Ultrasimple Single-Cell Detection of Multiple Cytokines by a Nanowell Chip Integrated with Encoded Microarrays

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Supporting Information

ABSTRACT: Cytokine production is often regarded as the marker of immune cells’ activation status. The spectrum and temporal secretion of cytokines are dramatically varied between cell phenotypes and even within the same phenotype. Multiparameter analysis of individual immune cell’s cytokine secretion has always been a challenging and complicated process that needs special facilities in a laboratory setting. Herein, we present an ultrasimple method with high sensitivity and high robustness to quantify cytokine expression at the single-cell resolution. A microchip is developed based on poly(dimethylsiloxane) nanowells on sticky tape, while each nanowell is integrated with a DNA–antibody convertible microarray. Only pipetting is needed for the whole single-cell analysis process. The sensitivity of the assay is evaluated by measuring various concentrations of six recombinant cytokine proteins, which was found comparable to conventional methods. Once single cells are loaded to nanowells and incubated there, a Fluorinert FC-40 is used to isolate nanowells; so, cytokines from those cells are captured by separate microarrays. The rest of the sandwich enzyme-linked immunosorbent assay detection process is also executed simply by pipetting of various reagents. This method is validated by measuring cytokine production from hundreds of single cells. It has simplified a typically sophisticated multiplex single-cell assay into an instrument-free, point-of-detection technology, and thus it may find a broad utility in clinical diagnostics.

KEYWORDS: single-cell analysis, simple method, point-of-care diagnosis, multiplex, microchip

Almost all human diseases directly or indirectly involve the activation of the immune system, which is mediated by cytokines. They are produced by a broad range of cells including immune cells, endothelial cells, fibroblasts, and stromal cells. Particularly for immune cells, the spectra and kinetics of cytokine expression are the hallmark of their phenotypes and functions. Decades of studies have built a solid connection between immune system status and disease progression. However, the current practice in the clinic still only relies on white blood cell counting or cytokine quantification in the blood serum. The physiological activities of immune cells have not been considered yet in clinical diagnosis, mainly due to the lack of appropriate technologies for functional analysis of cells.

Immune cells are naturally the most diversified population in the body to carry on a variety of tasks. The vast heterogeneity of cytokine expression by immune cells is not revealed until recently after the advent of highly multiplexed single-cell tools. Unfortunately, most, if not all, of them are complicated and require extensive practice and training for the operators. Conventional enzyme-linked immunospot is a simple, robust method, but it only measures one to three cytokines at a time with fairly low efficiency (24–48 h). The prevailing single-cell technology for protein profiling is flow cytometry and mass cytometry (CyTOF). Although they can measure dozens of proteins for each cell, they are barely used for cytokine analysis. This is because cytometers only assay proteins on the membrane or inside of a cell, while cytokines...
Microfluidics is the major technology for single-cell cytokine detection. However, compared with standard molecular biology techniques, microfluidics is usually tricky and hard to operate by first-time users. To date, there is no microfluidic chip routinely used in the clinic and across various locations for single-cell protein assay yet. Recent large-scale-integrated microchips can profile up to 40 cytokins and measure the cytokine secretion kinetics in single cells. However, those chips either need many pneumatic valves or involve microchannels that require pretreatment to remove air bubbles. These processes can be automated with the assistance of a complex setup. Other microchips for single-cell cytokine detection generally possess low multiplexity and thus retrieve very limited information from biological samples. The valve-less microengraved single-cell chips integrate a layer of antibody on the glass slide surface for sensing secreted proteins. The operation of the nanowell-based microchip is relatively simple; however, the assay multiplexity is only up to three. Emerging multiplexed methods can also measure a few cytokines simultaneously, however, they have not been tested yet for single-cell cytokine analysis.

The primary approach to improve multiplexity is to integrate a miniaturized antibody array with a microch. The conventional microarrays printed by spotting are not compatible with single-cell analysis since the spot size at about 100 μm is too large. Flow-patterning technique was developed to fabricate large-scale microarrays with a pitch of 20–50 μm on a glass slide. In this technology, a few DNAs of different sequences or antibodies of different species are injected into different microchannels with air pressure. However, the relatively large dimension of microarrays and the complexity of fabrication limit its multiplexity and popularity. Microbeads are in a much smaller size and can be coated with various probes on the surface. The assay multiplexity using microbeads relies on fluorescence spectrum, diameter, or DNA encoding. In our recent work, we introduced a microbead array-based technique to measure single-cell proteins. The physical approach of air/water interfacial self-assembly is highly subject to surface property, particle size, and particle material. The detection procedure involves some steps uncommon in a molecular biology experiment. A universal method for fabricating microbead array and a simplified single-cell detection strategy are highly demanded to popularize multiplexed single-cell analysis.

In this report, we introduce a highly sensitive yet ultrasimple method to measure multiple cytokines from single cells. A general technique is developed using an architect involving a sticky tape to simplify patterning of a large-scale microbead monolayer. This microbead-based multiplex in situ tagging (MIST) array is integrated within a nanowell microchip for sensing proteins. The MIST array is in a much smaller size compared with conventional microarrays of similar multiplexity capacity. Besides, the fabrication of MIST arrays is fairly simple once a mold is ready and does not require instruments. Single cells can be conveniently sealed within nanowells by applying Fluorinert FC-40 oil where secreted proteins are captured and detected by the MIST array. With the combination of those elements, the operation of the single-cell chip is no more complicated than that on a 96-well plate, whereas various formats of conventional well plates are far not sensitive enough to measure single-cell proteins. No additional setup that is frequently associated with microfluidics is required in the procedure. Our technology has made a typically sophisticated multiplex single-cell assay into a technique that can be grasped by a researcher in a common biomedical laboratory.

## RESULTS AND DISCUSSION

### Design of the Simple Single-Cell Multiplex Detection Chip

The chip design aims to make the on-chip operation as simple and robust as possible and to minimize the tricky processes typically associated with microfluidics. We adopt the well-plate-like platform in our design so that all of the operation steps simply need pipetting. The assembly of the microchip is shown in Figure 1a. A pressure-sensitive tape is attached on a glass slide and also holds a poly(dimethylsiloxane) (PDMS) membrane with through holes. A monolayer of 3 μm microbeads is attached on the sticky tape inside of the 0.625 nL nanowells (50 μm × 50 μm × 50 μm). The microbeads carry DNA probes that can be grafted with antibodies for sensing proteins. These nanowells can be sealed by a water-immiscible Fluorinert FC-40 oil, which is frequently used in microchip polymerase chain reaction for the isolation of nanowells. This oil is clear, colorless, chemically inert, and,
more importantly, does not absorb proteins and influence cell physiological activities as other oils do. With the integrated detection system on the bottom of the nanowell and sealing with FC-40 oil, any proteins from single cells are contained within the nanowell and can be detected by the microbead MIST array. Since all of the microbeads within a nanowell are randomly distributed, a decoding process is necessary to identify the type of protein detected on each of the microbeads (Figure 1b). We employ three consecutive cycles to find the fluorescent color sequence of each microbead. Through the consecutive labeling with Cy3- or Cy5-tagged complementary DNAs, each DNA-carrying microbead shows changing fluorescence, and the ordered fluorescent color on a particular microbead is the “code” to retrieve the DNA sequence that was used to coat this microbead. Theoretically, M cycles with N cycles can result in $M^N$ multiplexity. With only two colors (Cy3 and Cy5 dyes) and three cycles, at most $2^3 = 8$ different types of microbeads can be distinguished. Extension to five cycles or more is possible since no obvious loss of fluorescence was observed after five cycles (Figure S-4).

The decoding process is optimized to faithfully assign each microbead an ID on the MIST array. A cohort of pre-designed oligo DNAs tagged with either Cy3 or Cy5 is added to the MIST array for the labeling of microbeads, and fluorescence images are taken on the same array for each cycle. NaOH solution (0.5 M) is used to dissociate the hybridized DNAs (quenching step) until no fluorescence signal is detectable. While this report only detects six cytokines and focuses on the simple, instrument-free detection technique, the multiplexity of our assay can increase exponentially to reach a much larger number. For the research purpose, the decoding process was executed after protein detection. However, this process can be performed beforehand without affecting protein detection sensitivity; so, the users basically only need to perform the sandwich enzyme-linked immunosorbent assay (ELISA) experiment like on a 96-well plate.

**Characterization of the MIST Array.** We have developed a universal method for fabricating large-scale microbead monolayer on a substrate. The MIST array is compactly and uniformly patterned within nanowell to contain only one layer of microbeads. The whole process is shown in Figure 1. The pressure-sensitive tape is naturally sticky to any objects including microbeads and PDMS. The current cleanroom tape has been selected due to low leaching of chemicals, flat surface, high transparency, and low autofluorescence. Since the PDMS membrane on the device is deformable, the microbeads can be contact-printed onto the sticky tape surface at the bottom of nanowells. Only the bottom layer of the microbeads can survive sonication, and thus a monolayer of microbeads is conveniently formed. After sonication, the microchip is completely clean without excess microbeads or particles on the PDMS membrane or the walls of nanowells. This method is extremely efficient and superior to the existing methods including the one in our prior report using air/water interfacial self-assembly strategy to fix beads on a cleaned glass coverslip. Upon completion of the on-chip experiment, no obvious influence by the tape property has been observed. The MIST array has been fully characterized to ensure high quality before protein detection. Six types of DNA-encoded microbeads are mixed equally in number for patterning, resulting in an average of 393 microbeads per array with a variation of 32 microbeads (Figure 2a). The number of microbeads carrying the same DNA sequence is quantified to $65 \pm 31$ per array (Figure 2b). This variation was found insignificant to influence assay quantification and reproducibility in our previous work. The detection sensitivity is mainly determined by the loading density of DNA probes on the microbeads. We have optimized the DNA coating by amplifying amine groups on the surface through polylysine mediation (Figure S-1). Such a technique can enhance detection sensitivity significantly to surpass conventional ELISA assays in 96-well plates.

**Crosstalk Examination and Assay Sensitivity.** All of the antibodies and DNAs were examined to be orthogonal to each other and have no unwanted fluorescence background. One type of capture antibodies is conjugated with one sequence of oligo DNA through succinimidyl-6-hydrazinonicotinamide (S-HyNic) and succinimidyl-4-formylbenzamide (S-4FB) cross-linking (Figure S-2). The MIST array is converted from a DNA array to an antibody array through hybridization of DNA–antibody conjugates. The six proteins in our assay include interleukin 1β (IL-1β), interleukin-8 (IL-8), interleukin-6 (IL-6), migration inhibitory factor (MIF), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor (TNF)-α, which are the critical cytokines involved in the inflammatory response of the immune system to infection. No crosstalk was found in the detection of any of those proteins (Figure 3a,b). The detected signal is influenced by the incubation time. The signal with a short duration of 6 h is significantly higher than that of 12 and 24 h, whereas temperature has a negligible effect on the detected signal (Figure S-5).

The dynamic range and the limit of detection (LOD) are determined by the calibration curves where a series of recombinant proteins with known concentrations were applied to the antibody array. The dynamic range of $10^4$ is comparable to that of conventional ELISA assays. The LOD is determined to be $9.83, 7.72, 3.50, 4.03, 1.35$, and $3.38$ pg/mL for IL-1β, IL-8, IL-6, MIF, MCP-1, and TNF-α, respectively (Figure 3c). These LOD values are comparable to those by conventional ELISA methods that give LOD values of $0.5, 4.4, 4, 17.4, 1.6$, and $3.5$ pg/mL for those antibodies. The use of quantum dots (QD) 605 for labeling may have contributed to the high sensitivity. The other contributor should be the ultrahigh
parking of DNA probes and thereafter capture antibodies on the surface of the microarray. In consideration of the small size of nanowells at 125 pL, the detection sensitivity can achieve as low as 20 molecules per cell when a cell secretes cytokines within the nanowell (Table S-4).

**Analysis of Single-Cell Cytokine Secretion.** The steps for the simple on-chip assay are shown in Figure 4a. Before any test, the single-cell MIST chip remains dry with the DNA array to maintain long shelf life. Filling the chip with 70% alcohol kills microorganisms and removes air bubbles within the nanowells. A cocktail of conjugates is added to the chip to convert the DNA array into the antibody array, before loading lipopolysaccharide (LPS)-stimulated Tamm–Horsfall protein THP-1 cells in the nanowells. We used THP-1 cells to validate our system since they rapidly produce a large number and wide spectrum of cytokines upon stimulation.34,35 Of nanowells, 30% was installed with single cells when about 10 000 cells in a solution were loaded. Immediately after cell loading, a layer of Fluorinert FC-40 oil covers all of the wells and isolates the single cells. No crosstalk between nanowells was found, as is shown in Figure 4b. Only the nanowells with single cells have fluorescent signals. Cells were incubated in the nanowells only for 6 h to maintain their normal physiological activities and to minimize the influence of limited nutrition. Monocytes generally produce a significant amount of cytokines within 6 h,36 whereas longer incubation may lead to alteration of cytokine secretion.37

During the incubation, some cells slowly release 5-chloromethylfluorescein diacetate (CMFDA) CellTracker dye even after 1 h, which was also observed with cover slide sealing instead of oil (Figure 4b). Filling of the dye in individual nanowells coincidently validates the good sealing. The rest of the procedure of detection is the same as the operation on a well plate. The viability of cells was checked by Calcein AM dye and propidium iodide dye. It was found that 82.6% of cells were still alive after 6 h incubation (Figure S-6).

The decoding process is used to identify the protein detected on individual microbeads of an array. Figure 4c shows the typical decoding results by successive imaging of Cy3 (green color) and Cy5 (red color) of the same array. Each microbead is labeled with one color and is distinguishable from the others. Different from successive labeling from RNA...
imaging, our decoding method is naturally error-free as each microbead may carry hundreds of thousands of probes and signal is sufficiently higher than noise.

Microarrays with a cell are detected with significantly high signals than those without cell (0 cell) for all of the six proteins (Figure 5a). The control cells without stimulation were also measured by the same system. No signal was detected at the single-cell level (data not shown). This result is confirmed at the bulk level where supernatants of stimulated cells are found to contain much more cytokines than those of control cells (Figure 5b). The heatmap in Figure 5c shows the high heterogeneity of individual cells in expressing cytokines. The stimulated THP-1 cells tend to produce more MIF than the other five cytokines. The high MIF secreting cells are an independent subpopulation since they simultaneously express a very little amount of other cytokines.

![Figure 5.](image)

**Figure 5.** (a) Vertical scatter plots of IL-1β, IL-8, IL-6, MIF, MCP-1, and MIF protein signal from one-cell nanowells compared to background levels from zero-cell (0 cell) nanowells. Every dot represents the average fluorescence intensity of all of the microbeads of the same type in a nanowell that contains either one cell or no cell (0 cell). Blue bars are median values of the population. (b) Cytokine expression level in the supernatants of stimulated culture. (c) Heatmap of single-cell cytokine measurement result. Each row represents the complete protein profile from one single-cell measurement and each column corresponds to a protein of interest. The expression level is visualized with a color gradient from black (not detectable) to red (high expression).

**EXPERIMENTAL DETAILS**

**Coating of Single-Stranded DNAs (ssDNA) to Microbeads.** Polystyrene microbeads with aldehyde groups (Life Technologies) were coated with pol-y-lysine (PLL; Ted Pella) first to amplify functional groups on the surface. Microbeads (100 μL of 3 μm) of 4% w/v were incubated with 1.5 mL of 0.1 w/v PLL in phosphate-buffered saline (PBS; pH 8.5) overnight on a mixer (Figure S-1). The quality of PLL coating is validated by the nonspecific attachment of any ssDNA tagged with Cy3 fluorescence dye. Then, microbeads were washed by PBS containing 0.05% Tween 20 and centrifuged three times. ssDNA (10 μL) ended with primary amine (the DNA sequences and antibodies used in this work are listed in Table S-1; Integrated DNA Technologies) at 1 mM was mixed with the PLL-coated microbeads and 0.85 mM of bis(sulfosuccinimidyl)suberate (BS3; Life Technologies). The mixture was incubated for 4 h on a mixer before washing and centrifugation for three times.

**Preparation of DNA–Antibody Conjugates.** The conjugation process was detailed before.38 Complementary DNAs at 400 μM were reacted with succinimidyl-4-formylbenzamide (S-4FB; Trilink Biotechnologies) of 200 times excess for 4 h to fully install formylbenzamide group on DNAs. An antibody of 1 mg/mL was reacted with succinimidyl-6-hydrazinonicotinamide (S-HyNic; Trilink Biotechnologies) of 20 times excess for 4 h (Figure S-2). After desalting by 7K zeo spin column (ThermoFisher Scientific), the chemically modified DNA and antibody were mixed at pH 6 and incubated overnight before fast protein liquid chromatography purification. Six capture antibodies including IL-1β, IL-8, IL-6, MIF, MCP-1, and TNF-α (Table S-2) were conjugated with D', E', F', G', H', and I' oligo DNAs (Integrated DNA Technologies), respectively.

**Fabrication of the Microchip with Nanowells.** The through-hole poly(dimethylsiloxane) (PDMS; Dow Silicons) membrane was fabricated by soft-lithography method.39 Features on a chrome mask (Front Range Photomask) were transferred with a thickness of 50 μm on a 4 in. silicon wafer (Microetch using photoresist SU-8 2025 (MicroChem)). The membrane was made by an open capillary method. The PDMS prepolymer (Sylgard 184; Dow Silicons) was diluted with hexane at a 4:1 ratio, and a drop of the prepolymer was applied to the corner of the 1 cm × 1 cm feature area. After 1 h in room temperature, the prepolymer filled the entire area with microgrooves. The PDMS was cured by baking at 70 °C for 1 h and was peeled off from the mold.

**Patterning of Large-Scale Microbead Monolayer.** All six DNA-conjugated microbeads were mixed in equal portion and concentrated to be 50% w/v in Milli-Q water. The microbeads were spread to a confined area on a PDMS slab and dried. A pressure-sensitive tape (TPA cleanroom adhesive tape; Texwipe) was attached on a plain glass slide with the sticky side face up. A PDMS through-hole membrane was carefully pressed on the tape. Microbeads, the dried PDMS slab were contact-printed into the nanowell. The whole chip was sonicated for 2 s to remove excess microbeads and retain the monolayer in contact with the tape (Figure S-3).

**Decoding, Calibration, and Validation of the MIST Array.** The decoding process is to identify which DNA or protein is detected on a particular microbead on an MIST array. A cocktail of complementary DNAs tagged with Cy3 or Cy5 (Integrated DNA Technologies) fluorescent dyes (Table S-3) at a concentration of 200 nM was applied to the array and incubated for 1 h at 37 °C before imaging. Subsequently, the hybridized DNAs were dissociated by 0.5 M NaOH for 5 min. After blocking for 1 h with 3% bovine serum albumin (BSA; Fisher Scientific), the second cycle started with the same procedure, and the fluorescence images of the same arrays were taken. Totally, three cycles were executed. A bright-field image of microbeads was also taken for data processing.

The DNA array was converted to the antibody array before system calibration and sensitivity measurement. A cocktail of six complementary DNA–antibody conjugates at concentrations of 2.5 μg/mL in 3% BSA/PBS was incubated on the MIST array for 1 h before washing by 3% BSA/PBS. To obtain the calibration curves, recombinant proteins at various concentrations were added to the array and incubated for 2 h. After that, the array was washed and...
incubated with biotinylated detection antibody at 2 μg/mL for 1 h. Finally, streptavidin-QD 605 (Invitrogen, Inc.) at 20 nM was used to label the array. The same process was repeated three times. To validate no crosstalk of the detection, each of six recombinant proteins at a concentration of 5 ng/mL was added to the MIST array, incubated for 1 h, imaged, and quenched. The calibration curves were made by measuring the fluorescence intensity of microbeads when various concentrations of recombinant proteins were applied. Each condition was repeated three times, and the intensity of 10 microarrays was collected and averaged to represent each data point on the calibration curve. The limit of detection (LOD) was calculated based on the formula LOD = 3(SD/Slope), where SD is the standard deviation of the blank sample, and Slope is the slope of the calibration curve. All of the fluorescence images were taken on the same array and compared to check if the locations of any fluorescent microbeads overlapped.

Cell Culture and LPS Stimulation. THP-1 (ATCC TIB-202) human monocytes were cultured in a Roswell Park Memorial Institute Medium-1640 (Invitrogen) complete medium containing 10% fetal bovine serum (Coring), 100 U/mL penicillin G, 100 μg/mL streptomycin (Invitrogen), and 0.05 mM β-mercaptoethanol (Sigma-Aldrich) at 37 °C in an incubator supplied with 5% CO2. The THP-1 cells were subcultured every 2 days to keep cell concentration lower than 5 million cells/mL. Cells were stained with CellTracker CMFDA (Invitrogen, Inc.) at 0.2 μM for 10 min in a serum-free medium and washed by a complete medium as per the manufacturer’s instruction. Before the microchip experiment, lipopolysaccharide (LPS, Sigma-Aldrich) at 1 μg/mL was used to stimulate the monocytes for 6 h to differentiate into macrophages and produce cytokines.

On-Chip Single-Cell Procedure and Multiplexed Detection. The whole on-chip process only needs successive pipetting. The assembled microchip was rinsed with 70% alcohol to remove bubbles. After the conversion of the antibody array by the aforementioned method, 0.5 mL THP-1 cells at 10,000 cell/mL in a complete medium with 1 μg/mL LPS were loaded on the nanowells. Fluorinert FC-40 oil (Sigma-Aldrich) was added dropwise to the array to seal the nanowells. The whole setup is incubated for 6 h at 37 °C in a 5% CO2 incubator. The secreted proteins from the activated cells were captured by the antibody array. The oil was easily removed by rinsing with 3% BSA in PBS. Detection antibodies and streptavidin-quantum dots 605 (QDs; Invitrogen, Inc.) were added to the array by the method detailed above. After images of protein detection were taken, the fluorescence and DNA–antibody conjugates were removed by 0.5 M NaOH for 5 min. The decoding procedure is followed to match protein signal to the protein ID.

Fluorescence Microscopy Imaging and Data Processing. All of the images were taken on an Olympus IX73 inverted fluorescence microscope equipped with a digital camera (Zyla 4.2 sCMOS, Andor). A UV light-emitting diode of 405 wavelength (M405L3-C1, Thor Labs) was used to image QD signal in protein detection. The green fluorescence and red fluorescence were taken with a Cy3 filter set (U-FF, Olympus; excitation filter 528–553 nm, dichroic 565 nm long pass, emission 590–650 nm) and a Cy5 filter set (U-FF, Olympus; excitation filter 590–650 nm, dichroic 660 nm long pass, emission 665–740 nm). Microscope objectives (UPlanApo, Olympus, 4X/0.16; UPlanFLN, Olympus, 10X/0.30/Ph1; UCPlanFLN, Olympus, 20X/0.70/Ph2) were used to collect light. Laboratory-developed MATLAB programs were used to quantify the fluorescence intensity of individual particles, find their identities, and generate a cytokine profile for each cell. The digitalized images (6 × 6 microarrays per image) were aligned for each dataset that include a protein detection image, a bright-field image, and six decoding images (two for each cycle with totally three cycles). The order of fluorescent colors for each microbead on a microarray was extracted and then matched with the predesigned decoding map (Table S-3). The microbeads detecting the same protein were grouped and their fluorescence intensities were averaged for each array. The same procedure was repeated for each of ~300 arrays, which also carried the information of cell number per microcell or array. To the end, a dataset in the table was generated including cell number (zero or one cell) and the corresponding protein detection signal. The protein detection data in zero-cell microwells/arrays are taken as the background, and the average intensity across all microarrays was obtained. Prism (GraphPad) was used to statistically compare zero-cell data versus one-cell data and generated plots.

Cluster 3.0 (Stanford University) was used to hierarchically cluster single-cell cytokine data of the sample with stimulation. They were clustered by the Euclidean distance similarity metric method with a centroid linkage algorithm. Clustered single-cell data were visualized by heatmap using Java TreeView 3.0.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssensors.9b00765.

DNA coating on microbeads, conjugating oligonucleotides with antibody, procedure of fabricating microbeads monolayer, fluorescence intensities of using Cy3 tagged F-cDNA of five successive cycles, a bar chart for the IL-6 detection signal, image of cell viability test on MIST array, list of oligo DNAs, and list of antibodies in the study (PDF).

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Notes

The authors declare no competing financial interest.

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