Magnet Patterned Superparamagnetic Fe$_3$O$_4$/Au Core–Shell Nanoplasmonic Sensing Array for Label-Free High Throughput Cytokine Immunoassay

Yuxin Cai, Jingyi Zhu, Jiacheng He, Wen Yang, Chao Ma, Feng Xiong, Feng Li, Weiqiang Chen, and Pengyu Chen*

Rapid and accurate immune monitoring plays a decisive role in effectively treating immune-related diseases especially at point-of-care, where an immediate decision on treatment is needed upon precise determination of the patient immune status. Derived from the emerging clinical demands, there is an urgent need for a cytokine immunoassay that offers unprecedented sensor performance with high sensitivity, throughput, and multiplexing capability, as well as short turnaround time at low system complexity, manufacturability, and scalability. In this paper, a label-free, high throughput cytokine immunoassay based on a magnet patterned Fe$_3$O$_4$/Au core–shell nanoparticle (FACSNP) sensing array is developed. By exploiting the unique superparamagnetic and plasmonic properties of the core–shell nanomaterials, a facile microarray patterning technique is established that allows the fabrication of a uniform, self-assembled microarray on a large surface area with remarkable tunability and scalability. The sensing performance of the FACSNP microarray is validated by real-time detection of four cytokines in complex biological samples, showing high sensitivity ($\approx 20$ pg mL$^{-1}$), selectivity and throughput with excellent statistical accuracy. The developed immunoassay is successfully applied for rapid determination of the functional immunophenotype of leukemia tumor-associated macrophages, manifesting its potential clinical applications for real-time immune monitoring, early cancer detection, and therapeutic drug stratification toward personalized medicine.

1. Introduction

The ongoing revolution in fundamental immunology and clinical discovery critically hinges on the availability of diagnostic tools capable of decentralized point-of-care measurements\cite{1} to provide immediate quantitative information at the bedside or in the clinic.\cite{2} In particular the case of immune monitoring for practical medical treatment,\cite{3} fast, accurate, and high throughput analysis of multiple immune cells secreted cytokines using a small sample volume\cite{4} is highly required to precisely determine the rapidly changing immune status of patients in different inflammatory disease conditions.\cite{5} The current “gold standard” clinical technology is mainly based on enzyme-linked immunosorbent assay (ELISA). The complex labeling and washing processes require a total assay time up to more than 72 h and a sample volume of 0.5–2 mL per test per patient, which greatly hinders its application for immune monitoring at the point of care.\cite{6}

Label-free optical biosensing platforms, where the optical responses are measured at real-time without the need for secondary labeling, offer unique advantages in rapid analysis of complex biological samples.\cite{7} Among these techniques, inclusive of photonic crystal,\cite{8} optical ring resonator,\cite{9} surface plasmon resonance (SPR),\cite{10} fiber optics and interferometry,\cite{11} the nanoplasmonic biosensing based on the localized surface plasmon resonance (LSPR) of noble metal nanoparticles (NPs) has shown exquisite levels of precision and selectivity.\cite{12} In this paper, we present a label-free, high throughput cytokine immunoassay based on magnet patterned Fe$_3$O$_4$/Au core–shell nanoparticles (FACSNPs) that offers unprecedented sensor performance with high sensitivity, selectivity, and throughput, as well as excellent statistical accuracy. The developed immunoassay is successfully applied for rapid determination of the functional immunophenotype of leukemia tumor-associated macrophages, manifesting its potential clinical applications for real-time immune monitoring, early cancer detection, and therapeutic drug stratification toward personalized medicine.
of sensing performance. Recent advances in nanomaterials and nanotechnology have spurred the design and fabrication of next-generation nanoplasmonic biosensors with various nanostructures, such as nanorod, nano-bipyramid, nanoflower, nano core–shell structure, and nanohole arrays. The nanoplasmonic structures offer remarkable potential in sensor sensitivity, tunability, miniaturization, high throughput capability, and large-scale fabrication. The integration of these platforms into highly functional microfluidic devices has provided novel biological interfacing opportunities and promising features for practical biomarker detection. However, the implementation of such devices for real clinical and pharmaceutical settings has still been prohibited due to the deficiency in throughput and manufacturability without necessarily compromising the desirable sensitivity, multiplicity, and reliability. Various microarray nanoplasmonic sensing platforms are flourishing owing to the rapid technology evolution in nanofabrication. They hold great promise in massively parallel quantification of multiple analytes by immobilizing specific antibodies in separate spots on a single array. The versatility of parallel detection has significantly increased the throughput of the nanoplasmonic biosensors for multiplex label-free analysis. Yet, majority of these sensing arrays were fabricated using electron beam lithography, direct laser writing, chemical electrodeposition, and dip pen nanolithography, which often require dedicated instrumentations, labor intensive fabrication procedures and are extremely costly for large scale production. While there are a few multi-analyte high-throughput nanoplasmonic sensing platforms being developed at a fast pace, it has become clear that the cost, operation complexity, sensing performance, throughput, and scalability are equally challenging issues that must be addressed before the technology can be widely accepted in medical practice.

Here, we developed a high-throughput, label-free, multiplex LSPR immunoassay based on a facile magnet assisted fabrication method for Fe3O4/Au core–shell nanoparticles (FACSNPs) microarrays. By harnessing the unique super-paramagnetic property of the iron oxide nanocore, we demonstrated an easy-to-implement, scalable nanoparticle surface patterning technique for generating regular-shape, well dispersed, individual sensing spots over a large area. Moreover, the strong plasmonic coupling afforded by the decorated gold nanoparticles (AuNPs) on the FACSNPs exhibit superior sensitivity to the local refractive index change upon cytokine binding. Incorporating our previous developed LSPR dark-field imaging technique, the FACSNPs microarray biosensors can conduct 384 of tests on four different cytokines for each sample with 16 replicates per cytokine-test. The integration of the FACSNPs microarray sensors into a simple optofluidic device allows real-time, massively parallel detection of multiple cytokines with a low limit of detection to ~20 pg mL\(^{-1}\) using 1 \(\mu\)L of real biological samples. The stability, accuracy, and reproducibility of the immunoassay are further confirmed by standard ELISA and validated through successful demonstration of its practical use for functional immunophenotyping of leukemia tumor-associated macrophage (TAM) phenotypic polarization.

2. Results and Discussion

2.1. Synthesis and Characterization of Fe3O4/Au Core–Shell Nanoparticles

The FACSNPs were synthesized according to the fabrication procedures as illustrated in Figure 1a. The detailed synthesis processes are described in the Experimental Section. Briefly, the monodisperse Fe3O4 nanospheres (Fe3O4 NS) were prepared by a modified solvothermal reduction method. The Fe3O4 NS were then functionalized with 3-aminopropyl-triethoxysilane (APTES) to allow the covalent attachment of Au nanoseeds (AuNS) on the Fe3O4 nanocore. Finally, the FACSNPs were formed through in situ seed growth by directly reducing the Au4+ on the Au nanoseeds. We used centrimonium bromide (CTAB) to modify the surface of our FACSNPs. This provided the core–shell structured Fe3O4/Au nanoparticles with a positively charged surface showing a zeta potential of +46 mV (Zeta-sizer Nano ZS90, Malvern). Figure 1b shows the representative scanning electron microscope (SEM) images of the obtained Fe3O4 NS, Fe3O4 NS decorated with AuNS (Fe3O4-AuNS), and FACSNPs. The Fe3O4 nanocore displayed a structure composed of a cluster of many small Fe3O4 nanoparticles with diameters around 20 nm as shown in the high-contrast dark part of the core region. The AuNS and AuNPs (bright dots circled in yellow of the SEM images) were immobilized on the surface of the Fe3O4 nanocore with sizes around 3–5 and 20 nm, respectively. Multiple-crystalline porous structures were observed for the core–shell nanomaterial with an average diameter of 50–60 nm. We determined the composition (major elements: Fe, O, and Au) of the FACSNPs by energy-dispersive X-ray spectroscopy (EDX, bottom right panel of Figure 1b). Characteristic spectra of Fe, O, and Au were observed in the plot, confirming the presence of the major elements in the FACSNPs. Other signature peaks in the EDX spectrum were resulted from the silicon substrate. The morphology, structure, and size of the FACSNPs were further confirmed using transmission electron microscopy (TEM) (Figure S1a, Supporting Information). The size distributions of the FACSNPs and the embedded AuNPs were analyzed and showed good agreement with the SEM results (Figure S1b, Supporting Information). We also performed the X-ray diffraction (XRD) measurements on the synthesized FACSNPs (Figure S1c, Supporting Information). The results clearly showed the corresponding spectrum peaks of Fe3O4 phase, in consistent with those reported previously.

The UV–vis spectroscopy on the aqueous dispersions of the Fe3O4 NS, Fe3O4-AuNS, and FACSNPs was performed as displayed in Figure 1c. The Fe3O4 NS did not show any characteristic peak in 520–600 nm wavelength range (gray dashed line, Figure 1c). The addition of AuNS on the Fe3O4 resulted in a broad absorbance spectrum with resonance peak at around 550 nm. This can be explained by the large variation of the AuNS size distribution and their random deposition positions on the Fe3O4 NS. The growth of AuNS to AuNPs on the Fe3O4 core gives rise to a resonance peak at around 574 nm (Figure 1c, red line), indicating an absorbance red-shift of the AuNPs through enhanced plasmonic coupling due to the smaller interparticle distances. The much sharper resonance peak suggests a uniform immobilization of AuNPs on the Fe3O4 core.
We theoretically calculated (COMSOL Multiphysics) the extinction resonance spectrum of the FACSNPs based on the characteristic parameters from SEM and TEM results (Figure 1c, blue dash line). A highly enhanced localized electromagnetic (EM) field can be observed surrounding the AuNPs with strong coupling between the adjacent nanospheres (inset of Figure 1c). As a result, the optical response of the FACSNPs was determined by the superimposition of the plasmonically coupled neighboring AuNPs, which can be effectively utilized for the detection of local analyte binding events on the nanoparticles.

We further examined the magnetism of Fe3O4 NS and FACSNPs by measuring the magnetic hysteresis loop using a vibrating sample magnetometer (Figure 1d). The remanent magnetizations and coercivities were measured to be close to zero, which demonstrates that both the Fe3O4 NS and FACSNPs exhibited superparamagnetic characteristics. Modification of Fe3O4 NS with AuNPs slightly weakened the magnetization saturation value. But the FACSNPs can still be easily magnetized under external magnetic field, and demagnetized and redispersed immediately in the solution when external magnetic field was removed (inset of Figure 1d). Similar phenomenon was also observed under the dark-field microscope, confirming the superparamagnetism of the FACSNPs (Figure S2, Supporting Information). It should be noted that the conventional single-phase solid Fe3O4 NPs also exhibit superparamagnetic properties when the diameters of the NPs are in the range of 8.0–30 nm.[32] However, this smaller NP size makes the growth of 20 nm AuNPs onto the core much less energy favorable as compared to the nanocore of FACSNPs (∼60 nm) with a cluster of small Fe3O4 NS. As such, the entire FACSNPs afford both the superparamagnetic and strong plasmonic coupling characteristics, which allow tracking, manipulation, and patterning the FACSNPs without losing the advantage of the stable colloidal suspension, rendering them a well-suited material for microarray plasmonic biosensor fabrication as shown below.

### 2.2. Magnet-Assisted Fabrication of the FACSNP Microarray Biosensor

Using the physical and chemical properties of the FACSNPs that arise from both the intrinsic properties of constituent nanoparticles and their interparticle interactions, we adopted a magnet-assisted self-assembly process to pattern uniform antibody functioned microarray on a glass substrate. Figure 2a
presents the schematics of the fabrication processes (details can be found in Figure S3 in the Supporting Information). We first treated a polydimethylsiloxane (PDMS) microwell mask (20 µm × 20 µm well size) and a glass substrate with oxygen plasma to make the surface hydrophilic and negatively charged. Two of the microwell-shape PDMS masks were placed into a 3D-printed plastic frame to fix the pattern positions for subsequent antibody function and sample loading. FACSNPs (10 µL) dispersion was then dropped onto the plasma-treated PDMS masks and degassed for 25 min to ensure that all the microwells were fulfilled with the particle dispersion. The treated glass substrate was then attached to the FACSNPs loaded PDMS mask. Ceramic magnets were mounted on the bottom side of the glass substrate to exert a strong magnetic force on the superparamagnetic FACSNPs in the microwells. After overnight incubation, the positively charged FACSNPs were bound to the glass substrate by electrostatic attraction and assembled into uniform square-shaped microarrays over a large surface area as presented in the dark-field image (Figure 2b). We compared the patterning results with and without the assistance of the external magnetic field (Figure S4a,b, Supporting Information). Here, the magnet served as a concentrator that significantly enriched the local concentration, thus increasing the binding possibility of the FACSNPs to the glass substrate and avoiding the “coffee ring” effect. The release of the magnetic field allowed the redistribution of the FACSNPs owing to the superparamagnetic characteristic. As a result, we obtained a clearly better pattern quality of the FACSNPs with the magnet, showing a much stronger scattering intensity under dark-field imaging with regular microarray shapes (Figure 2c) and well-dispersed particle deposition as shown in Figure 2d,e.

Following the microarray patterning, we functionalized the FACSNPs with a panel of four cytokine antibodies using parallel microfluidic channels. The detailed function steps based on standard EDC/NHS chemistry are described in the Materials and Methods. The functionalized FACSNPs were imaged under SEM showing a thick layer of antibody coating on the NPs (Figure 2f). We further confirmed the antibody functionalization by measuring the zeta potential on the aqueous suspensions of the FACSNPs before and after the process. The functioned FACSNPs showed a neutralized zeta potential of +15 mV, indicating a partially coated surface of the FACSNPs with the antibodies. The successful antibody functionalization yielded four physically separated sensing regions with each consisting of large numbers of microarray sensors targeting specific cytokines (Figure 2c). This permits the multiplex...
detection of four cytokines in a massively parallel manner with high statistic accuracy. It should be noted that the shape, dimension, periodic distance, pattern area, and the number of the target analytes of the microarray biosensor can be readily tuned by changing the design of the PDMS mask (Figure S4c,d, Supporting Information), suggesting excellent flexibility, scalability, and manufacturability of our fabrication technique.

2.3. FACSNP Microarray Imaging and Calibration for Label-Free High Throughput Cytokine Detection

The clinical and immunological relevance of cytokine detection requires real-time, high throughput, and sample efficient analysis while simultaneously achieving a low limit of detection (LOD). For instance, the early diagnosis and screening of inflammatory diseases, such as sepsis, cancer, and graft-versus-host disease (GVHD), would need to identify multiple serum cytokine profiles at cutoff values less than 100 pg mL\(^{-1}\).[33] To provide such discriminatory power for valuable clinical outcomes, we integrated our previously developed LSPR dark-field imaging technique[34] with the FACSNP microarray biosensor into an optofluidic immunoassay for rapid, sensitive, and high throughput detection of cytokines in real biological samples. The optical setup and the principle of LSPR microarray imaging are illustrated in Figure 3a. Briefly, the prepared FACSNP microarray chip was mounted on a standard dark-field microscope (Nikon Eclipse Ni-U). Binding of the analyte cytokines onto the FACSNPs induces an increase in the intensity and a spectral red-shift of the scattering light. The collective

Figure 3. FACSNP microarray imaging schematics and intensity mapping of massively on-chip biosensing of multiple cytokines. a) Schematic of the dark-field microscope setup for FACSNP microarray imaging. The prepared microarray chip was fixed on the motorized stage with the other side in contact with the dark-field condenser via silicon oil. A sample was injected from the inlet of the PDMS channel covered on the prepared pattern, flown through the sample channel, and collected from the outlet. Different cytokines in the sample were captured by the antibody-conjugated FACSNPs on microarray. The light scattered from the FACSNPs was collected by 20× objective lens and imaged by the EMCCD. The right panel shows the principle of the FACSNP microarray imaging. The binding of specific cytokines onto the FACSNPs results in an intensity increase and a spectrum red-shift. b) Mapping of intensity variations of FACSNP microarray for four different types of cytokines (IL-6, MCP-1, TNF-α, and TGF-β) at different concentrations. c) Bar graph showing the scattering intensity variations obtained by the FACSNPs microarray sensing spots within each microfluidic detection channel (horizontal direction) when loaded with one specific recombinant cytokines spiked to the device. The concentration of the specific cytokine in the detection channel is 1000 pg mL\(^{-1}\).
light intensity shift was then recorded in real-time by an electron-multiplying charge-coupled device (EMCCD) camera. A customized Matlab program was used to automatically select the regions of interest through an edge detection/background subtraction algorithm and quantify the intensity change for each microarray sensing spots.

The intensity-based LSPR microarray imaging provides unique advantages over the traditional plasmonic biosensors based on the spectrum-shift detection schemes. The obtained optical signals from the assembled microarrays contain statistic information over a large amount of the FACS_arrays, which minimizes the variances in particle structure and spatial distribution. This distinct feature of LSPR microarray imaging offers unprecedented opportunities for high throughput immunoassay with inherently excellent statistic accuracy. As such, we first performed the calibration of the FACS_arrays microarray chip through parallel detection of four different cytokines: interleukin-6 (IL-6), monocyte chemoattractant protein 1 (MCP1), tumor-necrosis-factor alpha (TNF-α), and transforming growth factor beta (TGF-β) in Roswell Park Memorial Institute (RPMI) cell culture medium. The spiked samples with known concentrations (50, 100, 250, 500, 800, 1000 pg mL\(^{-1}\)) of cytokines were loaded into the sample loading channels and the quantified signal changes of the microarrays were translated into intensity maps as shown in Figure 3b. Here, we selected a 4 x 4 sensing array for each cytokine and recorded the real-time intensity shift of all the sensing spots in one sample channel (Figure S5a, Supporting Information). Thus, we can simultaneously acquire 64 real-time binding curves for four cytokines in one loading channel and achieve a total number of 384 (64 x 6) measurements for the whole immunoassay. The sensing matrix can be easily scaled up by increasing the numbers of sensing arrays and sample loading channels. We established the calibration curves for each cytokines based on the concentration dependent fractional intensity change (\(\Delta I/I_0\)) (Figure 3c, Figure S5b, Supporting Information). The intensity shift (\(\Delta I/I_0\)) was averaged over the 16 LSPR sensing microarrays by calculating the signal difference before (\(I_0\)) and after (\(I_0 + \Delta I\)) the sample incubation. We further determined the LOD as defined by \(3\sigma/k_{\text{slope}}\), where \(\sigma\) is the standard deviation of the control medium and \(k_{\text{slope}}\) is the slope of the linear regression of each calibration curve. The calculated LODs for the four cytokines were 18.96 pg mL\(^{-1}\) for IL-6, 14.57 pg mL\(^{-1}\) for MCP-1, 32.62 pg mL\(^{-1}\) for TNF-α, and 22.08 pg mL\(^{-1}\) for TGF-β. As a result, our FACS_arrays microarray immunoassay shows comparable sensing characteristics in terms of sensitivity, assay time, and sample volume to the most state-of-art LSPR biosensors,\(^{[35]}\) while offering unique advantages in selectivity, throughput, and manufacturability toward practical applications.

### 2.4. Selectivity and Stability of FACS_arrays Microarray Immunoassay

To validate that our microarray immunoassay can specifically detect target cytokines in a complex biological medium, we performed measurements on a set of samples with each containing only one specific type of the cytokines (IL-6, MCP-1, TNF-α, and TGF-β) at the concentration of 1000 pg mL\(^{-1}\). Figure 3c presents the fractional intensity changes of FACS_arrays microarrays after incubating with the prepared spiked samples in RPMI. With the presence of the target cytokines, only the corresponding antibody functioned sensing spots yielded significantly increased optical responses, proving the excellent selectivity of the microarray immunoassay. The intensity shift was then converted to analyte concentration based on the calibration curves (Figure S5c, Supporting Information), showing no significant difference with the expected concentration of 1000 pg mL\(^{-1}\). The microarrays targeting unspiked cytokines in the cell culture medium exhibited signals below the LOD as anticipated (Figure S5c, Supporting Information). These results suggest that our multiplex immunoassay demonstrated minimum cross-reactivity and negligible nonspecific adsorption among the four cytokine biosensor arrays. We believe that the spatial confinement of the antibody functioned sensing area and physically separated individual sensing arrays are the key factors that contribute to the pronounced selectivity as compared to conventional multiplex immunoassays. In this study, all the microarray immunoassays were used within the same day after antibody functionalization. We anticipate that the functionalized chips in proper storage condition at 4 °C could last for 1–3 weeks and believe that the stability of the immunoassay is highly dependent on the chemistry and physics of the antibody and the storage condition.\(^{[36]}\) Since the FACS_arrays microarrays without antibody can be stored for months and the antibody functionalization can be readily achieved within 2 h, it would be more preferable to use the functionalized FACS_arrays microarray on an as-needed basis for practical application, which has already been widely implemented in commercial immunoassays.

### 2.5. FACS_arrays Microarray Immunoassay for Functional Immunophenotyping of Tumor-Associated Macrophage

TAMs are the most prominent immune cells in the tumor microenvironment composed of leukocytes, fibroblasts, and vascular endothelial cells. They play a vital role in nonresolving inflammation in tumor microenvironment, which is known as a hallmark of cancer.\(^{[37]}\) In general, macrophages show a high degree of plasticity in response to local environments and can be polarized to pro-inflammatory (M1) or anti-inflammatory (M2) macrophages. The M1 phenotypes are well adapted to promote a strong immune response by secreting high levels of pro-inflammatory cytokines. In contrast, the M2 phenotypes are activated by T helper cell 2 and tumor derived cytokines (IL-4, IL-10, and IL-13), which are well-suited for the promotion of proliferation, invasion, and angiogenesis of tumor cells and thus the tumor development (Figure 4a). With the unraveling relationship of the apparent dual nature of macrophages to the tumor development, the M2-like TAMs are now being recognized as potential diagnostic biomarkers and therapeutic targets for cancer.\(^{[38]}\)

To explore the practical use of our FACS_arrays microarray immunoassay for clinical diagnosis, we performed the functional immunophenotyping of macrophages exposed to leukemia tumor cell (LTC) microenvironment. Here, we measured
the cytokine secretion profiles of macrophages under stimulation or treated using LTC-conditioned media (Figure 4b). The original unpolarized macrophages (M0) expressed no significant secretion for pro-inflammatory cytokines (IL-6 and TNF-α) and anti-inflammatory cytokine TGF-β. The stimulation with lipopolysaccharides (LPS) turned M0 into the M1 phenotype macrophages, where strong IL-6 and TNF-α expressions were observed in the cell culture medium with a negligible amount of TGF-β released. In contrast, the M2 phenotype macrophages polarized by IL-10 showed a significantly increased concentration of anti-inflammatory cytokine (TGF-β) expression, while the inflammatory responses by IL-6 and TNF-α secretion were largely suppressed. The macrophages treated with LTC-conditioned media displayed a similar cytokine secretion profile with that of M2 macrophages, indicating their transformation into M2-like TAMs. The measurements of LTC conditioned media showing low levels of all cytokines further confirm that the measured TGF-β and MCP-1 were secreted by the polarized TAMs. Here, the MCP-1 is known as a potent chemotactic factor for monocyte trafficking, thus was detected in all the macrophage culture media. The relatively higher level of MCP-1 released by M2 and TAMs could be attributed to the promoted macrophage recruitment in the tumor microenvironment. All the results obtained from the FACSNP microarray immunoassay were validated by the singleplex ELISA for all four cytokines across the cell medium samples prepared above. An excellent linear correlation ($R^2 = 0.9252$) was obtained between the results measured by both methods (Figure S6, Supporting Information). As such, the FACSNP microarray immunoassay showed discriminative power for immunophenotyping of macrophages in a biomimetic tumor environment, which could be potentially applied as a rapid and high throughput method for point-of-care clinical cancer diagnosis.

3. Conclusion

In conclusion, we have demonstrated a high-throughput, label-free, multiplex immunoassay that enables the analysis of multiple immune biomarkers in a rapid, accurate, and sensitive manner. The key to the success of this platform is by synergistically utilizing both the superparamagnetic and nanoplasmonic properties of the FACSNP for large-scale array patterning and high-throughput sensing. The magnet assisted patterning approach shows great advantages over many other technologies for large-scale array fabrication in terms of feasibility, flexibility, scalability, and cost-effectiveness. The multiplex immunoassay based on the patterned microarray can generally serve as a powerful tool for routinely monitoring a wide variety of biomarkers with rapid turn-around time and high statistic accuracy, which
can be readily implemented for point-of-care clinical diagnosis. This platform possesses unique characteristics that do not exist with currently available technologies in clinical settings, which can be further expanded by integrating with nano- and microfluidic systems to provide multiscale measurements from whole-blood level to single-cell level for comprehensive functional analysis of the immune system.

4. Experimental Section

Fe₃O₄/Au Core–Shell Nanoparticle Synthesis: First, 0.15 g of FeCl₃·6H₂O was added into a 4 mL mixed solution of 3 mL ethylene glycol and 1 mL ethanolamine under vigorous magnetic stirring to form a stable light brown solution. 0.1 g of polyethylene glycol 2000 and 0.4 g of NaAc was added into the above solution. After 30 min of vigorous magnetic stirring, the solution was transferred to a Teflon-lined stainless-steel autoclave and was heated to 200 °C for 8 h. The products (Fe₃O₄ NS) were collected and washed with ethanol and deionized water three times and dispersed in ethanol. Then, 40 µL of APTES was added into the dispersion under continuous magnetic stirring for 3 h at room temperature. The APTES modified Fe₃O₄ NS were harvested and washed with ethanol and deionized water three times. The Au nanoseeds were prepared by adding 0.6 mL ice-cold and fresh 0.1 M NaBH₄ solution into 10 mL aqueous solution of 2.5 × 10⁻⁴ M HAuCl₄ and 2.5 × 10⁻⁴ M trisodium citrate. The solution color changed into pink immediately. Then, after 5 min of APTES-modified Fe₃O₄ NS aqueous dispersion was mixed with 3 mL of gold nanoseeds solution. The mixture was quickly ultrasonicated and then stayed under magnetic stirring for 2 h. After washing with ethanol and deionized water, the products (Fe₃O₄ NS-AuNS) were dispersed in deionized water. 150 µL of 1% HaurCl₄ solution was added to 10 mL of 2 × 10⁻³ M potassium carbonate solution under vigorous magnetic stirring. After 30 min, the solution aged at 4 °C overnight to form Au(OH)₄⁻ ions solution. To further grow the AuNS to AuNPs on the surface of the Fe₃O₄ NS, 2 mL of Au(OH)₄⁻ ions solution and 8 mL of formaldehyde was added to the Fe₃O₄ NS-AuNS aqueous dispersion. The FACSNPs were harvested by a magnet and incubated with 0.1 µL CTAB solution at 4 °C for 48 h. The final products were washed with ethanol and deionized water.

FACSNP Microarray Fabrication and Functionalization: The FACSNP microarray was fabricated based on a magnet-assisted patterning technique. The detailed fabrication procedures are described in Supporting Information Section 4. To functionalize the FACSNP microarray, a PDMS layer with microfluidic flow channels was fabricated. The microfluidic channels can be easily aligned onto the FACSNP microarray, a PDMS layer with microfluidic flow channels was fabricated. The microfluidic channels can be easily aligned onto the FACSNP microarray, a PDMS layer with microfluidic flow channels was fabricated.

Between 1L PBS (depends on various conditions) at 2 µL min⁻¹. During all the process steps, the reagent solutions was loaded using a syringe pump (LEGATO101, Kd Scientific) at 1.5 µL min⁻¹. Between every step, the microarray sensing spots were thoroughly washed to remove any excessive solutions or molecules using 20 µL of deionized water or 1× PBS (depends on various conditions) at 2 µL min⁻¹.

Optical Setup and FACSNP Microarray Immunoassay: After the FACSNP microarray antibody functionalization, the microfluidic channel layer was removed from the glass substrate and immediately replaced with another PDMS layer with parallel sample-flow microfluidic channels. The sample-flow microfluidic channels (500 µm (W) × 2.5 cm (L) × 50 µm (H)) were bonded perpendicular to the direction of the antibody functionalization channels. The constructed FACSNP microarray chip was subsequently mounted on a motorized X-Y stage (ProScanII, Prior Scientific, Rockland, MA), and Roswell Park Memorial Institute (RPMI) medium was loaded into each of the on-chip flow channels using a syringe pump at 1.5 µL min⁻¹ to stabilize the initial light intensity of sensing spots. After signal stabilization, 1 µL sample was loaded into each of the sample-flow channels using a syringe pump at 0.1 µL min⁻¹ and the images of sensing spot arrays were captured real-time by the EMCCD camera and recorded them using NIS-Elements BR analysis. A customized Matlab program was used to analyze and quantify the scattering intensity increase for each microarray pattern.

Cytokine Measurement with ELISA: ELISA kits (Mouse IL-6 ELISA MAX Deluxe, Mouse IL-10 ELISA MAX Deluxe, and Mouse TNF-α ELISA MAX Deluxe, Biolegend, Mouse MCP-1/CCL2 Uncoated ELISA kit, Mouse TGF-beta 1 ELISA kit, Invitrogen) were used in this study. According to the manufacturer’s protocol, capture antibody solution was first added into all wells of a 96-well plate provided in the kit. The plate was subsequently sealed and incubated at 4 °C overnight. After washing and blocking, 100 µL of each standard and cell culture supernatant sample was added into the wells and incubated for 2 h at room temperature. The wells were then incubated with detection antibody for 1 h, followed by washing and incubation with HRP-labeled Avidin for 30 min. TMB mixture (1:1) was then added for 20 min in the dark to visualize chemiluminescence. Then, 100 µL of 2 µL sulfuric acid solution was added in each well to stop the reaction. The reading of ELISA results was carried out using a plate reader (SpectraMax i3, Molecular Devices) by recording the absorbance at 450 nm within 15 min after adding the stop solution.

Cell Culture and Macrophage Polarization: Mouse macrophage cells (RAW264.7, ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), and 1% penicillin/streptomycin (Gibco). All cell lines were grown no more than 20 passages in a 37 °C (5% CO₂) incubator. Macrophage polarization was induced in vitro. Briefly, untreated RAW264.7 cells were labeled as M₀ macrophages. RAW264.7 cells (10⁵ cells per mL) were seeded in 12-well plates overnight before polarization. M₁-like macrophages were obtained by treating RAW264.7 cells with 100 ng mL⁻¹ lipopolysaccharide (LPS, Sigma-Aldrich) for 24 h. M₂ macrophages were obtained by stimulating RAW264.7 cells with 50 ng mL⁻¹ of IL-10 (BioLegend) for 24 h. Subsequently, the media were replaced by fresh complete cell culture media for M₁ and M₂ macrophages. The cell culture supernatants for M₁ and M₂ macrophages were then collected after another 24 h. B-cell acute lymphoblastic leukemia (B-ALL) cells was isolated from a well-characterized model of pediatric Ph+ B-ALL, in which lethally-irradiated C57BL/6 mice are reconstituted with retrovirally infected hematopoietic stem and progenitor cells ectopically co-expressing the B-ALL-associated P190 BCR-ABL1 isoform, as well as GFP for fluorescent cell tracing. Following the isolation, B-ALL cells were cultured and expanded in Iscove’s Modified Dulbecco’s Medium (IMDM, Invitrogen) supplemented with 10% FBS, 1% penicillin/streptomycin, 100 × 10⁶ M L-glutamine, and 50 × 10⁶ M β-mercaptoethanol in a 37 °C (5% CO₂) incubator. The culture supernatants of leukemia tumor cells were collected after the concentration of tumor cells reached 13 × 10⁶ cells per mL and centrifuged at 2000 × g for 10 min to remove cell debris.
Biomimetic TAMs were obtained by culturing M0 RAW264.7 cells in complete culture media supplemented with conditioned media of leukemia tumor cells (1:1) for 24 h.

**Statistical Analysis:** In Figure 1c, the data of UV–vis absorbance spectra of Fe₃O₄-AuNSs, FACSNP, and the simulation results of FACSNP extinction spectrum were normalized to 0–1 range to clearly show the spectrum shift. In Figure 1d, the magnetization curves of the Fe₃O₄ NS and FACSNP were also normalized to 0–1 range. The normalizations in both figures were processed by Microsoft Office Excel. In Figure 3b, all the measured intensity values from the 16 FACSNP microarray spots for four different types of cytokines at different concentrations were transformed to “Heat Map” by Microsoft Office Excel. All the statistical analysis of the measured cytokine concentrations were obtained by calculating the mean and standard derivation of the signal responses from a set of 16 microarray sensing spots. The standard deviations (SD) were presented as error bars in the bar charts. \( p \) Values were calculated using the One-Way ANOVA, \( * p < 0.05, ** p < 0.01, *** p < 0.0001 \) using Microsoft Office Excel.

**Supporting Information**
Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**
The authors declare no conflict of interest.

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