

Integrated Microfluidic Device for Functional Secretory Immunophenotyping of Immune Cells

Roberto Rodriguez-Moncayo, Rocio Jimena Jimenez-Valdes, Alan Mauricio Gonzalez-Suarez, and Jose Luis Garcia-Cordero*



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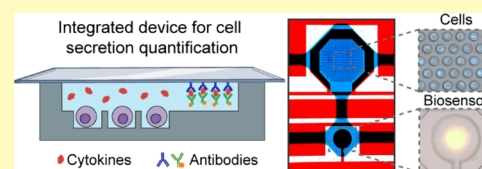
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Supporting Information

ABSTRACT: Integrated platforms for automatic assessment of cellular functional secretory immunophenotyping could have a widespread use in the diagnosis, real-time monitoring, and therapy evaluation of several pathologies. We present a microfluidic platform with integrated biosensors and culture chambers to measure cytokine secretion from a consistent and uniform number of immune cells. The biosensor relies on a fluorescence sandwich immunoassay enabled by the mechanically induced trapping of molecular interactions method. The platform contains 32 cell culture chambers, each patterned with an array of 492 microwells, to capture and analyze both adherent and nonadherent immune cells. Multiple stimuli can be delivered to a set of culture chambers. Per chamber, we were able to capture consistently 1113 ± 191 of blood-derived monocytes and neutrophils and 348 ± 37 THP-1 monocytes. Good occupancy efficiencies of $\sim 70\%$ with a uniformity of $\sim 90\%$ across all of the culture chambers of the device were achieved. Furthermore, we demonstrate that up to 96% of cells remain viable for the first 48 h. The employment of epoxy-modified glass substrates and active mixing enhanced the biosensing performance compared to the use of bare glass and simple diffusion. Finally, we performed functional secretory analysis of interleukin-8 and tumor necrosis factor alpha from human neutrophils and monocytes, stimulated with various doses of lipopolysaccharide and phorbol 12-myristate 13-acetate–ionomycin, respectively. We foresee the employment of our microfluidic platform in the diagnosis of different pathologies where alterations in cytokine secretion patterns can be used as biomarkers.

KEYWORDS: cytokine secretion, immune cells, fluorescence biosensors, MITOMI, microfluidic immunoassays, single cells



The survival and function of a cell depends to a great extent on the information received and processed from the microenvironment, including communication with other cells. There are several inter- and intracellular signaling mechanisms for the transmission of relevant biological information, one of them is the secretion of proteins, including cytokines, chemokines, growth factors, and hormones.¹ The immune system is mainly regulated by cytokines and chemokines, which can be divided into proinflammatory and anti-inflammatory proteins. These proteins are responsible for regulating the function and trafficking of immune cells and thus are essential for mounting adequate responses to infectious or inflammatory processes.¹ The pathways of release of these proteins are finely regulated spatially and temporally because they promote processes such as proliferation, activation, differentiation, and migration of multiple cell lineages. An inadequate regulation of the cytokines and chemokines can promote inflammatory responses, causing different chronic and autoimmune inflammatory diseases.^{1,2}

Because certain cytokines, chemokines, and their respective receptors are overexpressed in various diseases, they have been proposed as potential biomarkers of disease activity as well as to evaluate therapy effectiveness. Multiple diseases, such as cancer,³ tuberculosis,⁴ asthma,¹ systemic lupus erythematosus,⁵

lupus nephritis,⁶ among others, have been correlated with abnormal cytokine and chemokine profiles. The identification and detection of these biomarkers using specific antibodies, also called immunophenotyping assays, can be performed using several methods with high sensitivity (pg/mL);⁷ including, bead-based multiplex assays, enzyme-linked immunosorbent assay (ELISA),⁸ and enzyme-linked immunospot assay (ELISPOT).⁹ These assays are performed by analyzing the molecules secreted by the leukocytes or by a specific cell type depending on the type of disease; however, these methods require long incubation times and several manual handling steps. Furthermore, in the case of ELISA, it is not possible to perform the assay in the presence of the secretory cells; thus, it is necessary to collect the supernatant and then analyze it separately. On the other hand, the ELISPOT can analyze the secretion of proteins from individual cells; however, cells have to be removed before performing the immunoassay.

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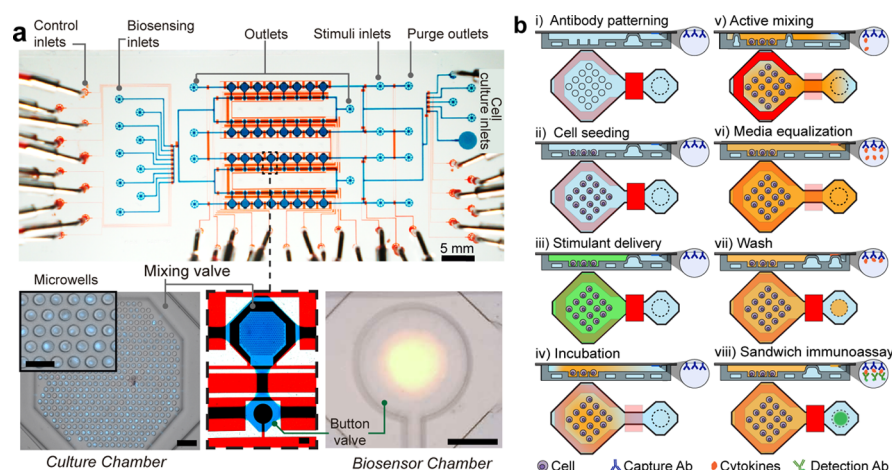


Figure 1. Device overview and experimental workflow. (a) Microfluidic platform consists of an 8×4 array of cell culture and biosensing chambers with 18 inlets and 6 outlets. The control and flow layer are colored in red and blue, respectively. Each cell culture chamber contains an array of 492 μ wells to trap cells, while each biosensing chamber has a MITOMI button for antibody patterning. All scale bars are 150 μ m except for μ well zoom-in inset which is 50 μ m. (b) On-chip functional secretory assessment of immune cells is performed by: (i) patterning capture antibodies on biosensing chambers, followed by (ii) cell seeding and (iii) stimulation with various inductors. (iv) Cells are incubated for 12–18 h, and (v) active mixing is performed for 2–4 h to achieve media equalization (vi) with cytokine binding to the biosensing surface. Next, (vii) all of the biosensing chambers are washed. Finally, (viii) immunoassays are developed with fluorescently labeled detection antibodies. Note the sequence of the actuation of valves.

Strategies for integrating cell culture, stimulation, and detection of secreted cytokines in a single platform will advance the automation of diagnosis, prognosis, and therapy evaluation of various diseases. Integrated microfluidic platforms for immunophenotyping cell populations consist of a cell capture chamber situated next to a biosensor. In these platforms, cells are captured either with hydrodynamic traps,^{10,11} porous membranes,^{12,13} or antibody spots;^{14–17} however, these strategies are difficult to implement, are not very efficient, and present high heterogeneity in the number of captured cells.

Most common biosensors integrated in these platforms include the mechanically induced trapping of molecular interactions (MITOMI),¹⁰ hydrogels with embedded antibodies,^{14–17} functionalized beads,^{11–13,18,19} and in open configurations, slides covered with antibodies.^{20–24} Novel optical biosensing approaches to assess cell secretion include photonic crystal resonant hyperspectral imaging²⁵ and localized-surface plasmon resonance (LSPR),^{26–28} both of which provide label-free cytokine detection and real-time monitoring of secretion dynamics. These approaches still lack the integration with microfluidic cell culture to perform in situ secretion detection, although some advances have been attempted to overcome this challenge, most platforms still require additional handling steps to stimulate off-chip, collect supernatant samples, and deliver it to the chip to measure cytokine concentration. Furthermore, LSPR platforms necessitate the integration of external hardware (e.g., optic fibers, prisms), patterning of gold nanofilms on the substrate, and additional expertise in their design, setup, and data analysis, which limits their application and wide adoption.

Overall, microfluidic platforms for functional secretory analysis of immune cells do not permit a multiplexed stimulation^{12–14,18,19,22–28} or lack the capability to perform biological and technical replicates for statistical inference^{12,13,26–28} (i.e., only a single condition is tested). Although microfluidic devices able to assess cytokine secretion at different timepoints have been reported,^{10,11} these require

several manual handling steps, making it an intricate process to setup. In addition to these disadvantages, a microfluidic platform capable of performing multiplexed stimuli on chambers with controlled and deterministic populations of both adherent and nonadherent cells has not been reported yet. This lack of determinism in the number of cells per given area (also known as confluence) produces a high variability on the levels of cytokines secreted because of paracrine communication effects. For example, macrophages have been shown to produce higher levels of tumor necrosis factor α (TNF- α) and a higher basal level of RelA expression during high confluency culture conditions,²⁹ which leads to highly activated cells. This effect is attributed to paracrine communication and was coined quorum licensing, although the authors were not able to identify specific molecules that mediated this process. In contrast, neutrophils at high-density confluency levels secrete less proinflammatory cytokines upon lipopolysaccharide (LPS) stimulation with either high (1 μ g/mL) or low (10 ng/mL) doses.³⁰ This effect is also attributable to paracrine communication, where neutrophils secrete antagonist of the IL-1 receptor (IL-1RA), which inhibits the production of interleukin-8 (IL-8) and TNF- α .

Here, we present an integrated microfluidic device to detect cytokine secretion from a deterministic and constant population of cells. Our device consists of an array of integrated cell culture and biosensing chambers, with multiplexed stimulation capabilities. Biosensing relies on the MITOMI method, while cell culture chambers consist of an array of microwells (μ wells). To demonstrate the performance of our device on a secretory immunophenotyping assay, we assessed the secretion of IL-8 and TNF- α from blood-derived neutrophils and monocytes when stimulated with various concentrations of phorbol 12-myristate 13-acetate (PMA)—ionomycin and LPS, respectively.

MATERIALS AND METHODS

Materials. A complete list of all materials and reagents employed are provided in the [Supporting Information](#).

Device Microfabrication. The device was fabricated by multi-layer soft lithography. For detailed information regarding mold fabrication, device fabrication, and setup, refer to the [Supporting Information](#).

Cell Culture Module Characterization. Complete explanation of how cells were cultured, and their viability measured can be found in the [Supporting Information](#).

Immunophenotyping Assays. Refer to the [Supporting Information](#) for details regarding isolation and culture of primary immune cells, as well as secretory immunophenotyping assays.

RESULTS AND DISCUSSION

Microfluidic Device Design. Our microfluidic device consists of 32 cell culture microchambers (μ chambers) integrated with a biosensor module divided in 4 rows, [Figure 1a](#). Each μ chamber (~ 20 nL) contains an array of 492 μ wells (~ 10 pL each).³¹ Reagents needed for the biosensor modules are introduced through eight inlets. Cells are injected through an inlet patterned with an array of posts designed to prevent cell clumps entering in the μ chambers. Three additional inlets are used to deliver reagents to the μ chambers. In addition, each row of μ chambers has its own inlet, through which stimuli are introduced. Microvalves are strategically placed not only between the biosensor module and μ chamber but also between μ chambers to prevent any contamination while incubating or performing immunoassays. A 150 μ m valve in the form of a horseshoe flanks the μ well array. These valves, through repeated activation and deactivation, are used to accelerate mass transfer between culture and biosensing chambers. Secreted cytokines are detected and quantitated with a fluorescence sandwich immunoassay ([Figure 1b](#)), implemented with the MITOMI, a technique previously employed to quantitate cytokine levels from serum,³² cell culture supernatants,³³ and from on-chip cell cultures.¹⁰

We chose to implement μ wells over hydrodynamic trapping because of the following: (i) its straightforward design: unlike hydrodynamic traps there is no need to calculate the hydrodynamic resistance or flowrate ratio between traps and the main channel;³⁴ (ii) its smaller footprint, which increases the number of trapped cells on a given area by at least a factor of two,³⁵ and (iii) the low shear-stress cells experience during stimulation. Also, our approach is compatible with adherent and nonadherent cells, [Figure S1](#). While capturing cells using antibodies against cell surface markers immobilized on a substrate is another strategy widely employed in microfluidics,^{14–18} this method lacks the ability to capture a precise and constant number of cells and would require a surface area ~ 2 times larger than our approach to trap as many cells, [Table S1](#). Our integrated platform was designed to allow automation, parallel processing, multiplexing of different conditions, rapid exchange of solutions without the need for pipetting, and evaluation of cellular secretory phenotypes if needed.

Cell Seeding and Culture Characterization. To characterize the efficiency of our traps, we used the human monocyte cell line THP-1. This cell line is a proxy for primary monocytes and has been used to investigate the monocyte structure and function in both health and disease,³⁶ while being easy to use, compared to isolating blood-derived monocytes. THP-1 cells at different cell densities were injected in the device and the number of occupied wells in each μ chamber was quantified, [Figure 2a](#). We observed that the percentage of occupied μ wells increases with higher seeding cell densities: $\sim 70\%$ of occupied wells for 1×10^7 cells/mL, reducing to ~ 32 and $\sim 7\%$, for 5×10^6 and 2.5×10^6 cells/mL,

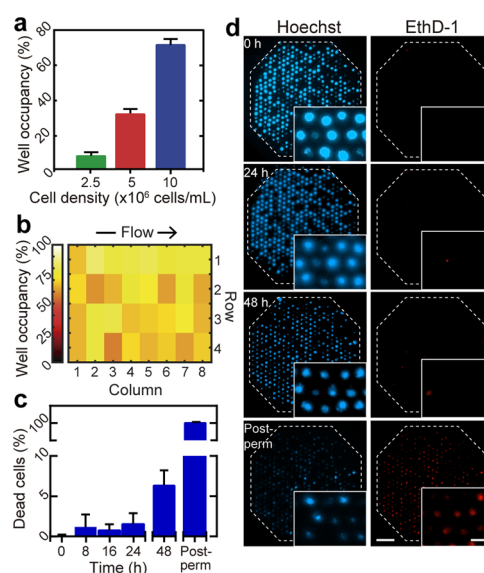


Figure 2. Cell culture module characterization. (a) Percentage of occupied μ wells with respect to cell seeding density. (b) Heatmap shows the distributions of occupied wells across the device. (c) Plots show percentage of dead cells at 0, 8, 16, 24, and 48 h, and post-permeabilization with Tween-20. (d) Representative fluorescence micrographs of Hoechst and EthD-1 stains, showing viability of cells at 0, 24, and 48 h and after cell permeabilization. Error bars represent one standard deviation. Scale bars are 150 μ m for the culture chambers and 50 μ m for the insets.

respectively. These results are on par with several studies; however, μ well dimensions can be optimized for each particular cell line, leading to occupancies of ~ 50 – 75% ,³⁷ and up to ~ 84 – 92% ,³⁸ while we have previously attained $\sim 80\%$.³⁹

For the highest THP-1 cell density tested (1×10^7 cells/mL), we found that cell capture is uniform across the cell culture chamber array, with a coefficient of variation (CV) of 0.106, [Figures 2b](#) and [S2](#). This is equivalent to a capture occupancy per chamber of 348 ± 37 THP-1 cells, [Figure S3a](#). However, when employing primary monocytes, this occupancy increased to 1162 ± 153 cells per culture chamber, [Figure S3b](#). Monocytes are smaller than THP-1 cells, and it is possible for more than one cell to reside in a single μ well. This observation is in agreement with results previously reported by our group,³⁹ in which neutrophils (similar in size to primary monocytes) seeded at high densities increase the number of μ wells containing multiple cells.

Next, we assessed the culture capabilities of our device. Hoechst-stained THP-1 cells were maintained in culture conditions for 48 h. The membrane impermeable dye, EthD-1, was used to monitor cell death by quantifying the percentage of cells that were stained positive, which reflects loss of plasma membrane integrity. We found that ~ 97 – 99% of trapped cells remain viable throughout the culture period, with a low percentage of cells testing positive for EthD-1 (~ 1 – 2%) for the first 24 h, and only increasing to 6% at 48 h, [Figure 2c,d](#). Reports assessing cellular viability of immune cells in microfluidic devices are highly variable. For example, in some instances 96% of THP-1 monocytes¹³ and 89% of peripheral blood mononuclear cells (PBMCs)¹⁹ remained viable after 2 and 24 h, respectively, whereas we have previously reported that under static culture conditions up to 85% of THP-1 cells and 80% of neutrophils are alive after 2

and 3.5 h,³⁹ respectively. These reports show that the different designs of microfluidic platforms can alter cellular viability. In addition, one must consider the heterogeneity in viability of different immune cell types; for example, well plate-based studies have shown that neutrophils can present up to 80% of apoptosis after only 20 h in the absence of exogenously added proteins;⁴⁰ whereas monocytes can be maintained for up to 4 months.⁴¹ In our case, primary monocytes under culture conditions on-chip showed a relatively high degree of dead cells after 24 h, in contrast to THP-1 cells, with 7% of cells being positive for EthD-1 and increasing to ~23% after 48 h, Figure S3c,d. These results are comparable to previous reports, in which the viability of human monocytes—maintained under standard culture conditions—drastically decreased from ~100% at day 0 to ~12% after 5 days.⁴²

Although, typical functional secretory assays are run for a period of a few hours,^{10,13,16,17,19} the fact that only ~7% of the cells have lost viability after 24 h suggest that our device is capable of maintaining cells in optimal culture conditions throughout the functional secretory experiments. However, long-term cell culture capabilities could provide certain advantages. For example, blood-derived monocytes could be immobilized and differentiated to macrophages on-chip, which could then be polarized to a pro- or anti-inflammatory phenotype by the addition of exogenous factors, a process which can take up to 7 days.⁴³ By optimizing culture conditions, such as increasing media volume or replacing periodically nutrient-depleted media, cells can be cultured for weeks in microfluidic devices.⁴⁴ Hypothetically, our platform could provide similar long-term cell culture capabilities.

Biosensing Characterization. A robust method to covalently immobilize antibodies to a surface is important to achieve low limits of detection in an immunoassay.⁴⁵ We compared the density and stability of adsorbed molecules between epoxy-modified and bare glass, Figure 3a. Fluorescent-labeled NeutrAvidin (NA650) was immobilized under the MITOMI biosensing surface area, at concentrations ranging from 0 to 100 ng/mL. Next, fluorescence intensities on the biosensing areas were monitored for 7 h on both epoxy-modified and bare glass devices. As shown in Figure 3b, the epoxy-modified substrate displayed an order of magnitude greater fluorescence intensities on all conditions, suggesting a higher concentration of molecules immobilized on the biosensing surface. Additionally, on the bare glass substrates, fluorescence intensities decreased as time increased, suggesting molecular desorption, while fluorescence intensities on epoxy-modified glass slides remained constant for 7 h, which suggests covalent molecule immobilization. It is well established that epoxy-activated surfaces allow stable covalent linkages with protein groups such as amine, hydroxyl, and thiol.⁴⁶ Furthermore, epoxy-modified surfaces have been shown to increase the density of patterned molecules with high uniformity;⁴³ these characteristics, in tandem with the ease to perform epoxylation by silanization, makes them ideal substrates to implement immune-based biosensors.⁴⁷ Although other surface functionalization protocols (e.g., aldehyde and carboxylate⁴⁸) provide good stability (i.e., capture antibodies are not prone to be desorbed from the substrate during assay reagent perfusion) and covalent immobilization of antibodies, they have their own shortcomings. In aldehyde modification, capture antibodies show a lower binding affinity and reduced specificity, whereas carboxylate modification is highly depend-

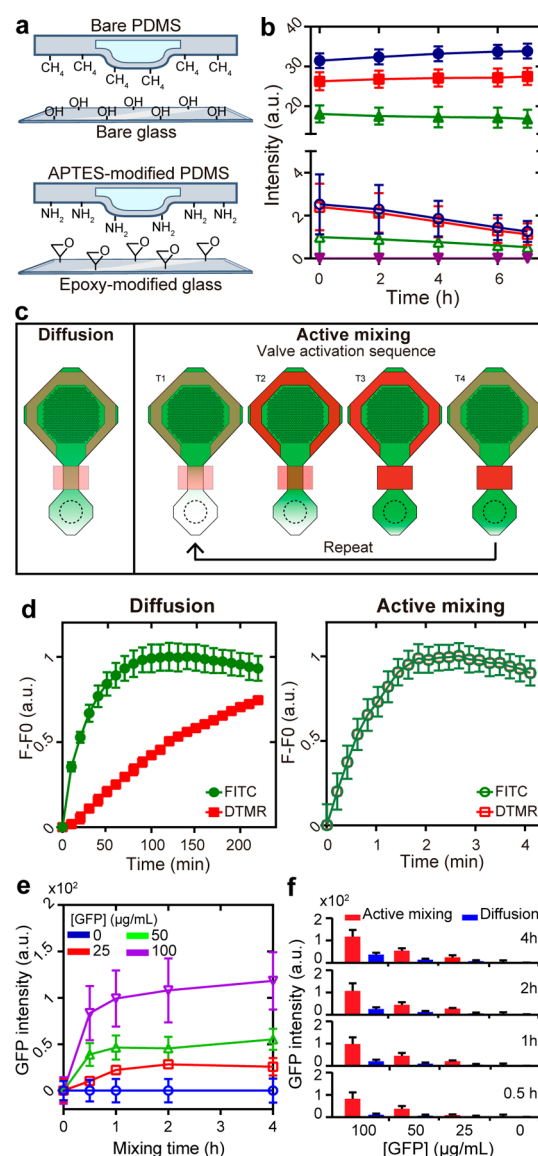


Figure 3. Biosensing characterization. (a) Schematic shows the functional groups present in (top) polydimethylsiloxane (PDMS) bonded to bare glass and in (bottom) (3-aminopropyl)-triethoxysilane-modified PDMS bonded to epoxy-modified glass. (b) Comparison of molecule immobilization stability between epoxy-modified and bare glass substrates. Fluorescent NeutrAvidin (NA650) was immobilized at 100 $\mu\text{g/mL}$ (blue circles), 50 $\mu\text{g/mL}$ (red squares), 25 $\mu\text{g/mL}$ (green triangles), and 0 $\mu\text{g/mL}$ (purple triangles) on epoxy-modified (filled symbols) or bare glass (empty symbols). Fluorescence intensity was monitored for a period of 7 h. (c) In the diffusion-based mass transfer regime, cytokines are transported by diffusion to the biosensing chamber. In the active mixing regime, the valves are actuated (red color) in a sequence of four steps and repeated for several cycles. (d) Plots showing the change in fluorescence intensity (F) with respect to the base intensity level (F_0), reached between the two regimes. Note the time scale. (e) Plots showing the fluorescence intensity levels for 100, 50, 25, and 0 $\mu\text{g/mL}$ of recombinant GFP after performing active mixing. (f) Comparison of GFP intensities obtained between active mixing and diffusion modes after 0.5, 1, 2, and 4 h. All error bars represent one standard deviation.

ent on pH, concentration, ionic strength, and reaction times, which reduces reproducibility.⁴⁵

Next, we characterized the mass-transfer process from the cell culture chamber to the biosensing chamber. In microfluidics, reagent homogenization is achieved by either diffusion or active mixing. Active mixers stir fluids mechanically;⁴⁹ in our case, this is enabled by the actuation of two microvalves, one of them surrounding the culture area and the other one separating the biosensor chamber from the culture chamber, Figure 3c. We compared diffusion-based and active mixing-based mass transfer; fluorescent biomolecules of different weight, fluorescein isothiocyanate (FITC) (MW = 398 Da), and DTMR (MW = 40 kDa) were loaded in the cell culture chambers (mimicking cytokine secretion), and changes in fluorescence intensity were monitored in the biosensing chamber. Under the diffusion regime, FITC intensity showed an exponential increase, reaching a plateau after 95 min, while DTMR increased linearly and did not plateau even after 4 h, Figure 3d. In contrast, in the active mixing regime, a plateau was reached after only 2 min for both molecules that are 2 orders of magnitude different in their molecular weights, Movie S1. Active mixing not only shortens the mass-transfer time between the culture and biosensing chambers but also might improve the immunoassay performance by enabling a faster encounter between capture antibodies and their antigens.⁵⁰ Previous microfluidic devices employed peristaltic pumps to homogenize solutions in biosensing chambers, requiring 30 s to 6–18 min^{19,51} of active mixing to achieve complete concentration equalization; however, the time to transport the sample into these chambers was not specified. In contrast, our approach allows complete mixing of the solutions in as little as 2 min.

Next, we assessed the effect that the active mixing time has on the amount of immunocomplexes formed on the biosensor, using green fluorescent protein (GFP) to monitor these formations over time. Antibodies against GFP were immobilized on the biosensing surfaces, and four different concentrations of GFP were delivered to the culture chambers. After enabling the communication between biosensing and culture chambers, active mixing was performed. Fluorescent images were acquired at different time points and the GFP intensities under MITOMI buttons were measured. As shown in Figure 3e, intensities sharply increase for the first 30 min of active mixing, reaching a plateau after 1 h. These results suggest that once the antigen–antibody binding reaction reaches equilibrium, extended periods of active mixing will not significantly increase the amount of immunocomplexes; indeed, the maximum intensities for mixing periods from 0.5 to 4 h show no significant differences, Figure S4a. Thus, the total immunoassay incubation time for our device can be set to 30 min, consistent with previous microfluidic approaches that report that 30 min is sufficient to discern between different cytokine concentrations.^{10,51} In contrast, in the diffusion regime after 30 min of incubation, the fluorescence intensity measured for the highest concentration of GFP is one-third of that reached in the active mixing mode, with the two other concentrations barely discernible after 1 h, Figures 3f and S4b,c. These results further support the idea that active mixing shortens the time to reach media equalization and to achieve antigen–antibody binding equilibrium.

Finally, we assessed the performance of our MITOMI biosensing module. First, we obtained calibration curves for the proinflammatory cytokines TNF- α and IL-8 (Figures 4a and S5). In both cases, known concentrations of recombinant proteins were delivered into the culture chambers, simulating

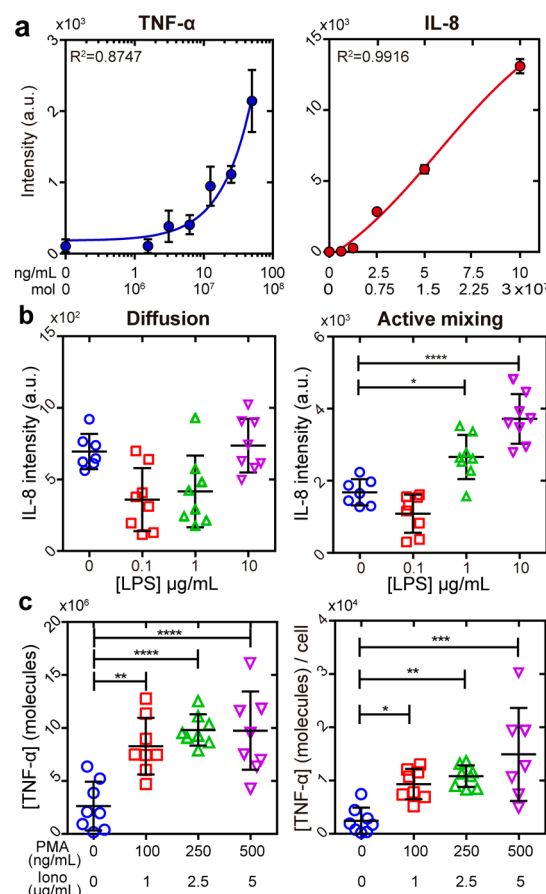


Figure 4. Calibration curves and functional secretory assays for immune cells. (a) On-chip calibration curves obtained for TNF- α and IL-8. (b) Human neutrophils were isolated, captured, and stimulated with various doses of LPS, to assess IL-8 release. After 12 h of secretion, no statistically significant differences were observed in the diffusion regime. Subsequently, active mixing was performed for 2 h, and immunoassay was performed again. Mixing allowed the observation of statistically significant differences between IL-8 secretion levels by neutrophils stimulated with 1 and 10 $\mu\text{g/mL}$ of LPS, compared to negative controls. (c) Quantification of TNF- α secretion from human blood-derived monocytes induced with PMA–ionomycin. The left panel shows the quantification of TNF- α molecules in each culture chamber, while right panel shows the average number of released molecules per individual cells. Data correspond to mean and one standard deviation. n.s. $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

secretion from cultured cells. After solution equalization, antibodies were incubated with the antigens with constant mixing. Finally, the bound antibody–antigen complexes were developed with a fluorescent-labeled antibody. Calibration curves for TNF- α and IL-8 showed linearity with coefficients of determination $R^2 = 0.8747$ and $R^2 = 0.9916$, respectively, and a limit of detection (LOD) estimated to be 902,955 molecules for TNF- α and 920,172 molecules for IL-8. Kaestli et al. reported a microfluidic approach to quantify secreted TNF- α from macrophages using the MITOMI method on bare glass and demonstrated a LOD of $\sim 155,000$ molecules.¹⁰ Although epoxy surface modification increases surface molecule density, discrepancies between our results and those reported by Kaestli et al. can be attributed to the secondary anti-IgG antibody they used, which amplifies the signal. These amplification strategies could be applied when

evaluating secretion of low-abundance cytokines. An additional factor to be considered in our reduced LOD is the surface area of the antibody spot, while we generated MITOMI spots of $\sim 200\ \mu\text{m}$ in diameter and Kaestli et al. created spots of $\sim 65\ \mu\text{m}$. All immunoassay measurements are based on the fractional occupancy of analytes to capture antibodies, this fraction of bound sites is governed by the law of mass action and is determined by both the equilibrium constant and concentration of unbound antigen.⁵² Hence, by increasing the spot area, the fraction of bound antibodies will decrease, leading to a reduced sensitivity. Although it has been shown that a reduction of the antibody spot in a protein microarray leads to an increase in the readout signal,⁵¹ commercially available protein microarrays used in laboratory settings range in spot diameters from 100 to 300 μm ;⁵³ therefore, our decision is to employ detection spots of comparable size. In addition, this effect could be exploited to our advantage, by preventing signal saturation when assessing the release of highly abundant cytokines from larger population of cells.

Cell Secretory Assays. We performed secretory immunophenotyping assays from neutrophils and primary monocytes isolated from peripheral blood, Figure S6, to assess their secretion profiles for IL-8 and TNF- α upon stimulation with LPS and PMA-ionomycin, respectively. IL-8 is a chemo-attractant for neutrophils, induces degranulation, and is the inflammatory mediator of diseases such as psoriasis, Chron's disease, and cancer.² TNF- α , on the other hand, is a proinflammatory cytokine, mainly released by macrophages, which stimulates the acute phase of the immune response and plays a pathological role in conditions such as cancer, infectious diseases, and autoimmunity.² Neutrophils isolated from human blood were seeded in the device and stimulated with different doses of LPS (0, 0.1, 1, 10 $\mu\text{g/mL}$) to evaluate their secretory profile of IL-8. First, we assessed their functional status by employing the diffusion-based mode of mass transport by allowing communication between culture and biosensing chambers for a period of 12 h, immediately after stimuli delivery. After developing the immunoassay, we did not observe any significant differences between the various stimuli conditions. Biosensing chambers were refilled with culture media and an active mixing period of 2 h was performed and the immunoassay was developed once again, showing that fluorescence intensities increased by 2.8-, 2.9-, 6.5-, and 5-fold from the lowest to highest concentration of the stimulant, Figures 4b and S7. Furthermore, significant differences in the fluorescence levels between the stimuli concentrations of 1 and 10 $\mu\text{g/mL}$ and the negative control arose only after the mixing process, which were not evident before. In addition, we observed a high-level secretion of IL-8 from nonstimulated neutrophils. This high basal secretion is in agreement with previous observations, which is attributed to a constitutive secretion process or to neutrophil activation during the isolation process.⁵⁴ Overall, the implementation of an active mixing protocol enhances the biosensing performance because affinity-based biosensors are dependent on the rate at which analytes are transported to the sensing surface, where they are captured by the antibodies. Moreover, mixing contributes to the replenishment of analytes in the biosensing chamber, which would otherwise generate a depletion layer of analyte that would limit the capture rate.⁵⁵ The performance enhancement due to active mixing has been exploited in microfluidic platforms; for example, Kaestli et al. showed that

biosensor signal increased over extended periods of media mixing.¹⁰

Finally, isolated primary monocytes were seeded to the device and stimulated with different combinations of PMA (500, 250, 100, 0 ng/mL) and ionomycin (5, 2.5, 1, 0 $\mu\text{g/mL}$), respectively, to induce TNF- α secretion for a period of 18 h. As shown in Figure 4c, we were able to quantify the release of TNF- α for all of the stimuli. We observed that increasing doses of stimuli do not correlate with an increase in the number of released TNF- α molecules per culture chamber and that this secretion saturates at $\sim 10^7$ molecules. Nonetheless, the amount of TNF- α produced by all stimuli conditions was significantly different from that of the negative control. This is in concordance with a previous report in which PBMCs were stimulated with various doses of PMA to induce TNF- α secretion.⁵⁶ Next, we averaged the amount of secreted molecules per cell by normalizing with respect to the number of immobilized cells. In this case, an increase in the stimuli dose correlated with an increase in the significant difference of the secretion level when compared to the negative control. In addition, a higher variability in the number of released proteins is observed for the highest dose; this variability could be due to a cytotoxic effect of the cytokine itself by autocrine or paracrine signaling, as has been previously reported.⁵⁷ This effect was shown in additional independent experiments, as shown in Figure S8. We analyzed the amount of TNF- α secreted at low ($<1\ \text{cell}/\mu\text{well}$) and high ($>1\ \text{cell}/\mu\text{well}$) cell densities challenged with 500 ng/mL of PMA and 5 $\mu\text{g/mL}$ of ionomycin, Figure S9. Low-cell densities showed a linear relationship between levels of TNF- α secreted and the number of cells captured per chamber. However, at high cell density levels this relationship disappears and seems to be inversely proportional. The effect of these variations could be attributed to paracrine communication because at high cell densities, more than one cell occupies a single μwell , allowing neighboring cells to uptake TNF- α . It has been established that monocytes have TNF- α feedback loops that play an important role in pro- and anti-inflammatory processes.⁵⁸ It has been shown that TNF- α signaling spreads to distances of only a few cell diameters.⁵⁹ Overall, this study supports the idea that our device could potentially be employed to perform paracrine signaling studies. Overall, our results demonstrate the ability of our device to (i) perform functional immunophenotyping assays on a variety of cells, (ii) stimulate cells with a range of chemical compounds, and (iii) quantify the secretion of diverse cytokines.

CONCLUSIONS

We presented a microfluidic device that can perform functional secretory immunophenotyping assays on adherent and non-adherent cells. Our device integrates cell culture and biosensing modules. The cell culture module employs arrays of μwells patterned on a microfluidic chamber to immobilize different immune cell types: meanwhile, the biosensor module relies on the MITOMI method. Once immobilized, cells are subjected to a wide variety of stimuli to assess their immune response by quantifying the secretion of cytokines of interest. We demonstrated the high efficiency ($\sim 70\%$) and uniformity ($\text{CV} = 0.106$) of cell seeding. Moreover, we were able to capture a variety of cells, from immortalized cell lines (THP-1 cells) to primary blood-derived cells (monocytes and neutrophils) and kept them in culture conditions for a period of 48 h with negligible cell death. Although our capture

efficiency is low, ~ 1 out of 1000 cells are captured from a seeding density of 10^7 cells/mL; we only use $\sim 10^6$ cells to perform 32 assays in parallel. In contrast, conventional well-plate assays to quantify cytokines using ELISAs require $\sim 10^6$ cells per condition.⁶⁰

Additionally, we showed that employment of epoxy-modified glass as biosensing substrates enabled a high-density immobilization of molecules and that when coupled with active mass transfer, an enhancement of immunoassay performance is achieved. Experiments with green-fluorescent protein, allowed us to confirm that active mixing shortens the time for the antigen–antibody binding to reach equilibrium under 2 h. Last, we proved the versatility of our platform by performing functional immunophenotyping of neutrophils secreting IL-8 upon LPS challenge, as well as TNF- α released from monocytes stimulated with PMA–ionomycin. Furthermore, previously we had shown that the devices that integrate μ wells for cell cultures reduce the shear stress on the cells during stimuli delivery, so that our platform has the advantage of being able to assess cytokine secretion in response to chemical cues and not by activation due to shear stress. In the future, our device could be integrated with an upstream module to capture leukocytes directly from a blood sample, facilitating its employment in disease diagnosis, where altered profiles in cytokine secretions are used as biomarkers, such as tuberculosis or sepsis, as well as prognosis and therapy evaluation. Furthermore, the ability to control the number of seeded cells would allow the employment of our platform in research laboratories to weigh paracrine effects on overall cytokine production.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssensors.9b01786>.

Complete list of materials and reagents employed, mold and PDMS device fabrication protocols, slide surface modification, device assembly and setup, THP-1 cell line culture, monocyte and PMN isolation and culture protocol, PMN immunostaining, cell viability assays, biosensor characterization methodology, capture antibody immobilization, calibration curve generation, immunophenotyping assays, and image analysis protocols, platform compatibility with adherent and non-adherent cells to perform functional immunophenotyping, uniform well occupancy in μ chambers across entire device, cell occupancy and viability of blood-derived monocytes, GFP immunoassay under diffusion regime, representative images obtained from calibration curves against TNF- α and IL-8, brightfield micrographs of monocytes and fluorescence micrographs of PMN stained with CD66b, signal comparison from secretory assays under diffusion and active mixing regimes, results from independent functional secretory assays from primary monocytes challenged with PMA–ionomycin, relationship between TNF- α signal intensity and number of trapped cells, and comparison between integrated immunophenotyping microfluidic devices (PDF)

Solution concentration equalization between culture and biosensing chambers employing active mixing (AVI)

■ AUTHOR INFORMATION

Corresponding Author

Jose Luis Garcia-Cordero – Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Apodaca, Mexico; orcid.org/0000-0002-3868-7405; Email: jlgarcia@cinvestav.mx

Other Authors

Roberto Rodriguez-Moncayo – Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Apodaca, Mexico

Rocio Jimena Jimenez-Valdes – Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Apodaca, Mexico; orcid.org/0000-0001-7124-5599

Alan Mauricio Gonzalez-Suarez – Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Apodaca, Mexico

Complete contact information is available at: <https://pubs.acs.org/doi/10.1021/acssensors.9b01786>

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

TNF- α , tumor necrosis factor α ; IL-8, interleukin-8; PMN, polymorphonuclear neutrophils; PBMCs, peripheral blood mononuclear cells; LPS, peripheral blood mononuclear cells; LP-Slipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; MITOMI, mechanically induced trapping of molecular interactions

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