trace amounts of biotoxin. Mechanical, biochemical, and electrical probing techniques at the nanoscale have the potential to elucidate single, subcellular-level events. Second, enabling microchip technologies are required both to present a spatial array of nanoprobes at the molecular to cellular length scale and to provide a reliable structure to relay nanoprobe output to detectors. Although current microchip technologies provide the foundation for engineering a nanotool interface with single cells, both the integration of multiple measurement techniques and the robust automation of output and analysis of complex biological data remain to be accomplished in a self-contained analytical chip. Thus, this Perspective concludes with a discussion of future challenges for engineering the cell–chip interface to create an integrated system capable of high-resolution analysis and control of cellular physiology.

What Can Be Gained from Nanoscale Measurement and Control in Living Cells?

Traditional experiments in cell biology characterize parameters that govern the behavior of organs and tissues in terms of spatial averages from millions of cells acquired at a single point in time. Although recent improvements in instrumentation and the development of fluorescent probes have enabled tracking real-time spatial dynamics of proteins and second-messenger molecules at a cellular to subcellular length scale, often these measurements serve to illustrate only specific examples within a population; conclusions continue to depend on sample statistics. In fact, examination of individual cells often reveals heterogeneous gene and protein expression among regions that are separated by only a few cell diameters (9). Furthermore, the physiological functions of many tissues depend on the spatial arrangement and biochemical communication among a variety of cell types, and disruption of these patterns leads to pathological abnormalities. For example, proliferation of the innermost layer of smooth muscle cells in the artery wall increases risk of stroke or occlusion after balloon angioplasty procedures, and the architectural transition of epithelial sheets to amorphous cell masses indicates dysplasia or cancerous tumor formation. From the clinical perspective, the design of novel biomaterials that promote proper spatial arrangement, phenotype, and communication at the cellular level would be relevant to the design of single cell, microchip-based medical diagnostics applications or toxicity detection assays. For single cell-based diagnostics, biosensing materials must be able to sense functional changes in only a few cells that may be predictive of either subsequent tissue disease or quality of healing. Toxicity sensors should be capable of rapidly detecting and quantifying small numbers of bacteria, viruses, or biotoxin agents to warn of exposure risks.

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To accomplish these goals, cellular bioengineers must first develop technologies that enable understanding and, more importantly, prediction of mechanisms by which a cell responds to local cues in its environment. Three main approaches can be used to accomplish this outcome. First, single cells can be modified by means of cellular markers or chemical tags that report physical interactions or biological signaling among subcellular components. Second, nanotechnology tools can be used to probe the properties of unmodified cells or protein complexes in their native environment. Third, biological systems analysis or systems bioengineering approaches can implement computational modeling, control theory, or decision-making algorithms to predict emergent behaviors of molecular complexes or populations of cells based on local stimuli, such as changes in protein conformation or bond energy landscapes. In all cases, nanotechnology-based tools are providing insight for the first time into these biophysical and biochemical interactions at the length scale of individual proteins and molecular scaffolds.

Novel nanotechnology-based tools for measuring and manipulating mechanical, biochemical, and electrical events at a subcellular length scale could serve as candidate platform technologies for subcellular analysis. Subcellular mechanical interactions, including cell and cytoskeletal geometry, and intracellular force distribution govern an array of physiological processes such as cell division and death (10). Variations in viscoelastic moduli from one region of the cytoplasm to another emerge in response to forces acting at the cell surface (11). Mechanical stiffening in lamellae and edge ruffles correspond to the spatial and temporal distribution of small GTPase activity (12) and the polarized generation of traction forces against the substrate (13). At the molecular scale, tension exerted on extracellular matrix (ECM) proteins may induce protein unfolding to expose binding sites that improve adhesion strength (14). This behavior suggests a novel mechanism by which ECM proteins could serve as molecular force sensors independently of direct cell traction forces, but direct evidence supporting this hypothesis does not yet exist. Spatial control of these adhesive interactions is possible by fabricating patterns of ECM proteins using contact-printing techniques (15). To generate submicrometer protein features that specify the distribution of individual focal adhesion complexes, polymer stamps have been cast from molds fabricated by electron beam lithography and reactive ion etching (16). Alternatively, proteins can be directly printed onto the substrate by using a modified inkjet printhead, thereby enabling creation of closely spaced arrays of protein dots with spatially varying composition (17). In addition to protein nanopatterns on flat substrates, cell adhesion and migration are sensitive to nanometer-scale topography (18). Topographical cues in nanophase polymer/titania composites improve osteoblast and chondrocyte adhesion, perhaps because of modulation of tension transmitted through adhesion sites to the cytoskeleton, leading to bone or cartilage formation that is enhanced compared with that on conventional polymeric or ceramic biomaterials. Polymeric nanofiber matrices mimic the topography and material structure of the ECM *in vivo*. Osteoblasts seeded in these synthetic matrices promote mineralization reminiscent of bone formation (19). These approaches show promise for controlling physical interactions between cells and biomaterials, but methods for directly measuring forces at the scale of a few adhesion bonds remain elusive.

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Strategic presentation of biochemical cues could guide cell differentiation for regenerative medicine and wound healing. A growing body of evidence suggests that guiding cell function and differentiation requires precise timing and spatial arrangement of biochemical stimuli rather than a continuous concentration gradient of diffusible factors. For example, caged growth factors, which are released on demand by cells migrating through a synthetic polymeric matrix material, control morphogenetic processes such as angiogenesis and bone formation (20). Incorporation of bioactive hydroxyapatite crystals promotes mineralization and bone formation in polymeric matrices for tissue engineering (21), whereas effects of growth factors alone are limited by diffusivity to regions near the surface of the polymer. These novel biomaterial designs offer opportunities to control phenotype in living cells, but measurement of differentiation markers still depends on histology techniques. Although discrete spatial domains of pharmacological stimuli can be presented to a single cell by using microfluidics, it remains a challenge to determine which of the stimuli triggers a cellular response based on stimulus–response location. High-resolution, real-time measurement of biochemistry in living cells is crucial for detecting biological responses to pharmaceuticals or biotoxins but is much more difficult. Progress toward establishing this type of "high content screening" currently depends on indirect readouts derived from fluorescence-based techniques. For example, effects on intracellular rheology have been measured by tracking the displacement of cytoplasmic organelles (22) or fluorescence-labeled cytoskeletal elements (23). More general tracking techniques have detected nuclear translocation of transcription factors associated with onset of inflammatory processes (24). Perhaps more important to the drug discovery process is the utilization of FRET to determine where in the cytoplasm an extracellular stimulus triggers activation of signaling molecules associated with inflammatory processes (12). Other approaches depend on molecules whose fluorescence quantum yield changes according to physical interactions with adjacent molecules or after binding a target second messenger molecule such as nitric oxide or calcium. More recently, imaging of lipid trafficking without disturbing the lipid structure (and thus potentially altering function) has been accomplished (25). Although these techniques provide some spatial information regarding activation of biochemical signaling networks, significant challenges remain to achieve the time resolution necessary for high-throughput screening of candidate drugs or suspected biotoxin activity.

Measurement of electrical properties in cell culture has been limited to bulk impedance across layers of cells or to current–voltage relationships across cell membranes using micropipette electrodes. The impedance between the substrate and an electrode in the growth medium is useful for evaluating the integrity of intercellular junctions in a monolayer but cannot pinpoint the spatial locations of events at the cellular length scale. In contrast, patch clamp experiments yield electrical properties of either whole cells or individual patches removed from the plasma membrane, but these measurements are often impractical in physiological configurations relevant to adherent cells in 3D tissues. Thus, arrays of nanoscale electrical contacts at the cell–substrate interface would be useful for a variety of applications. For example, cell biologists would like to determine mechanisms by which cell polarity and directional behaviors such as migration and biochemical signal propagation are derived from sensing spatial cues in the local matrix environ-

ment or from endogenous signals generated within the cell. Local changes in transmembrane voltage would also reveal subtle pathological behaviors, making the cell itself an exquisitely sensitive sensor for pharmacological or toxic agents. In addition, detecting adhesion or behaviors of small numbers of cells or bacteria requires high spatial resolution of small changes in electrical properties. A second major application area is micro- and nanoelectronics. Dielectrophoresis has been proposed for positioning cells, proteins, or DNA on the substrate (discussed in more detail below). This technique would enable fabrication of a cell-based biosensor array capable of rapid high content screening to identify and investigate pharmacological agents or toxins. Control of viral protein or DNA positioning shows promise for guiding self-assembly of molecular electronics circuits in the push for smaller, faster, more energy-efficient device designs.

These examples illustrate recent advances in the measurement and control of mechanical, biochemical, and electrical interactions at the interface between biomaterials and living cells. Thus, new tools are becoming available at a time when cell biologists are learning more about molecular mechanisms of cell physiology and pathology. However, the elucidation of disease mechanisms and increased sensitivity to detect small amounts of biological agents requires improved robustness and spatial resolution for fabricating nanostructured surfaces within microfluidic chips. A major challenge for the next 5–10 years is the reproducible placement and seamless interfacing of functionalized nanoscale features and messaging systems within a chip-based device to measure and interpret complex biological processes in real time.

Enabling Chip Technologies

As microfluidic chip technologies mature, future research will be directed toward increasing versatility and robustness of integrated nanodevices that will spatially and temporally probe cell function at length scales down to that of single protein complexes. Lab-on-a-chip and micro-total analysis systems (μTAS) have been advanced as tools to provide high resolution, low cost, and rapid analysis of diverse biological and chemical applications (26). Microchips with inclusive capabilities can translate complicated, multistep analytical laboratory assays into fully automated handheld devices available in non-laboratory settings. The pursuit of these futuristic, fully integrated analytical devices has spawned the development of microscale technologies to (*i*) isolate and immobilize specific cells of interest, (*ii*) distinguish/separate cells or subcellular organelles, (*iii*) amplify signals that fall below the sensitivity thresholds, and (*iv*) communicate quantitative results as outputs (27, 28). A variety of electrical, optical, and biochemical approaches have been explored for each of these stages. However, devices fully integrating all four steps are scarce, and those that exist, although innovative and ingenious, are only the beginning steps in a field with boundless opportunities (29, 30).

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Existing technologies to manipulate a single intact cell and to measure single-cell functional responses within a microchip environment include chemical, optical, and electrokinetic, each of which take advantage of different physical forces at the micrometer length scale (31). Biochemical approaches have been traditionally used by biologists and biochemists to characterize cell populations, and more recently these methods have been adapted for use in microdevices. The most prominent example is the development of a chip-based DNA purification (32) and PCR technique (33) that rapidly synthesizes thousands of copies of a DNA fragment, thus amplifying the amount of DNA above the detection threshold. Biochemical tags or cues or inhibitors of cellular functions can be delivered to a single cell either in soluble form or immobilized on a solid surface. The introduction of soluble biochemical cues within microchip systems is limited by micromixing and by characteristically slow diffusion of reagents in small channels (34). However, the surface area of a microchannel wall is large compared with channel volume, which enables presentation of controlled concentrations or spatial gradients of chemical stimuli or sensors over a relatively large area with respect to the cell surface. The quantitative measurement of cell responses from such chemical cues remains a challenge. Fluorescence-based techniques show promise for single-molecule detection in systems with ideal optical imaging conditions, but accurate quantification is difficult at such small length scales. In addition, advances in nanotopography fabrication on both horizontal and vertical surfaces in a microfluidic channel are required to accomplish chemical trapping of single cells at a given location. Finally, rapid throughput and analysis are required to determine whether results from only a few cells used in the microdevice are indicative of a population behavior.

Optical tools have been embraced due to the small and precisely controlled forces lasers can impart on cells (35) without impacting cell integrity or viability. However, this approach requires large, expensive, energy-intensive equipment that is external to the microchip. As a result, the microchip cannot be used in a non-laboratory setting. In addition, a microscope is needed to verify movement of the cell and to provide optical feedback for positioning and focusing the lasers that trap and move a cell within the channels of the microchip. The most attractive and versatile tools available are in the area of electrokinetics. Electric fields can be engineered to selectively trap a specific type of cell, subcellular organelle, protein, or DNA and can easily interface with microfabricated devices (36). Power generators for microchips are portable, miniaturizable, and translatable to non-laboratory conditions or remote clinical locations.

Electrokinetic tools for cell manipulations in microdevices fall into two broad categories: linear and nonlinear electrophoresis. Linear electrophoresis includes applications in which the electric field lines are unidirectional (direct current) and parallel. The first principles of linear electrophoresis were explored by Nobel laureate Arne Tiselius and have broadened and matured to the point where mapping of entire genomes is accomplished with DNA slab gel electrophoresis. Optimization led to capillary electrophoresis, in which charged particles are driven through fused silica microcapillaries by a uniform dc electric field. Particle movement is due to electroendosmotic flow induced by the charged capillary wall and by electrophoretic translation of the particle due to its inherent surface charges and size. Capillary electrophoresis and its offshoot technologies have been miniaturized on microchips and adapted for a variety of DNA detection applications (37). Whole-cell capillary electrophoresis microdevices have been developed, but robustness has been limited by the increased complexity of cells under electroendosmotic flow conditions (38). One major limitation of linear electrophoresis applications involving dc fields is that of large ionic gradients that form in the small volumes inherent within the microchip. Electrolysis reactions at the cathode and anode surfaces induce pH gradients between the electrodes to develop within

minutes (38). For subcellular constituents, this phenomenon is not a major concern because it impacts elution times only of the simply charged molecules. The dynamic response of intact cells to ionic gradients is far more complicated and results in physiological changes on the cellular level that impact flow direction within the capillary (38, 39).

Nonlinear electrophoresis circumvents the formation of ion gradients in the medium by alternating the orientation of the electric field at a rate that is greater than the reaction times at the electrodes (>10 kHz) (40). These alternating current fields are either uniform (parallel field lines) or nonuniform (curved, unequally spaced field lines) and allow for whole cells to be selectively manipulated based on conductivity and subsequent polarization of the cell and its membrane (41, 42). One subcategory of nonlinear electrophoresis, known as dielectrophoresis (DEP), has been used alone and in conjunction with field-flow fractionation (FFF) (41, 43) for the separation of live from dead yeast cells (44), live from dead bacteria cells (45), malaria-infected cells from healthy cells (46), and human leukemia cells from healthy blood cells (47). Dielectrophoretic technology has also aided in the detection of cellular responses to toxicants (48) and in the concentration of various cell suspensions, including bacteria (49) and viruses (50). Nonlinear electrokinetics has also been used to achieve particle levitation (51), alignment for the electrofusion of cells (52, 53), electroporation for the inclusion of molecules into cells (27, 54), and fabrication of dielectrophoretic field cages to trap single cells (55, 56). Micropatterned metal electrodes are typically used to create the nonuniform electric fields; however, carefully positioned insulated posts can achieve the same effect (57, 58). More recently, traveling wave dielectrophoresis in serpentine channels has been used to separate white blood cells from red blood cells (59).

Creative integration of microchip technologies and nanostructures is feasible. By tuning the dielectrophoretic frequency within a microdevice, nanoparticles can be manipulated with the same precision as cells (60–63) because a one-to-one correspondence exists between a given alternating current frequency and a nanoparticle interaction or biological event. Multiple biological events could be probed simultaneously provided that their corresponding frequencies are distinct. Although simultaneous analysis of multiple dielectrophoretic frequency responses remains relatively unexplored, such a multiplexing approach would enable single-cell analysis at a variety of length scales from protein complexes up to whole cells to give an overall profile of single-cell function. In addition, disparate frequencies could be used to manipulate reactants in free solution or to aid in targeted transport of reagents or biochemical indicators to the cell surface (27). Combined with electroporation, electrokinetics also enables inclusion of molecular complexes inside the cells (52, 53). Alternatively, functionalized nanoposts can be used to impale cells and relay information from the cell interior to nanoelectronic circuits, thus accomplishing stage 4 of an integrated portable μTAS (64).

By merging the fields of microfluidics, electrokinetics, and cell biology, microchips are capable of creating tiny, mobile laboratories (65). Although a broad spectrum of technological advances are progressing toward the goal of probing living cell populations, these tools are mostly limited to isolated assays. The challenge for the future of designing a nano-interface in a microfluidic chip to probe a living cell lies in seamlessly integrating techniques into a robust and versatile, yet reliable, platform.

Perspectives on the Future of Cellular Nanobiology

Although a growing number of nanoscale and microfluidics-based analysis tests are reported in the literature, the challenge remains integrating these technologies into comprehensive, multifunctional microchips with the sensitivity to detect cellular functions (Fig. 1*a*). Overcoming this barrier requires piecing together perhaps hundreds or thousands of stimulators and sensors to interrogate the physicochemical response profile of a single cell (Fig. 1*b*). Development of a patient-specific therapy based on this profile requires delivery of nanoprobes targeted specifically to molecules of interest without crosstalk to adjacent signaling networks (Fig. 1*c*). To address this challenge, which spans from the multicellular to subcellular length scale, discussions of the focus group[§] centered on several critical questions. What technology advances must be realized such that existing tools and future technology developments could be completely integrated on a chip? How could one assess whether the data from the single cell was meaningful?

Major challenges remain for probing cell biology at the nanometer scale. Because diffusion across the cell cytoplasm of second messenger molecules such as calcium ions occurs on a subsecond time scale, future self-contained, chip-based laboratories must be capable of reading out and interpreting a large array of multiplexed signals in real time. Detecting signals with adequate signal-to-noise

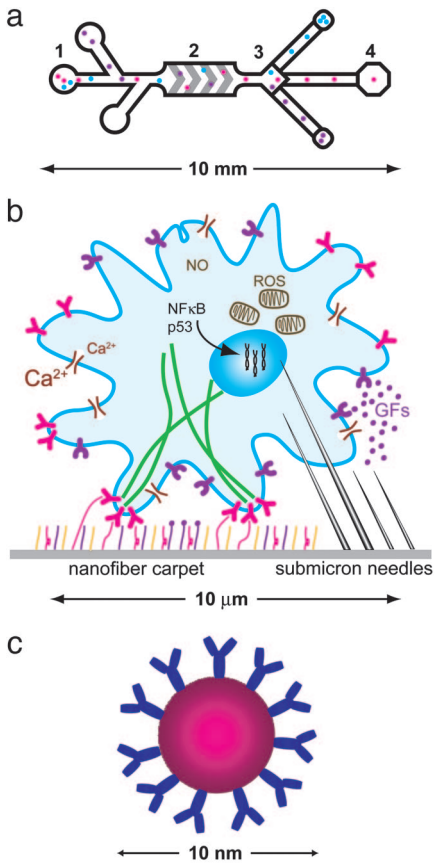

Fig. 1. Agents and length scales involved in designing a microchip-based nanoanalysis system to interface with living cells. (*a*) A typical microfluidic chip sequence illustrating circular sample/reagent introduction ports (label 1), reagent delivery to the main channel and subsequent mixing chamber (label 2), a sorting hub (label 3), and a cell isolation chamber (label 4). Colored dots represent living cells with different phenotypes that are treated, tagged, and/or sorted to stimulate or measure physiological activity profiles. (*b*) Cell isolation traps incorporate an array of submicrometer needles for stimulation or sensing in the cell nucleus (dark cyan), cytoplasm (light cyan), or extracellular space. A ''nanofiber carpet'' includes stretchable polymer chains to bind cell adhesion receptors and measure mechanical forces (magenta), filaments capped with growth factors (GFs, purple) that are either immobilized or released in soluble form, and nanowire electrodes (gold) to stimulate or measure electrical activity near the cell membrane. Data from these sensors comprise a ''cell activity profile'' consisting of mechanical events such as interactions within the cytoskeleton (green), production of reactive metabolites such as nitric oxide (NO) and reactive oxygen species (ROS), toxic gene regulatory responses such as activation of the transcription factors NF-κB and p53, and transport of Ca^{2+} ions that contribute to signaling networks and transmembrane potential. (*c*) Schematic of a conjugated nanoprobe that could be targeted to protein complexes on or in the cell of interest using the submicrometer needles or nanofiber carpet.

[§]Focus Group 4, The Second Annual National Academies Keck *Futures Initiative*, ''Designing Nanostructures at the Interface Between Biomedical and Physical Systems,'' November 18–21, 2004, Irvine, CA, pp. 31–37.

ratio is not currently feasible in real-time readouts from sensor arrays that are spatially dense at nanometer length scale. The delivery or placement of nanoprobes in close proximity introduces the potential for interference among adjacent sensors. In addition, the array of sensors or probes must be spatially addressable at a time resolution sufficient for measuring or stimulating the events of interest. Finally, a prototype chip must be engineered to be reproducibly manufactured. To overcome these challenges, future molecular electronics designs could take advantage of biological self-assembly processes to fabricate materials interfaces capable of communicating directly with protein complexes on the living cell surface.

Within the realm of electrokinetics to probe cell physiology, challenges arise in isolating physiologically significant readings from noise associated with factors such as thermal gradients, gravity, and buoyancy (66). For instance, dielectrophoresis forces must be closely correlated to physiologically important cellular parameters such as growth stage, protein expression, or disease abnormalities. Another major challenge is developing energy-efficient power sources that are portable and detection/sensing devices that are spatially sensitive and do not rely on expensive, nonportable equipment located external to the chip. To solve the energy management problem in a self-contained μTAS of the future, engineers could learn along with cell biologists how biological energy production through oxidative metabolism supports cell functions. Such an advance would take the field of molecular electronics and nanofabrication beyond the current focus on biological self-assembly to a revolutionary new approach of generating electricity by harnessing biological reduction–oxidation mechanisms.

The most important gap in the state of the art relates to the current (lack of) understanding of cells and populations of cells as physiological systems (67). The rational design of an array of inputs to engineer cell behavior requires creation of a spatiotemporal "cell activity profile" (Fig. 1*b*). One major challenge is to determine how much information is enough to specify a functional profile. The current approach for gene microarrays involves defining subsets of functionally related genes for expression profiling, but this model presupposes knowledge of interactions among gene groups. In the case of cell-signaling networks, the functional consequences of crosstalk among pathways are many-faceted or even unknown. Nanosystems on a chip would enable multifactor interaction analysis to help define a physiologically relevant reference state for isolated cells *in vitro*. This steady-state cell activity profile would replace static expression and activation measurements with dynamic interaction profiles. Thus, the difficult task of comparing diseased to healthy cells would be simplified by analyzing cell signatures during and after stimulation. Although it remains difficult to compare single cells *in vitro* to cell profiles *in vivo*, detection of cellular dysfunction associated with early stages of disease would be more robust using activity profiles than searching for a few molecular markers.

To understand and engineer cell function successfully, future analysis of physiological and pathological activity profiles will incorporate new developments in biological systems engineering. Using output of analysis from multidimensional data arrays generated by pattern recognition and neural network-type learning algorithms, control theory and decision-making processes can be applied online within a self-contained μTAS. Thus, future medical devices tailored to individual patients may consist of self-adapting robotic chips that are capable of sampling tissue function, reporting infection, and initiating healing processes automatically, thereby augmenting or replacing the body's innate response.

Potential benefits to society of personalized nanosystems on a microchip result from real-time detection of numerous events in parallel. In addition to early detection of cell-level dysfunctions, these systems will enable broad screening that encompasses not just a large number of toxic stimuli and disease processes but also population subgroups. Such activities will revolutionize patient-specific therapies and personalized medicine. Of course, reaching this goal will require advancing the knowledge base of cellular and subcellular functions, perhaps by designing nanosystems that operate in the tissue milieu.

The key to the future of nanoscale systems that interface with human physiology lies in this futuristic goal to scale up cell profile monitoring to communities of cells *in vivo* rather than depending on single cell analysis *in vitro*. A complex systems approach is required to predict emergent behaviors that result from the control of individual nanoscale components of cells and tissues. Current work in systems biology and metabolic network analysis is leading the charge toward realizing nearly noninvasive therapeutic approaches that are based on assessment of local cell activity profiles and targeted delivery of cell- and patient-specific agents.

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