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# Sensing cell-secreted molecules

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**Abstract** Fields of medicine and life sciences are constantly evolving and striving for improved understanding of how cells function at an individual level, small ensemble level, and tissue level. Such improved understanding will translate into developing therapeutic strategies as well as approaches for disease diagnosis. Behavior of cells at all levels is shaped in significant part by secreted molecules that serve as cues for proliferation, migration, death, and other cell life-altering events. Improved understanding of what signals released when by which cells requires novel tools for local detection of cell-secreted molecules. This paper reviews recent efforts by bioengineering and bioanalytical chemistry communities to develop biosensors for detecting molecules in extracellular space. Multiple topics including antibody-, enzyme- and aptamer-based biosensors for cell analysis as well as sensor miniaturization approaches are discussed.

**Keywords** Aptamer · Biosensor · Biomaterial · Cytometry · Electrochemical · Microfabrication · Metabolites · Microfluidic · Photolithography

## Introduction—detection of cell metabolites

Living cells transmit a variety of chemical and physical signals, such as changes in pH, oxygen consumption, ion concentrations, membrane potentials, and release of proteins and metabolites [1]. Given the appropriate signal transducer, these signals can be quantitatively measured to gain valuable comparative information about the cell response to different extracellular stimuli [2]. Cell-secreted metabolites are the end products of cellular regulatory processes [3]; therefore, monitoring cellular metabolism is critical to clinical diagnostics, development of new drugs, and assessment of biocompatibility.

Cellular signals can be measured and quantified in many ways. Traditional in vitro cell cultures employ large numbers of cells and reveal little information about metabolic dynamics. These methods are thus not suitable for analyzing scarce cells (e.g., primary cells, cancer cells, or stem cells) or monitoring metabolic events requiring high temporal resolution.

In addition, due to the limitations of existing instrumentation technology, traditional in vitro cell culture studies tend to be focused on monitoring large cell populations and rely on population averages to draw conclusions on cell function. The analysis of single cells or small groups of cells provides a more accurate representation of cell behavior; therefore, the biological research community is increasingly focused on the analysis of individual cells or small groups of cells [4, 5].

In order to develop new bioanalytical methods for cell analysis, it is essential to integrate miniaturized biosensors so as to measure analytes at the site of the cell [6]. Our review discusses some of the recent advances in developing microsystems for cell analysis, focusing on biosensor development and the integration of biosensors with cells. This review is not meant to be exhaustive and points out only a small fraction in the large pool of excellent articles on this subject.

## Microfabrication approaches for integrating cells and biosensors

The importance of placing sensors next to cells in order to detect molecules going in and out of cells has been recognized by the biosensing/bioanalytical community for at least two decades [7–9]. To a large extent, early efforts focused on detection of neurotransmitters such as dopamine using

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hand-machined microelectrodes for detection of amperometric/voltammetric signals [7, 10]. The use of microelectrodes was then extended from analysis of tissue to analysis of cells. Precise positioning of the microelectrodes near specific cells was initially ensured by micromanipulators [11–13] and has evolved to include the use of scanning probe approaches such as scanning electrochemical microscopy (SECM) [14–16]. While the use of SECM is gaining popularity for applications requiring precise placement of sensor along the cells [17, 18], external manipulation of sensors is somewhat complicated and is not amenable to high-throughput analysis.

Microfabrication represents a suite of techniques for the fabrication of integrated circuits and is therefore well suited for miniaturization. Importantly, starting in early 1990s, variants of microfabrication approaches have been applied to controlling cell adhesion to surfaces [19–22]. Microfabrication techniques such as photolithography and soft lithography have proven particularly well suited for designing and controlling microenvironments for living cell assays and studying cellular dynamics. Because these processes are parallel, they may be used to construct large number of micrometer-scaled objects, such as arrays of wells for cell analysis [21–24].

## Antibody-based biosensors for detection of cell-secreted molecules

A number of groups have employed microfabricated platforms integrated with antibodies for detection of cell-secreted proteins. These approaches may be broadly categorized into (1) single-cell arrays and (2) antibody spot arrays. Formation of single-cell arrays is enabled by the fabrication of microwells with dimensions of individual wells comparable to that of single cells. Photolithography and soft lithography approaches have been used to fabricate microwells in polymeric or glass substrates [25–27]. To enable detection of secreted proteins, antibody molecules may be immobilized inside or next to microwells housing cells. One approach described by the Love group employs arrays of polydimethysiloxane (PDMS) microwells housing single cells and covered with a glass substrate containing detection antibodies [28] (Fig. 1). This approach has been used to concurrently detect cytokines (IFN-γ and IL-6) and antibodies (IgG and IgM) secreted from individual human immune cells [29]. Other groups fabricated microwell arrays with antibody molecules immobilized inside or on top of the wells [30, 31]. Of particular note is the work of the Muraguchi lab which has demonstrated fabrication of microwell arrays in polystyrene and then employed these microarrays for high-throughput analysis of antigen-specific B cells [30, 32].

Yet another approach was developed by Heath and co-workers who micropattern antibodies against multiple analytes inside microfluidic channels, infuse immune cells into the channels, and then use pneumatic valving to segment channels and trap single cells in small volumes next to the antibody regions [33]. This approach allowed the analysis of the secretion of multiple proteins at a single-cell level and to query phenotypic heterogeneity in tumor-specific responses of cytotoxic T cells. Our lab has pursued a somewhat different approach to assembling single-cell arrays. This approach involves photopatterning of non-fouling poly (ethylene glycol) (PEG) hydrogel on glass or hydrogel substrates precoated with antibodies [34]. This design of the surface allows to capture pure leukocyte subsets from minimally processed blood and to carry out cytokine assays on these same cells [31].

Another method for detecting cell-secreted proteins involves the use of antibody arrays. These arrays are printed using robots originally designed for DNA hybrizidation and gene expression studies [35]. Arrays of printed antibodies have been used in the development of multiplexed immunoassays and in capturing cells to phenotype leukemia

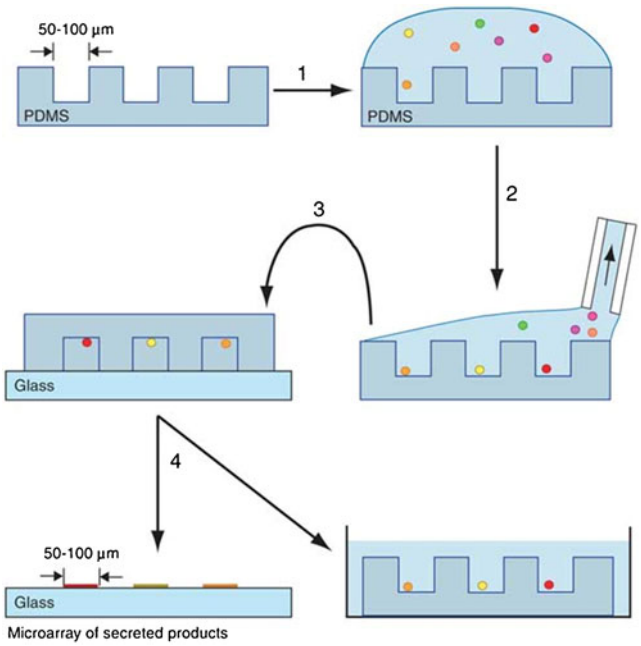


**Fig. 1** Schematic diagram depicting method for preparation of engraved arrays of secreted products from a mixture of cells. *1* A suspension of cells is deposited onto an array of microwells fabricated by soft lithography. *2* The cells are allowed to settle into the wells, and then, the excess medium is removed by aspiration. *3* The dewetted array is placed in contact with a pretreated solid support, compressed lightly, and incubated for 2–4 h. *4* The microwells are removed from the solid support and placed in a reservoir of medium. The figure is based on information presented in [28]

[36–38]. Davis and co-workers used array format to capture antigen-specific T cells and to detect, on the same spots, the release of an important inflammatory cytokine IFN-γ from captured cells [39]. Our lab was interested in creating arrays of protein spots with some spots being used for cell capture and other adjacent spots used for the detection of cell-secreted proteins [40, 41]. These microarrays have been employed to capture T cells and determine the levels of secreted cytokines. In another application, antibody spots were printed next to spots of extracellular matrix to culture hepatocytes and to capture, on the adjacent spots, proteins secreted by hepatocytes [42]. The liver-specific proteins, α1-antitrypsin and albumin, were detected using antibody arrays printed next to hepatocyte spots. We see this approach as a way to assay local cell function without having to collect large amounts of media for ELISA analysis. In addition to obvious diagnostic applications, we view microarrays of antibodies or other biorecognition elements as complementary to the high-throughput cell culture approaches being developed [43–45].

## Aptamer-based biosensors for detection of cell-secreted molecules

While antibodies serve as the main source of biorecognition elements for biosensors, immunoassays based on antibodies have a number of limitations; chief among them is lack of temporal data on binding of target analyte. In this regard, nucleic acid-based binding probes, aptamers [46, 47] have an advantage over antibodies.

Aptamer-based optical probes are short nucleic acid molecules and thus are more robust and simple than antibody-based optical probes. The selection of aptamers is termed Systematic Evolution of Ligands by Exponential Enrichment and involves the discovery of full-length aptamers from single-stranded DNA or RNA. The selection process results in highly robust and specific aptamers that are 25–40 nucleotides long [47].

The relatively simple chemical structure of aptamers allows the insertion of electrochemical or fluorescent reporter molecules [48] as well as surface-binding agents [49] in specific locations on the oligonucleotide [50]. During probe-target binding, the alteration of the fluorescence characteristics of the reporter group is exploited to generate an analytical signal [51].

The robustness and simplicity of aptamers have allowed for multiple uses of aptamer-based biosensors, and a number of direct detection strategies employing aptamers have been proposed [33, 52, 53]. Over the years, aptamers have been used for the detection of proteins, small molecules, and ions with high specificity and affinity [46, 54–58]. In addition, while there have been reports on aptamer-based techniques for multiplexed detection of biomarkers and cells from complex living samples [59–63], few studies have focused on utilizing aptamers for real-time detection of secretions from living cells. In one study, Karp and colleagues utilized previously established microwell arrays by the Love group [64] in conjunction with aptamers bound to the membranes of mesenchymal stem cells to detect platelet-derived growth factor from neighboring cells [65].

Our group has been micropatterning surfaces [40, 66] and using antibody arrays [40, 41] for the detection of cytokine release from leukocytes. While there have been reports describing continuous detection of cell-secreted proteins using antibodies, the complexity of devices used in these experiments is quite high [67, 68]. We sought to explore the use of aptamers for making continuous measurements from cells and have designed electrochemical and fluorescence aptamers for detection of IFN-γ [69–71]. We thus demonstrated micropatterned surfaces juxtaposing aptasensors and cells for continuous detection of cytokine production [72]. Figure 2 below

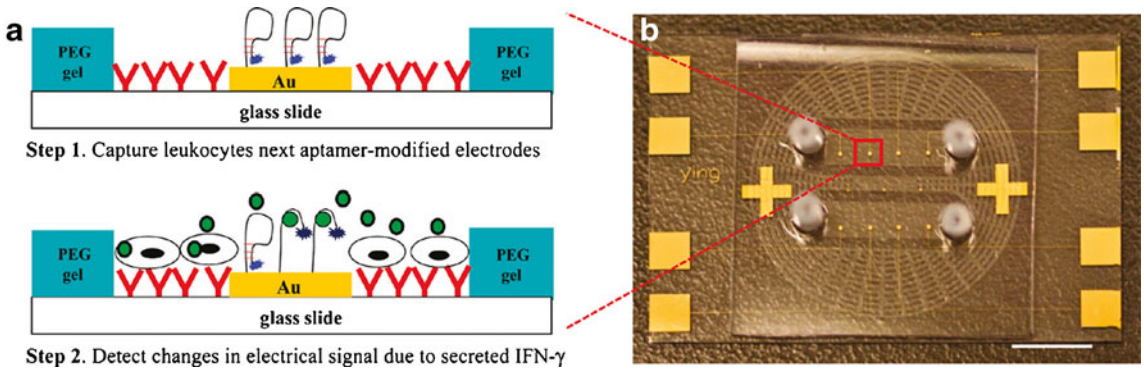


**Fig. 2** **a** Schematic of the sensing area layout. Au electrode arrays fabricated on glass slides are surrounded by PEG hydrogel and incubated with T cell-specific antibodies. Upon injection of blood, leukocytes are bound on Ab-modified glass regions next to aptasensors. Cytokine release is detected at the aptamer-modified electrodes by an aptamer recognition layer consisting of DNA hairpin-containing redox reporters (**b**). These micropatterned substrates are integrated inside PDMS microfluidic channels (*scale bar*, 4 mm). There are four individually addressable aptasensing electrodes per channel. The figure is based on information presented in [72].

shows a scheme for the electrochemical detection of cell-secreted IFN-γ. The microfluidics-based microsensor consists of an array of aptamer-modified electodes, each housed in PEG wells so as to define cell attachment sites. The attachment sites are modified with anti-CD4 Ab to promote binding of leukocytes. Upon infusing blood (lysed free of red blood cells) into the microfluidic channels, leukocytes are captured next to the sensing electrodes and stimulated to produce cytokines. IFN-γ released by cells is then detected in real time at the neighboring sensing electrodes using square wave voltammetry to determine the rate of IFN-γ production.

## Enzyme-based biosensors for monitoring cell metabolites

Enzyme-based biosensors can be divided into two main categories: (1) optical and (2) electrochemical. While optical transduction is widely used [73–75], electrochemical enzyme-based detection of cell metabolites remains prevalent due to the simplicity, robustness, and cost-effectiveness of electrochemical analysis. As an example, our lab has reported the development of an optical, enzyme-based biosensor for the quantitative local detection of hydrogen peroxide ($H_2O_2$) secreted by stimulated macrophages [76, 77]. In this method, the detecting enzyme, horseradish peroxidase (HRP), was immobilized inside PEG hydrogel. The HRP-containing PEG hydrogel was photolithographically patterned to create biosensing microstructures (Fig. 3) on the glass surface. When macrophages were introduced into the system, the cells selectively attached to the non-patterned areas on the glass surface, allowing for close proximity of the biosensing elements to the cells. Amplex® Red, an indicator fluorophore, was either immobilized inside the hydrogel elements along with the enzyme or added into the cell culture media during cell activation. Mitogenic simulation of the seeded macrophages resulted in $H_2O_2$ secretion, which was measured as fluorescence in the HRP-containing hydrogel microstructures, and fluorescence intensity was found to be correlated to analyte concentration [77]. In this study, cells were positioned in close proximity of sensing elements by utilizing PEG hydrogel as both a non-fouling agent as well as a matrix for immobilization of enzymes.

Another class of enzyme-based biosensors is electrochemical. Electrochemical enzyme-based biosensors have been used extensively to monitor cell function [7, 78] such as glucose and oxygen consumption by single Islets of Langherans [11, 78, 79] detection of exocytosis events from individual cells [80] and detection of nitric oxide release [81]. Microfabrication techniques have enabled miniaturization of electrodes and their integration into microdevices. However, several challenges remain, including: (1) how to position cells near the sensing electrodes and (2) how to ensure that, while reporting on cell function, these electrodes remain non-fouling and unaffected by cellular activity. To achieve this, microfabrication methods have been employed in combination with the non-fouling properties of some polymers to integrate sensing elements with biological components and enable the positioning of enzyme-based sensors in close proximity to live cells [1, 82, 83].

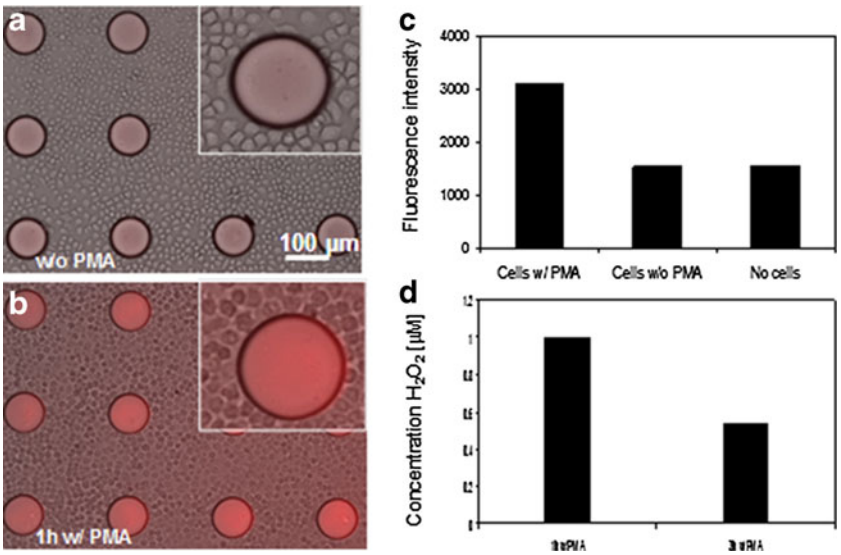


**Fig. 3** Detection of $H_2O_2$ from macrophages activated with phorbol 12-myristate 13-acetate (PMA). **a**–**b** Brightfield/fluorescence images of macrophages adherent around HRP-containing PEG microstructures without (**a**) and with (**b**) PMA incubation. Exposure of cells to PMA was expected to trigger $H_2O_2$. **c** Fluorescence signal from sensing hydrogel elements incubated with macrophages (+) PMA, macrophages (−) PMA, and no cells. These results show that minimal or no $H_2O_2$ release was observed in unactivated macrophages. **d** Using calibration curves, concentration of macrophage-secreted $H_2O_2$ was determined after 1 and 3 h of incubation with stimulant PMA. The figure is based on information presented in [77]

We have described the integration of miniature gold electrodes next to immune cells (macrophages) in order to detect cell-secreted $H_2O_2$ [84] (Fig. 4). Photopatterning of PEG hydrogels was used to both immobilize HRP molecules onto electrodes and to define regions for cell attachment in the vicinity of sensing electrodes [76, 85]. Electrodes micropatterned in such a manner were enclosed inside PDMS microfluidic conduits and incubated with macrophages. The cells did attach onto the exposed glass regions in the vicinity of the electrodes and nowhere else on the non-fouling PEG hydrogel surface. The microfluidic device was further converted into an electrochemical cell by placing flow-through Ag/AgCl reference and platinum wire counter electrodes at the outlet and inlet, respectively. This microdevice with integrated $H_2O_2$-sensing electrodes had sensitivity of 27 μA/cm2mM with a limit of detection of 2 μM. In a similar study, Matsue and colleagues described an indium-tin-oxide electrode coated with osmium-polyvinylpyridine gel polymer containing HRP enclosed in a PDMS device to monitor $H_2O_2$ release from leucocytes [86]. These recent reports provide examples of controllable seeding of cells next to electrodes, activation of cells, and on-chip monitoring of cell secretions in real time.

## Microfluidic approaches to enable cell sensing

Microfluidics has become an attractive tool for cytometry-based single-cell analysis because its dimensions are comparable with the size of single cells and also due to the potential for performing rapid multiplexed analyses. Several "lab-on-a-chip" devices have been reported that integrate cell transport, sorting, lysis, separation, and chemical derivatization [34, 87–91]; however, these devices rely on the lysis of cells, which is not suitable for real-time analysis.

To enable the analysis of live cells, the Kennedy group developed a microfluidic device that incorporates continuous perfusion and an on-line electrophoresis immunoassay for monitoring insulin secretion from single live Islets of Langerhans [67, 68, 92]. In this device, the cell chamber is perfused with cell culture media, and perfusate was continuously sampled at 2 nL $min^{-1}$ by electroosmosis through a separate channel on the chip. The sampled perfusate is mixed on-chip with fluorescein isothiocyanate-labeled insulin and monoclonal anti-insulin antibody and allowed to react for 60 s as the mixture travels down a 4-cm-long reaction channel. The reaction mixture is then separated under an electric field of 500 to 600 V $cm^{-1}$. Using this method, individual islets were monitored for up to 1 h while perfusing with different concentrations of glucose (3 to 11 mM).

In a more simplified approach, Easley and colleagues reported a novel passive microfluidic droplet sampling method combined with fluorescence imaging to quantify zinc secretions from live pancreatic islets [93, 94]. The microdevice (Fig. 5) was fabricated using standard soft lithography techniques, and the use of passive flow control within the device removes the need for syringe pumps, requiring only a single hand-held syringe [95]. Cultured and prepared islets were loaded onto the microdevice and zinc ions, which are co-secreted with insulin from β cells, were quantitatively measured from basal and glucose-stimulated individual islets with high temporal resolution using the FluoZin™-3 fluorescent indicator,.

In another example, Lee and colleagues developed an integrated microfluidic array plate for simple cell loading,

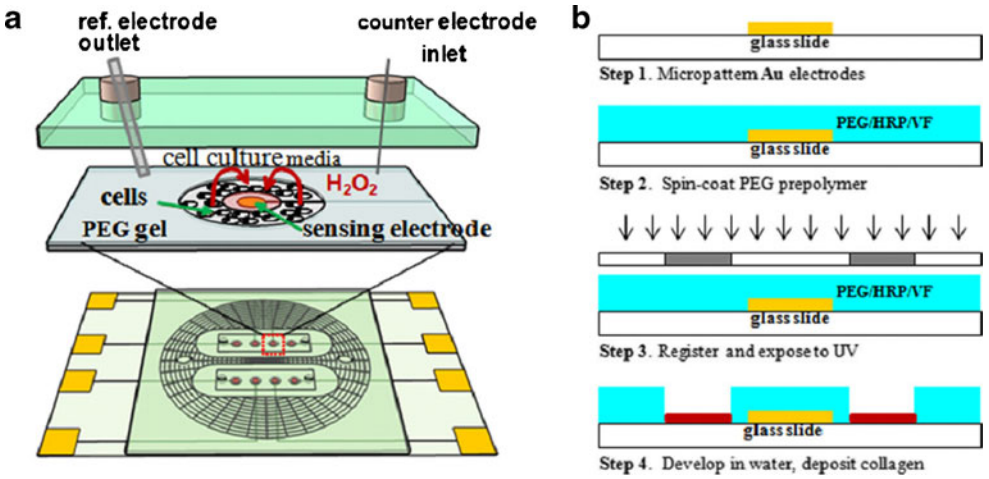


**Fig. 4** **a** *Upper panel*: schematic of a single electrode for detection of cell-secreted $H_2O_2$. Macrophages are captured on a micropatterned surface in the vicinity of sensing electrode. The sensor consists of an HRP-carrying hydrogel disc immobilized on top of a 300-μm-diameter Au electrode. Each channel constituted an electrochemical cell with Ag/AgCl flowthrough reference and platinum wire counter electrodes placed at the outlet and inlet, respectively. *Lower panel*: There were two parallel microfluidic channels in the device, with four individually addressable electrodes in each microfluidic channel. Each fluidic channel had a volume of 3 μl. **b** Step-by-step diagram for immobilizing HRP carrying hydrogel microstructures on top of Au electrodes. The figure is based on information provided in [84]

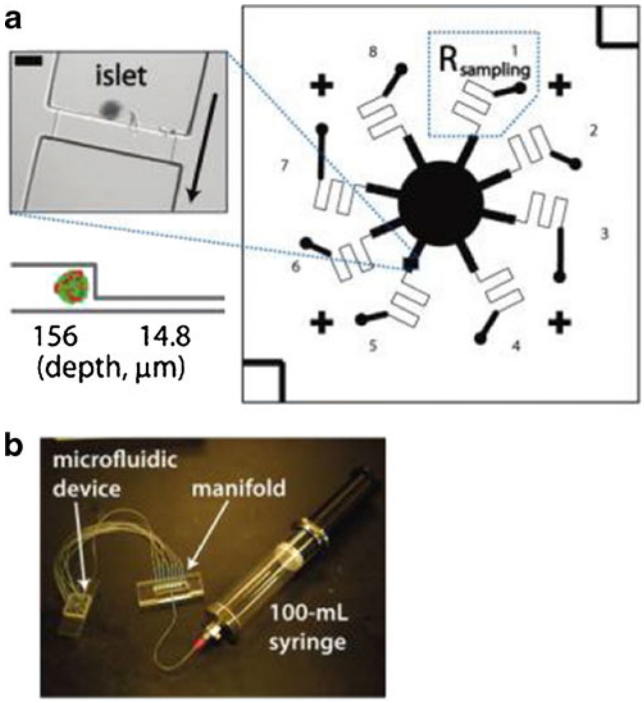


**Fig. 5** Passively operated microfluidic device for islet secretion sampling and imaging. **a** Device layout, including an image of a trapped islet. **b** Hand-held apparatus for secretion sampling with no electrical components. The figure is based on information presented in [94]

cell capture, reagent loading, reagent mixing, and on-chip processing for dynamic studies with several readout capabilities such as real-time Nucleic Acid Sequence-Based Amplification (NASBA) and immunofluroescent (IF) protein expression detection [96]. The microfluidic system is capable of performing 512 dynamically configurable integrated cell-based assays coupled with real-time NASBA and IF analysis. The device has an on-board gravity-driven loading and flow control of cells and reagents and utilizes a micro-trench to capture cells and particles for gravitational sedimentation making it independent of tubing, cumbersome syringe pumps, or other active macro-scale flow control components such as pressure sources and solenoid valves. In addition, a controlled flow over the top of the trench allows successive, diffusion-driven loading, mixing, and replacement of reagent while retaining the cells free of hydrodynamic shear stress within the trench. The required flow control is achieved by setting the height of a liquid in an attached vessel, e.g., a pipette tip, which generates a hydrostatic pressure head at the inlet.

## Future directions: biosensors for biocompatibility testing

Monitoring molecules secreted into the extracellular space is critical to the study of disease pathophysiology, development of new drugs and clinical diagnostics, and cell and tissue engineering. We see a bright future for cell function biosensors in all of these areas. In this section, however, we would like to focus on biocompatibility testing—an area that has not received much attention from the biosensing community. The International Standards Association (ISO) provides guidance for the selection of tests to evaluate the biological responses relevant to the safety of medical devices and materials [97]. These guidelines require that the materials used for the manufacture of body-contacting medical devices as well as any intended additives, process contaminants, residues, leachable substances, and degradation products be evaluated for cytotoxicity using in vitro cell culture methods [98]. In addition, the ISO guidelines require that the materials used in medical devices intended for long-term exposure in the human body to be evaluated for system toxicity using live animal subjects [99].

The traditional method for evaluating in vitro cytotoxicity, as recommended by the ISO standard, consists of direct exposure of cells to the material under evaluation or to extracts of the material followed by microscopical evaluation of cell morphology, cell lysis, detachment, and membrane integrity. A numeric scale is then employed to assign a cytotoxicity rating to the material, corresponding to noncytotoxic, mildly cytotoxic, moderately cytotoxic, significantly cytotoxic, and severely cytotoxic. While widely used in industry, this method is highly qualitative and does not provide an opportunity for granular comparison of material performance. Animal studies on the other hand are expensive and time-consuming.

Qualitative examination of cells is not the only method permitted by ISO for evaluating cytotoxicity. The standard states that any measurable indicator of cell viability may be quantified by objective means as long as the objective measure and response are fully described [98]. Few studies have described in vitro assessment of material cytotoxicity. For example, Bhatia and Yetter utilized mouse 3T3 fibroblast cultures in parallel with a tetrazolium-based colorimetric assay [100] to assess cytotoxicity after exposure to several biomaterials with different levels of biological reactivity [101]. Reichert and colleagues used a monocyte culture in parallel with protein array technology to detect cytokine expression following exposure to titanium particles or discs [102, 103]. These very promising studies of cell–biomaterial interactions may benefit from future development of biosensors at the site of the cells for local and continuous monitoring of cellular response. It may also be interesting to develop platforms with integrated biosensors for high-throughput screening of cell–biomaterial interactions [2]. Developing and validating such platforms would greatly reduce the reliance on qualitative assessment or expensive and time-consuming animal studies. To this end, electrochemical biosensors utilizing enzyme or aptamer sensing elements juxtaposed with cells, such as those described in this review, provide significant promise for real-time monitoring of cell-secreted molecules in evaluating cytotoxicity.

## Conclusions

This paper provides an overview of some recent advances in the development of biosensors for real-time monitoring of cell secretions. Novel technologies addressing the challenge of integrating biosensors with cells and positioning cells in close vicinity to sensing elements have been described. In addition, a few potential future applications of such devices are explored.

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