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Biosensors for immune cell analysis—A perspective

Alexander Revzin,[1] Emanual Maverakis,[2,3] and H.-C. Chang[4]
[1]*Department of Biomedical Engineering, University of California Davis, 451 Health Sciences Dr. #2519, Davis, California 95616, USA*
[2]*Department of Dermatology, University of California Davis, 3301 C Street, Suite 1400, Sacramento, California 95816, USA*
[3]*Department of Veteran Affairs Northern California Health Care System, Sacramento, California 95655, USA*
[4]*Department of Chemical Engineering, Center for Microfluidics and Medical Diagnostics, University of Notre Dame, Notre Dame, Indiana 46556, USA*

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Massively parallel analysis of single immune cells or small immune cell colonies for disease detection, drug screening, and antibody production represents a "killer app" for the rapidly maturing microfabrication and microfluidic technologies. In our view, microfabricated solid-phase and flow cytometry platforms of the future will be complete with biosensors and electrical/mechanical/optical actuators and will enable multi-parametric analysis of cell function, real-time detection of secreted signals, and facile retrieval of cells deemed interesting. © *2012 American Institute of Physics*. [http://dx.doi.org/10.1063/1.4706845]

I. IMMUNOLOGY BACKGROUND

Technologists are in constant search for application and we think that some of the best "killer apps" for microfluidics/microfabrication are in the rapidly evolving field of immunology. In addition to protecting the body from pathogenic organisms, such as fungi, protozoa, helminths, and bacteria, it is now known that the immune system also plays a major role in a diverse set of physiologic and pathologic processes, including wound healing,[1] cancer surveillance,[2] obesity,[3] atherosclerosis,[4] diabetes,[5] responses to sunburn,[6] and autoimmunity.[7] However, after decades of discovery, numerous functions of the immune system still remain to be fully characterized and this task seems as daunting as ever.

The fact that so much is now known about the immune system is quite remarkable. For example, as recently as few decades ago, all lymphocytes were thought to be the same, since they looked identical morphologically (small cells with circular nuclei). Subsequently, Niels Jerne, Frank Burnet, and David Talmage contributed to the development of the clonal selection theory.[8–11] This theory, originally developed to explain the formation of specific antibodies, states that B cells have unique antigen specificities. Upon exposure to an antigen, a specific-immune response is selected and then expanded. Gustav Nossal's discovery that a single B cell produces only one antibody provided insurmountable evidence in favor of the clonal selection theory.[12] As the tools for cell analysis improved, T cells were identified as a unique population distinct from B cells. Although the mechanism by which T cells respond to antigens was not elucidated until much later,[13,14] the clonal selection theory also applies to T cells. As one can surmise, generating a specific (adaptive) immune response requires thousands of antigen-specific T and B cells that originate from single cells.

There are multiple steps involved in clonal selection and expansion of lymphocytes. For T cells, these would include: (1) the digestion of a microbial pathogen by an antigen presenting cell (APC) (e.g., macrophage or dendtritic cell);[15,16] (2) the formation of an immune synapse, a contact between a peptide-bound major histocompatibility complex (MHC) molecule on the surface of an APC and the T cell receptor (TCR) of a T cell;[17] (3) proliferation of the T cell; and (4) its polarization to secrete a certain profile of cytokines.[18]

The complexity of the immune system lies in part in the heterogeneity of immune cells (leukocytes). Leukocytes reside in nearly all tissues of the body. At the most rudimentary level, they can be subdivided based on their nuclear morphology; for example, monocytes and lymphocytes are "mononuclear" cells, while neutrophils are "polymophonuclear" cells. Cell surface molecules can further subdivide the different leukocyte populations. The cluster of differentiation (CD) protocol is used for identification of cell surface molecules present on leukocytes.[19] Using this nomenclature, T cells can be divided into helper T cells that express CD4 and cytotoxic T cells that express CD8 molecules. There are currently 350 known CD molecules and all have different expression profiles.

In addition to using CD molecules to characterize leukocytes, T cells and other leukocytes can also be categorized based on the cytokines that they secrete. T helper type 1 (Th1), Th2, Th9, and Th17 cells have unique cytokine secretion profiles. Each cytokine secretion profile was likely developed to fight off a particular type of infection.

The need to characterize a large variety of immune cell types based on surface markers and nuclear morphology spurred the development of several immunological tools including flow cytometry—an approach for multi-parametric, single cell analysis and sorting that became widely adopted in biological fields outside of immunology.[20] The microfluidics/microfabrication community has begun to mimic and miniaturize flow cytometers a decade ago[21] and is still perfecting this technology today.[22,23] In addition, multiple other novel technologies are being developed for immunology applications including (1) protein microarrays,[24–28] (2) biosensors for detection of antibodies or cytokines secreted by single immune cells,[29,30] (3) platforms for creating heterotypic cell pairs,[31,32] and (4) cell sorting approaches.[33–35]

Ultimately, understanding the intricacies of an antigen-specific immune response will necessitate single cell analysis to evaluate gene regulation, expression of cell surface molecules, and signaling molecule secretion. Moreover, given how the immune response is orchestrated, it is important to monitor intercellular communication between immune cells of the same type (homotypic) and of different types (heterotypic). While analysis of cells based on gene expression and surface markers are important targets of the bioMEMS community,[36] our research interests and the focus of this perspective lie with cell function analysis and intercellular communications. Below, we discuss several novel microtechnologies that are being developed for the analysis of T- and B-cell function as well as for monitoring secreted inflammatory markers. These technologies have applications in infectious disease diagnosis, antibody screening, and detecting inflammation.

II. MICROTECHNOLOGIES FOR IMMUNE CELL ANALYSIS

A. Immune cell arrays

Development of immune cell arrays is a natural extension of biointerface design and surface micropatterning fields.[37,38] In a typical design, surfaces are modified in order to create periodic patterns of non-fouling and cell adhesive domains. Figure 1 shows one variant of such a surface with non-fouling regions composed of poly(ethylene glycol) (PEG) and cell adhesive domains containing antibodies or other ligands.[39] As highlighted by Figure 1, one advantage of these surfaces is the periodic and well defined-placement of single cells on a substrate such that individual cells have permanent address on the surface and may be monitored over time.

The micropatterned surfaces may be viewed as solid-phase cytometry platforms, which in contrast to flow cytometry, allow the investigator to work with cells affixed to surfaces and to monitor for temporal changes in the phenotype of specific cells. The utility of such surfaces was demonstrated, for example, by the Irvine group who studied interactions of T cells with membrane components of antigen presenting cells.[40]

Moving beyond the capture and culture of immune cells, technologies are being developed for massively parallel analysis of cell-secreted molecules (antibodies, cytokines, or small metabolites), real-time analysis of cell function, and cell sorting. In this perspective, we begin with a brief highlight of some of the available technologies, followed by our thoughts on where new

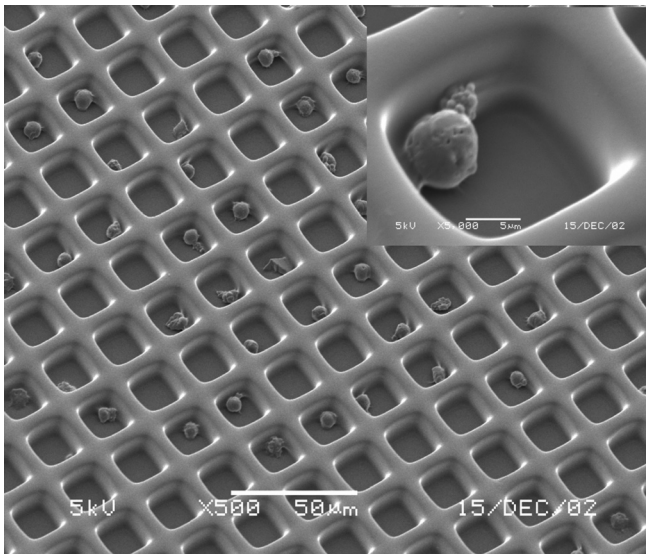

FIG. 1. An example of solid-phase cytometry platform. Microwells composed of non-fouling hydrogel (PEG) are fabricated on glass. Dimensions of the wells are made large enough to house individual cells. Approximately 1×10^6 wells may be packed onto a standard 3×1 in glass slide. Upon seeding onto this surface, a large fraction of wells (~70%) will contain single cells. Reproduced by permission from Revzin *et al*., Lab on a Chip **5**, 30–37 (2005). Copyright © 2005 by The Royal Society of Chemistry.

technologies are heading, and conclude with our vision of the "killer apps" for the integrated platform.

B. Detection of cell secreted molecules in the cell array

One of the more powerful microtechnologies for high-throughput single cell secretome analysis was developed by Love and co-workers who called their approach microengraving.[29,30,41,42] It employs microwells fabricated in PDMS to be large enough to house single cells. The PDMS mold is filled with cells and is pressed against a glass slide pre-coated with antibodies against secreted molecules. This way the immune cells become enclosed inside and secrete signaling molecules (antibodies or cytokines) into picoliter volumes. To determine secreting cells, the cover slip is removed and analyzed using a laser microarray scanner. Another variant of a platform for analyzing cell-secreted proteins was reported by Kishi and co-workers who developed microwells so as to place a single cell inside a well and then detect secreted proteins (antibodies) on its side walls.[43] This approach was used to identify production of influenza and hepatitis B specific antibodies.

The Heath lab had a different take on analyzing the immune cell secretome. This group micropatterned anti-cytokine antibodies on glass surfaces, enclosed these surfaces inside a microfluidic device, then captured immune cells within the microfluidic channels, and finally employed pneumatic actuation to segment channels into smaller, nl volume compartments.[44] Based on density of cell seeding into channels, one could ensure that a fraction of chambers contained single cells. When challenged with antigens, immune cells trapped in the microfluidic device released cytokines, which were captured in the same small volume next to secreting cells. This method allowed them to identify polyfunctional T cells capable of producing multiple cytokines.[44]

Our lab has taken a slightly different angle of attack in developing leukocyte cytometry platforms. Our original interest was in being able to capture specific leukocyte subsets (i.e., CD4 T-helper cells or CD8 cytotoxic T cells) from complex samples such as blood containing multiple extraneous cell types. Therefore, we began by developing surfaces and washing protocols for capturing CD4 or CD8 T cells[45] and demonstrating that the proportions of cells

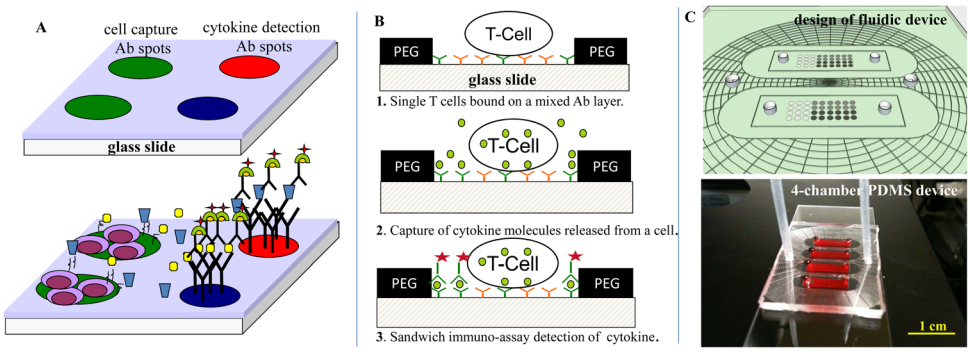

FIG. 2. Co-localizing leukocytes and secreted cytokine signals. (a) Arrays of Ab spots for capturing leukocytes and detecting secreted cytokines. Reproduced by permission from Zhu *et al.*, Lab on a Chip **8**, 2197–2205 (2008). Copyright © 2008 by The Royal Society of Chemistry. (b) Arrays of hydrogel microwells functionalized with Abs to enable capture of single cells and detection of secreted cytokine molecules. Reprinted with permission from Zhu *et al.*, Analytical Chemistry **81**, 8150–8156 (2009). Copyright © 2009 by American Chemical Society. (c) Integration of microarrays into microfluidic devices for analysis small blood volume.

captured on the surface reflected those in the blood.[46] Subsequently, we turned our attention to analyzing the function of the captured cells. The logic being that in addition to cell numbers and subset proportions, cell function could be used for diagnostic purposes. Thus, we proposed two types of immune cell capture and cytokine detection surfaces (shown in Figure 2): (1) arrays of antibody spots with cell capture spots printed next to cytokine detection spots[47,48] and (2) microwell arrays containing Abs for cell capture and cytokine detection.[49,50]

These surfaces were integrated into microfluidic devices (Figure 2(c)) and were used to capture CD4 or CD8 T cells from minimally processed blood and then detect secreted cytokines (IFN-γ, IL-2, and TNF) from the cells. Unlike approaches taken by other groups that require off-chip purification of T or B cells prior to analysis, we employ either whole blood or RBC-lysed blood and perform both cell capture and cytokine detection in the same device.[47,48] This may be advantageous for applications where only small amounts of blood are available, such as pediatric immunology or small animal research or in point of care applications where blood processing needs to be minimized. Figure 3(a) provides a typical result of immune cell analysis using antibody array (strategy 1 described above) where T-cells, stained for CD3, are juxtaposed with secreted signal for IFN-γ and TNF-α. Figures 3(b) and 3(c) show an example of microwell arrays (strategy 2 above) being used for capturing single T-cells and detecting secreted IFN-γ from the bound cells.

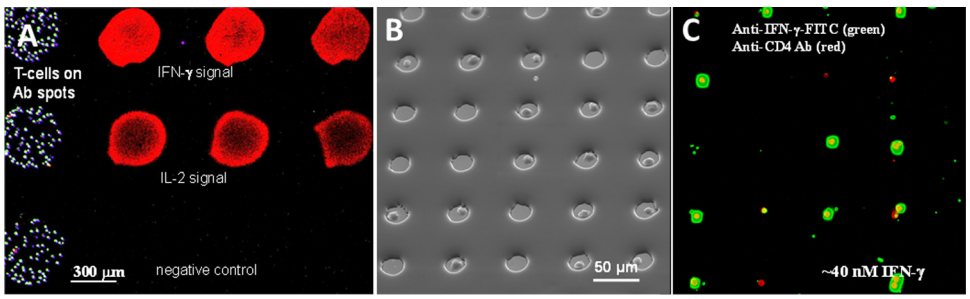

FIG. 3. (a) Mixed array of cell and cytokine specific Abs. T cells are captured on anti-CD4 Ab spots. Reproduced by permission from Zhu *et al.*, Lab on a Chip **8**, 2197–2205 (2008). Copyright © 2008 by The Royal Society of Chemistry; (b) single T cells captured in Ab-functionalized microwells. (c) IFN-γ signal co-localized with single cells. Green fluorescence is due to immunostaining for secreted IFN-γ whereas red fluorescence is for CD4 surface antigen. (b) and (c) Reprinted with permission from Zhu *et al.*, Analytical Chemistry **81**, 8150–8156 (2009). Copyright © 2009 American Chemical Society.

III. NEW DIRECTIONS IN IMMUNE CELL BIOSENSORS

A. Measuring dynamics of cytokine release

Microtechnologies for detecting cytokines and other proteins released by immune cells are evolving and improving. However, these technologies employ antibodies as biorecognition elements and therefore, reveal limited information about release dynamics. We see several reasons for dynamic monitoring of molecules secreted by cells. (1) At the present time, no tools exist that allow establishing cytokine release vs. time in activated immune cells. It is likely that potentiation of immune cells—how fast and how strong their secretory response is—carries valuable diagnostic information that is currently untapped. (2) Immune cells communicate with each other via release of cytokines and monitoring local changes in cytokine levels over time would shine light on cellular interaction in the context of injury or disease. (3) Intercellular communication may be asymmetric and directional[51] and thus, it is valuable to monitor where and when signals appear. (4) Frequently, intercellular communications are reciprocal and occur via the same signaling molecules. Deconvolving which cells secreted original signal and which cells reciprocated requires both spatial and temporal detection.

What is the way forward to dynamic and local monitoring of protein release in cells in general and immune cells in particular? Several groups are employing Ab-modified surfaces in conjunction with detection technologies, such as surface plasmon resonance (SPR) to monitor protein release from cells.[52,53] This is a promising direction for dynamic, label-free sensing, but the problem of expensive instrumentation will need to be addressed and detection of specific cell-secreted proteins has yet to be demonstrated. Other approaches utilize networks of microfluidic channels to sample extracellular space at different time points and detect cell secreted proteins using antibodies.[54,55] While this is an excellent approach, fluidic circuitry required for such analyses is fairly complex.

Aptamers provide an exciting opportunity to develop biosensors for continuous monitoring of cell function. Aptamers, first described in the early 1990s,[56,57] are nucleic acid molecules (RNA or DNA) that offer a number of advantages compared to Abs. The primary advantage, in our opinion, is the structural simplicity of aptamers that allow one to engineer beacons where molecular conformation changes upon analyte binding, providing the basis for signal generation.[58,59] Both optical and electrochemical aptasensors have been described in the literature.[59,60] Our team has become interested in immobilizing fluorescent and electrochemical aptamer beacons on surfaces for use in cell analysis.[61–63] Surfaces for cell analysis are built based on micropatterning approaches described in Figures 1, 3(b) and 3(c) where hydrogel photolithography was used to define non-fouling regions of the surfaces and Ab immobilization is used to capture cells. However, as shown in Figure 4(a), this new sensing surface includes aptamer-functionalized Au electrodes. Micropatterned sensing surfaces are enclosed inside microfluidic devices and are used to capture T cells from RBC lysed blood. As shown in Figure 4(a), T cells are activated to commence cytokine production. These cytokines molecules are then detected in

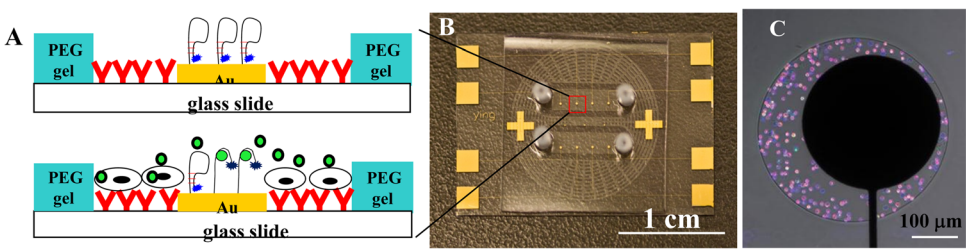

FIG. 4. (a) Individual Au electrodes fabricated on glass slides are surrounded by PEG hydrogel and incubated with T cell-specific antibodies. Leukocytes are captured on Ab-modified glass regions next to aptasensors. Cytokine release is detected at Au electrodes using aptamer recognition layer consisting of DNA hairpin containing redox reporters. (b) A microdevice with sensing electrode arrays integrated into microfluidic device currently used for cytokine detection. (c) Immune cells captured next to sensing Au electrode (black circle). Reprinted with permission from Liu *et al.*, Analytical Chemistry **83**, 8286–8292 (2011). Copyright © 2011 American Chemical Society.

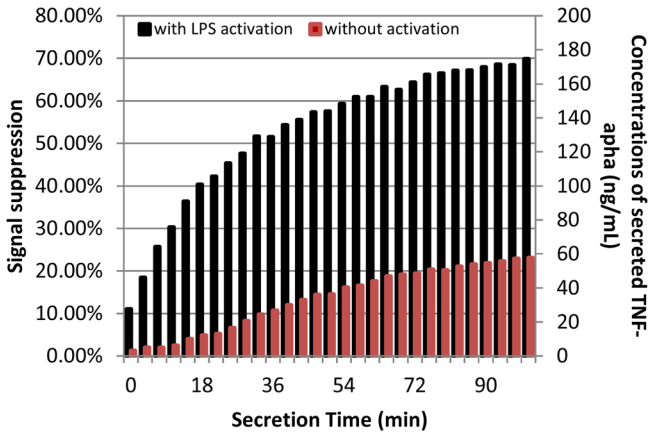

FIG. 5. Continuous monitoring of TNF from monocytes captured and cultured next to sensing electrodes analogous to those described in Figure 4. Measurements are taken every 4 min. Signal suppression—decrease in signal due to biding of cytokine molecules—is correlated to TNF concentration through calibration curve (not shown).

the form of redox signal at the neighboring electrode. Figure 4(b) shows a cytokine sensing device which consists of electrode arrays integrated into microfluidic channels with 4 electrodes per channel. As seen in Figure 4(c), a cell detection site contains T cells captured next to a cytokine sensing electrode. At the present time, we are able to detect IFN-γ (Ref. 64) and TNF (unpublished) using aptasensors.

The microdevice described in Figure 4 has been used for detection of IFN-γ and TNF from a small group of cells ($\sim$50 to 100 cells) with signals observed only minutes after activation of immune cells. Cytokine fluxes are present in the vicinity of the cells but are undetectable a short distance ($\sim$2 mm) away, highlighting the importance of placing sensors next to cells. Figure 5 shows an example of data collected from TNF aptasensor monitoring of monocyte activity. Because in this configuration, binding of the analyte causes a decrease in voltammetry signal, the results in the y-axis of Figure 5 are presented as a percent loss or signal suppression. This experiment highlights the continuous detection and the possibility of determining cytokine concentrations at different points in time afforded by aptasensors. We see immediate uses for this detection method in the analysis of cellular communication and for infectious disease diagnosis. Our team is currently assessing the suitability of atpamer-containing microdevices for detection of tuberculosis (TB) based on IFN-γ release.

B. Detecting small molecules released by immune cells

Molecules other than cytokines play an important role in signaling during immunoinflammatory response. Reactive oxygen species (ROS) are a class of important molecules that are secreted by immune cells to cleanse the site of injury from possible pathogens. In addition to destroying pathogens, release of ROS serves as a signal for stimulation of immune cells. While there are multiple types of ROS molecules, including hydroxyl radicals, superoxides, and peroxides, hydrogen peroxide is most stable/long-lived ROS. Our lab has been developing biosensors for local detection of hydrogen peroxide using both optical[65] and electrochemical[66] modes of signal transduction. The images in Figure 6 demonstrate one avenue for combining single cell analysis for secreted cytokines with peroxide detection. To obtain these results, we fabricated arrays of PEG hydrogel microwells on glass and functionalized anti-TNF Abs at the bottom of the wells. Sensing components for fluorescence-based detection of peroxide, including horseradish peroxidase (HRP) and Amplex Red were encased into the walls of the hydrogel wells. Macrophages were then captured and activated inside the microwells. Images in Figure 6 show TNF molecules released by activated macrophages were captured inside the microwells next to the cells (green fluorescence). To demonstrate the sensitivity of our ROS-

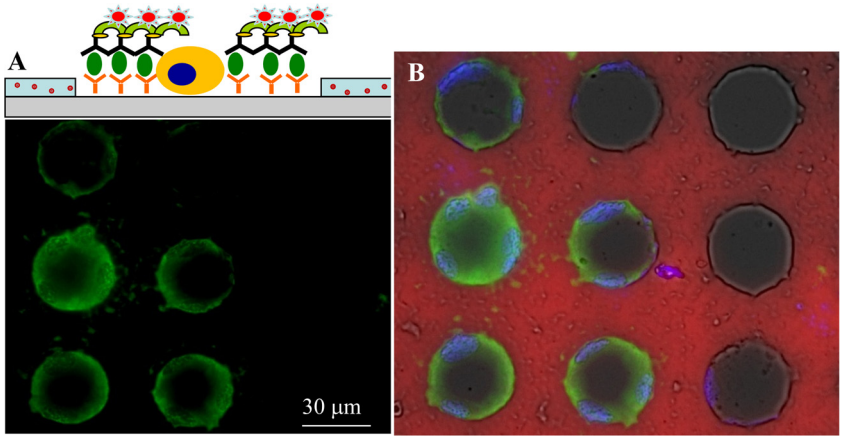

FIG. 6. Merged brightfield/fluorescence image showing integration of hydrogel-based H_2O_2 sensor with TNF sensor.(a) Macrophages were treated with LPS for 3 h and the sample was stained with biotinylated anti-TNF Ab and neutravidin-FITC. The green fluorescence indicates secreted TNF from cells. (b) The addition of 1 μM of H_2O_2 and Amplex Red resulted in appearance of red fluorescence signal in the PEG hydrogel walls.

detection system, we added exogenous hydrogen peroxide (1 μM), which resulted in appearance of red fluorescence signal. Figure 6(b) shows proof-of-concept sensing surfaces for simultaneous detection of cytokine molecules and ROS. Our goal in the future is to demonstrate the concept of simultaneous detection with endogenous (cell-secreted) peroxide and TNF.

C. Novel strategies for sensor regeneration and signal amplification

Ideally, biosensors should be responsive and regenerable on the time scales relevant for cellular communication and should be able to select an analyte of interest in a complex biological fluid out of a plethora of interfering substances. Sensor regeneration is particularly important. In Sec. III A of this perspective, we highlighted the need to develop biosensors for dynamic and continuous monitoring of signaling molecules secreted by the immune cells. One question that has yet to be effectively addressed is how to design affinity biosensors based either on antibodies or aptamers that would be sensitive down to nM or pM analyte concentration while simultaneously allowing for detection of fluctuations in the level of cell-secreted molecules. Sensitivity implies high affinity for the analyte of interest and slow dissociation kinetics. This means that while increase in concentration of cell-secreted molecules is detectable, downward trends in concentrations are difficult to monitor. We see one solution, whereby affinity detection will be combined with regeneration of the sensor in a sequence of detect-regenerate-detect steps.

What would these regenerable sensors look like? While optical, particularly SERS, sensing has matured significantly, its μM detection limit[67] does not allow it to detect signal proteins from single cells or small colonies. Its complex peripheral instrumentation also discourages integration with small wells like those in Figure 1. Finally, the nanocrystals may interact with the cells and trigger unwanted responses. Certain fluorescent detection methods have shown unprecedented resolution (single receptor binding event) and sensitivity (single cytokine)[68] but are not suitable for a practical assay platform. In contrast, electrochemical sensing represents a more promising platform from the stand point of low cost, simplicity, and practicality. Some of the widely used biosensors are based on field effect transistors and electrochemical impedance spectroscopy (EIS). While promising, these approaches suffer from multiple drawbacks including signal drifts, irreproducible signals, ionic strength sensitivity, and interaction of probes with counterions, all of which are common problems for these sensors.[69–71]

Cheng laboratory has developed a microfluidic regeneration strategy for any sensing platform involving probes.[72] Bipolar membranes with cation and anion-selective features are synthesized on silica chips using UV photosynthesis methods. The bipolar membrane behaves like

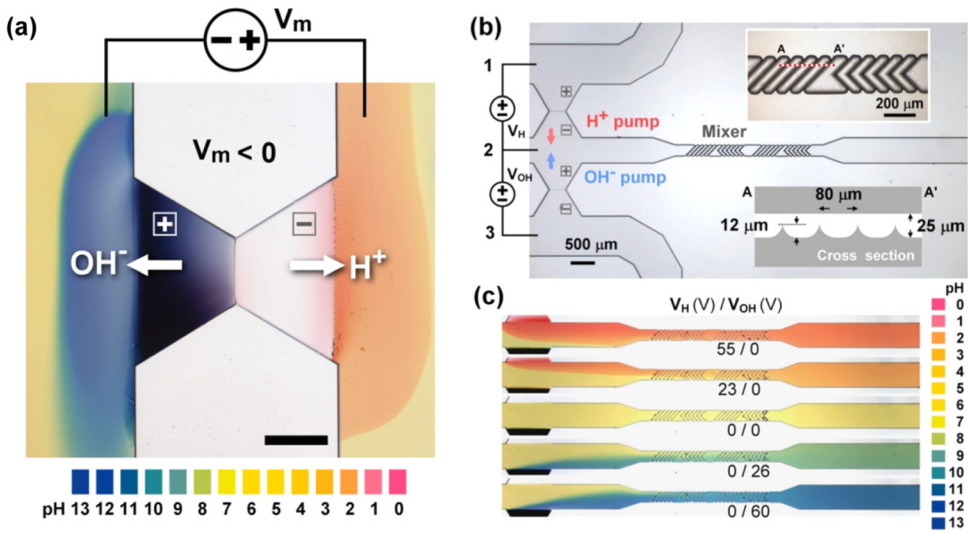

FIG. 7. An on-chip bipolar membrane *p*H actuator. The image in (a) shows how a DC field across a bipolar membrane can dissociate water and generate protons and hydroxyl ions at a controllable rate. Two such bipolar membranes are fabricated up stream of a flow channel and the protons/hydroxyl ions are mixed by a static mixer into a buffer of a specific *p*H between 2 and 10 in (b). The mixed streams are shown in (c) with a color chart for the universal *p*H dye.

a semi-conductor diode, with the electrons and holes replaced by anions and cations. Like a diode, when a reverse DC bias is applied across the bipolar membrane, mobile charges are depleted at the junction. Since both membranes are highly ion-selective, whose mobile ions are all counterions at equilibrium, the application of a DC field can deplete all mobile ions at the junction. This ion depletion effect reduces the electrolyte conductivity at the junction and, hence, amplifies the local electric field by orders of magnitude. In a recent paper,[72] we showed that the electric field can exceed 1 MV/cm at the junction such that water molecules at the junction can be dissociated into proton and hydroxyl ions by the so-called Wien effect.[73] The resulting proton and hydroxyl ions migrate through their respective ion-selective membranes and are therefore, separated to two sides of the bipolar membrane. By using two such bipolar membranes, we are able to produce two controllable proton and hydroxyl ion actuators on the chip and, with a down-stream static mixer, the generated ions are mixed into a solution of a specific *p*H between 2 and 11 with high precision as shown in Figure 7. This chip generated a high *p*H spike that can be used to dehybridize the captured molecules and regenerate the probe.

The quantification of molecules secreted is often necessary for immune cell analysis. However, signals from electrochemical sensors based on Faradaic reactions (or field-effect transistor sensors based on charge sensing[74]) are often sensitive to local *p*H and ionic strengths. The above *p*H actuation device can be used to control the *p*H near the sensor for a large culture. To

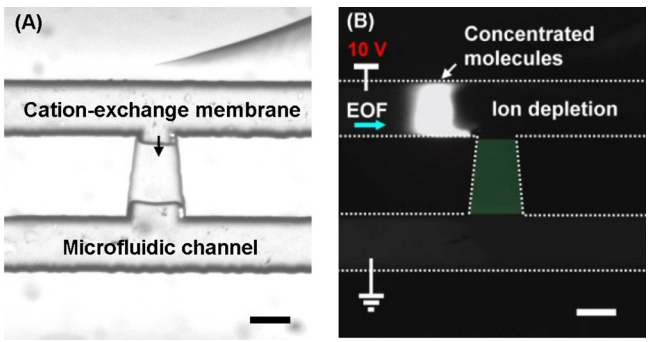

FIG. 8. (a) Optical microscopic image of a charge-selective membrane based preconcentrator. (b) Concentration of fluorescently labeled molecules taking place 10 s after applying a voltage bias of 10 V. The scale bars represent 50 μm.

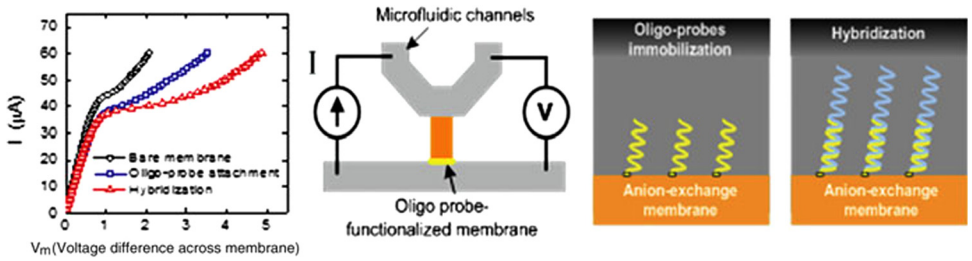

FIG. 9. Nonlinear PNP and PN membrane sensors for cytokine and mRNA. Molecular docking produces a surface membrane with an opposite charge. This nonlinear ion dynamics due to molecule docking produces hysteretic IV with nonlinear amplification of the ion current signal for the docking events. (Note that the signal is on the order of several volts instead of the usual mV in electrochemical sensing.) These membrane components can be integrated into the culture scaffold in Fig. 1.

control the local ionic strength, we employ other features of ion-selective membranes—ion concentration and depletion.[75–78] As shown in Figure 8, we are able to lower the local ionic strength to deionized water level and to increase it from DI water level to mM. The same technique can be used to enhance the sensitivity of the molecular sensor by increasing the local concentration of the target molecules.

Another intriguing strategy to enhance the sensitivity of molecular a sensor is to mimic excitable potential dynamics of a neuron cell—its response is much larger than the input when the input amplitude exceeds a threshold. Their IV curves do not possess linear Ohmic characteristics and in fact, often demonstrate negative differential resistance. Just as excitability amplifies input action potential signals, nonlinear oscillatory cardiac neuron dynamics acts like a clock and bistable dynamics in brain neurons allows for a binary memory device. Intracell sensors have confirmed the highly nonlinear nature of action potential dynamics during neuron signaling and cardiac oscillations.[74] Such "neuron" sensors can be utilized to detect antibody-antigen docking in our immune cell cultures, as a more direct sensor of antigen docking onto a single cell. In fact, one can imagine an artificial membrane unit that can communicate with a real immune cell by converting cytokine signals to electronic signals and vice-versa.

Analogously, a promising strategy for external signal protein detection is to replace the linear electrochemical sensing techniques with a nonlinear one based on ion currents instead of Faradaic currents from electron-transfer reactions. The usual paradigm of linear EIS spectroscopy must be modified in the process. For example, the phase-lag between input and output is locked at a frequency-independent value for nonlinear sensors. In Chang's lab, several on-chip nanoporous membrane and nanoslot real-time nucleic acid and signal protein sensors have been created with this design.[75–78] With molecular probes functionalized onto the membranes to mimic receptors, the membrane is designed such that its charge is opposite to the target molecules (see Figure 9). As such, target molecule binding produces a PN membrane for ions, akin to a PN semi-conductor diode for holes and electrons. This strategy produces a very specific sensor that generates an amplified ion conductance signal that does not rely on electron transfer. The ion current is picked up by electrodes in isolated chambers uncontaminated by the culture. Insofar as the nonlinear I-V features of a semi-conductor diode and cell membrane ion channels can be reproduced by the ion-selective membrane components, other features such as oscillatory and bistability should also be possible. If molecule capturing can induce oscillations of a specific frequency at one sensor, electrical signals from a large array of sensors can be monitored by a single electrical detector thus, simplifying the sensor network design. This is yet another promising future direction.

IV. APPLICATIONS

Analysis of leukocytes, including T and B cells can provide clinically relevant information about infections, allergic reactions, and vaccine efficacy. This information content is currently

"harvested" by sophisticated techniques such as flow cytometry, ELISpot, or ELISA or less sophisticated approaches such as skin tests for allergic reactions and TB detection. Complexity of the first group of approaches limits broader use of immune analysis, while simplicity and crudeness of the second category of approaches contribute to false results and poor patient compliance. We see several untapped applications for immune cell biosensors.

A. Analysis of antigen-specific T cell responses

Immunologists commonly query disease-specific T cell responses using flow cytometry and ELISpot in academic settings. However, in the clinic, these techniques are secondary or even tertiary to other detection methods that look for presence of pathogens or pathogen-specific antibodies. There are cases like TB detection where pathogen biomarkers are not yet available and interferon-(IFN)-γ production by T cells is used in diagnosis of latent TB.[79] If made simple and inexpensive, detection of antigen-specific T cell responses may be used for early pathogen detection in cases where pathogen-specific antibodies are not yet produced (sero-negative individuals) or when the pathogen has not yet proliferated in the host.

B. Analysis of antigen-specific B cell responses

B cells express antibodies against specific pathogens on their cell surface and secrete the same antibody molecules upon pathogen binding. The ability of B cells to recognize and respond to pathogens makes them ideally suited for biosensing. While there have been reports of genetically engineered B cells that "light-up" in the presence of specific pathogens,[80] recent advances in the field describe single cell platforms for detection of pathogen specific antibody production in unmanipulated primary B cells.[43,81]

C. Isolation of cancer-specific T cells

Immunotherapy for cancer has recently received significant attention in the medical literature. Initial studies demonstrated that the adoptive transfer of cancer-specific T cells can induce prolonged remission and newer strategies with engineered T cells appear very promising.[82,83] An important finding from these groundbreaking studies is that there is considerable heterogeneity in cancer-specific T cells, with some working much better than others. Thus, a microfluidic device that can isolate and characterize cancer-specific T cells prior to their labor-intensive expansion and adoptive transfer will have a profound clinical impact.

D. Characterization of the autoreactive T cells responsible for autoimmunity

T cells play a central role in many forms of autoimmunity.[7,84] However, the pathogenic population likely comprises only a small fraction of the peripheral T cell repertoire. Recently, protocols have been developed to directly isolate putatively pathogenic T cells from the peripheral circulation of patients with autoimmunity.[85] However, low cell numbers make analysis of these virtually clonal populations difficult. In addition, *in vitro* expansion of the T cells will likely lead to changes in their effector phenotype.[86] Thus, once isolated from the peripheral circulation, microfabrication and microfluidic technologies will be essential to rapidly characterizing these T cells.

E. Screening for potential allergens

There are numerous screening strategies for identifying food and environmental allergens. The very common radioallergosorbent test (RAST test) detects specific IgE antibodies to suspected allergens. In this test, the patients' serum is added to suspected insoluble allergens. If allergen-specific IgE antibodies exist in the serum, they will bind to the insoluble allergens. Bound IgE molecules are then detected with a secondary radiolabeled anti-IgE antibody. However, allergic responses can also have a cellular component that cannot be detected by the assays, which are focused purely on detecting anti-allergen IgE titers. Contact allergies are

often due to allergen-specific T cells, with no or low corresponding IgE titers. Novel approaches to identify allergin-specific T-cells will have considerable impact on the field.

F. Cellular communication via secreted signals

It should be noted that in addition to T and B cells there are other important cellular participants of the immune system including granulocytes, monocytes, dendritic cells, etc. These cells release cytokines, reactive oxygen species, or other signaling molecules in response to pathologies. However, because these cells are not designed to mount pathogen-specific immune responses, they are more challenging to use in disease diagnosis. This said, inflammation—general state of immune cell activation—has emerged as an important factor in cancer, tissue injury, and regeneration. Therefore, sensitive, temporal analysis of inflammatory markers will likely become more and more significant as understanding of biological mechanisms evolves.

In fact, we see biosensors and microfluidic platforms as being a key to improving mechanistic understanding of immuno-inflammatory responses. Given the key role of paracrine signals and the involvement of multiple cell types in generating these signals, there is a clear need to develop platforms for monitoring heterotypic cellular signaling.[87] While the development of first microfabrication approaches for heterotypic cell cultures dates back to over a decade ago,[88,89] the use of reconfigurable microfluidic chambers now allows to sequester individual cell types and to actively control how and when communication happens.[90,91] Integration of such cell culture systems with biosensors for local and continuous monitoring of secreted signals will help to elucidate which cell type the signals originate from and how reciprocal communication propagates.

V. CONCLUSION

This paper describes current trends in biosensors for immune cell analysis and suggests future directions for development of this field. Microfabrication, microfluidics, and multiplexing are particularly important in leukocyte analysis where individual cells are frequently unique in terms of function and response to stimulation. Exciting technologies to analyze immune cells have been proposed and developed, yet the opportunities for new technology development remain ample. These opportunities are outlined in the paper.

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