

Machine-Learning-Assisted Microfluidic Nanoplasmonic Digital Immunoassay for Cytokine Storm Profiling in COVID-19 Patients

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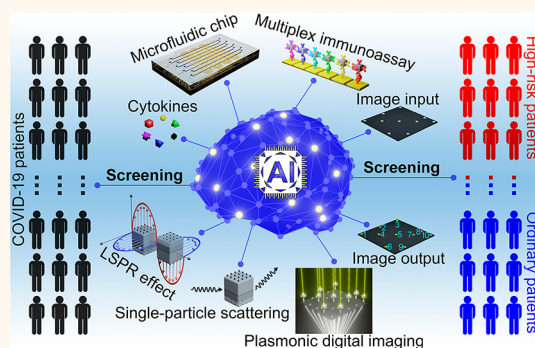
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ABSTRACT: Cytokine storm, known as an exaggerated hyperactive immune response characterized by elevated release of cytokines, has been described as a feature associated with life-threatening complications in COVID-19 patients. A critical evaluation of a cytokine storm and its mechanistic linkage to COVID-19 requires innovative immunoassay technology capable of rapid, sensitive, selective detection of multiple cytokines across a wide dynamic range at high-throughput. In this study, we report a machine-learning-assisted microfluidic nanoplasmonic digital immunoassay to meet the rising demand for cytokine storm monitoring in COVID-19 patients. Specifically, the assay was carried out using a facile one-step sandwich immunoassay format with three notable features: (i) a microfluidic microarray patterning technique for high-throughput, multi-antibody-arrayed biosensing chip fabrication; (ii) an ultrasensitive nanoplasmonic digital imaging technology utilizing 100 nm silver nanocubes (AgNCs) for signal transduction; (iii) a rapid and accurate machine-learning-based image processing method for digital signal analysis. The developed immunoassay allows simultaneous detection of six cytokines in a single run with wide working ranges of 1–10,000 pg mL⁻¹ and ultralow detection limits down to 0.46–1.36 pg mL⁻¹ using a minimum of 3 μ L serum samples. The whole chip can afford a 6-plex assay of 8 different samples with 6 repeats in each sample for a total of 288 sensing spots in less than 100 min. The image processing method enhanced by convolutional neural network (CNN) dramatically shortens the processing time $\sim$ 6,000 fold with a much simpler procedure while maintaining high statistical accuracy compared to the conventional manual counting approach. The immunoassay was validated by the gold-standard enzyme-linked immunosorbent assay (ELISA) and utilized for serum cytokine profiling of COVID-19 positive patients. Our results demonstrate the nanoplasmonic digital immunoassay as a promising practical tool for comprehensive characterization of cytokine storm in patients that holds great promise as an intelligent immunoassay for next generation immune monitoring.

KEYWORDS: microfluidic immunoassay, digital/single-molecule detection, nanoplasmonics, machine learning, cytokine storm, coronavirus disease 2019



INTRODUCTION

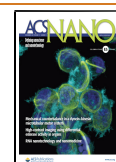
Coronavirus disease 2019 (COVID-19), an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has emerged as a mounting threat to global health since December 2019.^{1,2} COVID-19 infection is accompanied by an aggressive inflammatory response, known as a “cytokine storm”, resulting from complex interplays between lymphocytes and myeloid cells.^{1,3,4} Excessive production of proinflammatory cytokines in COVID-19 patients can further lead to acute respiratory distress syndrome (ARDS), multiple organ failures (MOFs), and death.^{1,3,4} Hence, early detection and close monitoring of cytokine storms are critical for rapid

identification of high-risk COVID-19 patients and the development of prompt guidelines for anti-inflammatory therapies to improve survival rates. Due to the dynamic and time-sensitive clinical features of COVID-19, characterization of cytokine storms requires fast analysis of cytokines across a wide dynamic

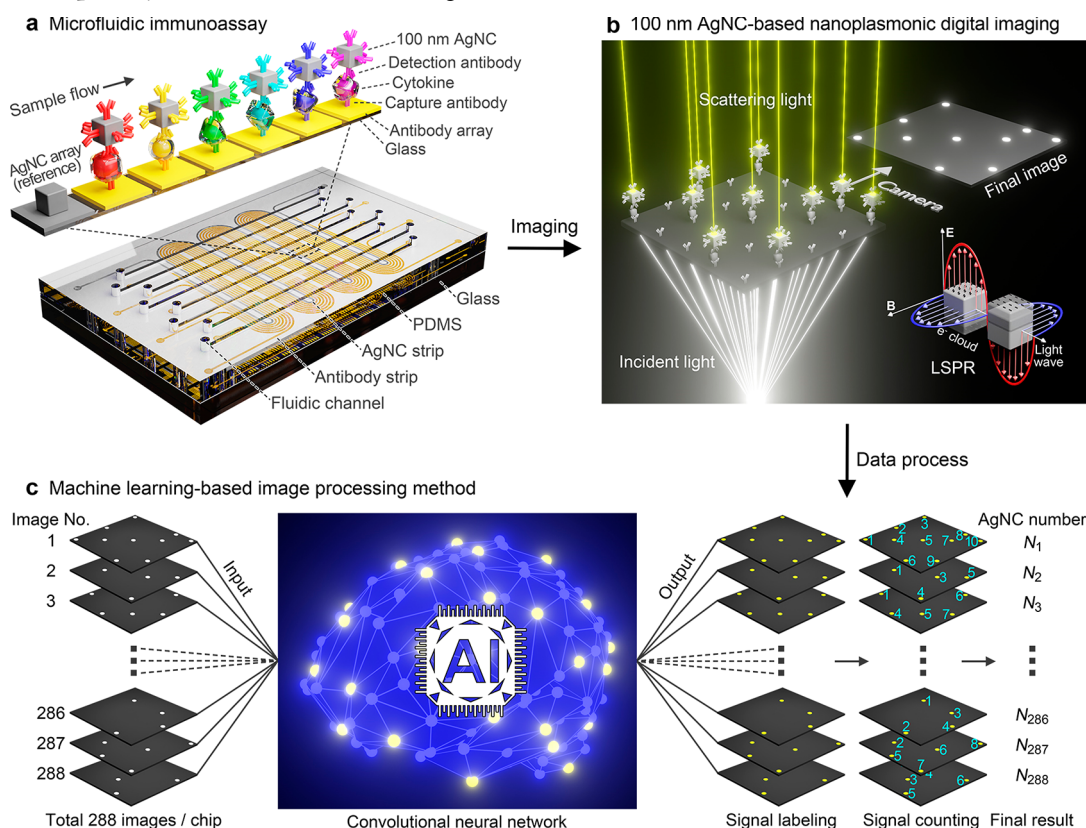
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Scheme 1. Schematic Illustration of the Machine-Learning-Assisted Microfluidic Nanoplasmonic Digital Immunoassay for High-Throughput, Multiplex Cytokine Detection (Including IL-1 β , IL-2, IL-6, IL-10, TNF- α , and IFN- γ)^a



^aThe immunoassay involves three key components: the microfluidic immunoassay platform (a), the nanoplasmonic digital imaging technology (b), and the machine-learning-based image processing method (c).

range in a small amount of patient serum with high sensitivity, selectivity, throughput, and multiplex capacity. Current gold-standard clinical tools to test blood circulating cytokines are based primarily on enzyme-linked immunosorbent assay (ELISA). The laborious and lengthy procedures, large sample consumption, and required centralized facility greatly impede its practical usage for monitoring cytokine storms in COVID-19 patients. As such, there is an urgent need for effective cytokine detection strategies that satisfy the stringent requirements to provide rapid and informative insights for COVID-19 diagnosis, monitoring, and treatment.

Over the past two decades, the microfluidic immunoassay has become an emerging technology for rapid analysis of biomolecules in complex biological samples.^{5–7} Microfluidics offer significant advantages in controllable fluid handling, low reagent consumption, and confined microenvironment analysis.^{5–8} The integration of immunoassays to the microfluidic scale shows greatly improved analytical performance at point-of-care, such as reduced assay time, small sample volume, high throughput and multiplexity, and semi-automation.^{5–8} Recent advancements in a variety of microfluidic immunoassays have demonstrated promising features for cytokine detection, including a sample-to-answer time shortened to ~ 30 min, a sample volume reduced to a few μL , a throughput improved to hundreds of parallel tests, a multiplex capacity up to dozens of targets, and so on.^{9–12} However, accumulating evidence suggests that the cytokine concentrations in plasma of COVID-19 patients span across a wide dynamic range (1–40,000 pg mL^{-1}) with a few key inflammatory cytokines at the

sub- pg mL^{-1} level.¹ Current microfluidic immunoassays rely mainly on conventional signal transduction technologies based on measurement of ensemble average signals, which often require numerous captured signaling molecules to generate a detectable signal over background noise. Hence, the majority of these platforms could not afford sufficient sensitivity in a multiplex, high-throughput scheme to accurately detect cytokines at ultralow levels and across a wide concentration range.

The ever-increasing demand for ultrasensitive and accurate detection has driven the development of advanced bioanalytical methods such as the digital (or single-molecule) imaging assay, where the binding of a single biomolecule to the corresponding affinity biomolecule can be individually visualized *via* a single signal label.^{13–17} In contrast to the conventional ensemble average measurements, digital imaging approaches enable the measurement of individual biomolecule binding events with extremely high signal-to-noise ratio, resulting in the highest class of sensitivity down to single-molecule level.^{13–17} Up to this time, various digital imaging technologies have been exploited based on different labeling agents (*i.e.*, enzymes, fluorescent dyes, upconversion nanoparticles (NPs), and plasmonic metal NPs).^{13,16–23} Among them, nanoplasmonic digital imaging has received considerable attention owing to the superior physicochemical properties of plasmonic metal NPs.^{20–33} The strong Rayleigh scattering of individual plasmonic NPs allows subdiffraction imaging with extremely high spatial resolution under dark-field microscope.^{20–30} The scattering intensity and the extinction spectra of the NPs can be tuned at will *via* precise

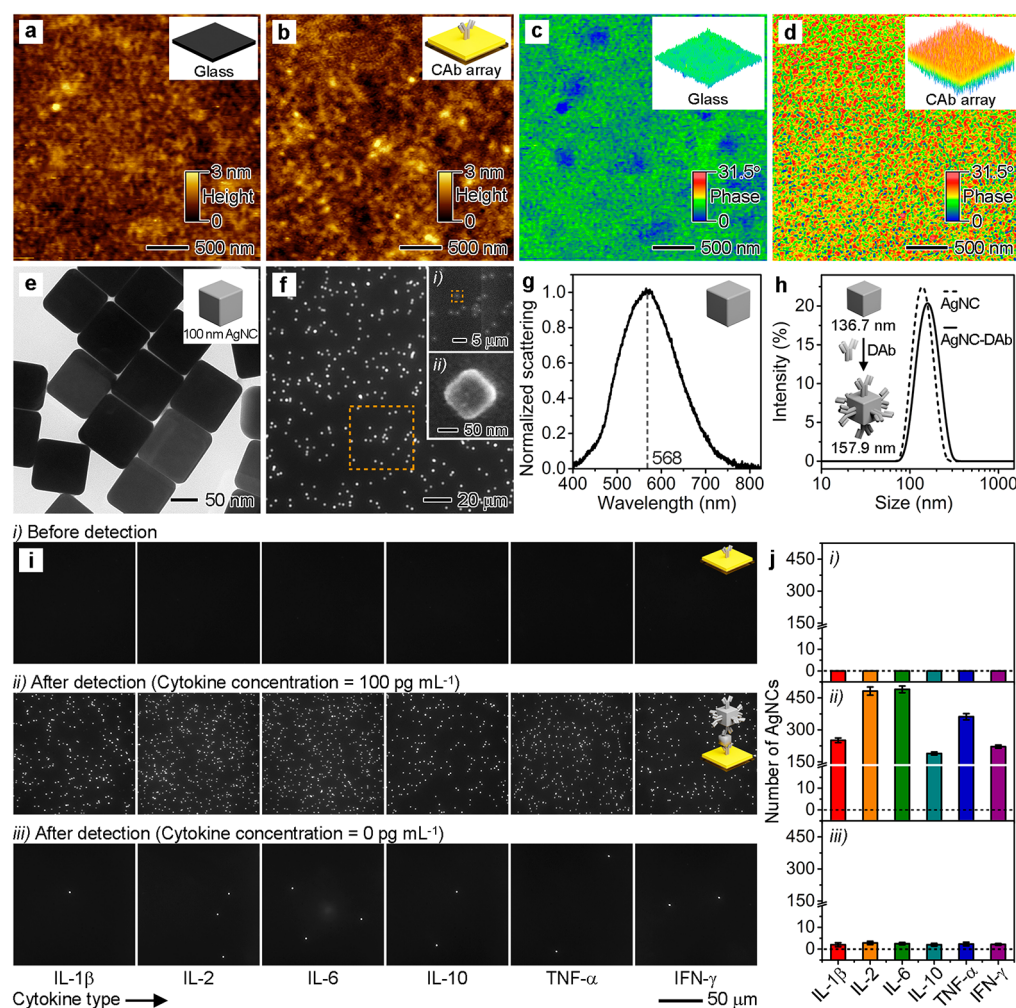


Figure 1. Characterization of the microfluidic nanoplasmonic digital immunoassay. (a–d) AFM topographic (a, b) and phase (c, d) images of the surfaces of glass substrates before (a, c) and after (b, d) the immobilization of capture antibodies. Insets in a–d show the corresponding images in three-dimensional (3D) models. (e) TEM image of AgNCs with an average edge length of 100 nm. (f, g) Dark-field image (f) and scattering spectrum (g) of 100 nm AgNCs deposited on a glass substrate. In (f), insets (i) and (ii) show the SEM images taken on the region marked by a yellow box in (f) and in inset (i), respectively. (h) DLS size distribution of 100 nm AgNCs (dashed curve) and AgNC-DAB conjugates (solid curve) dispersed in ultrapure water. (i) Dark-field images of antibody microarrays taken before the detection of cytokines (i) and after the detection of 100 pg mL^{−1} (ii) and 0 pg mL^{−1} (iii) cytokines. (j) Bar graph showing the intensity of the detection signal (i.e., particle number of AgNCs) obtained from (i).

control over NPs' size, shape, and dielectric properties.^{32–34} Since signal transduction is based on light scattering, plasmonic NPs display excellent photophysical stability, allowing continuous, intermittence-free measurement.^{20–33} While a few recent studies have demonstrated the use of nanoplasmonic imaging for digital biomarker detection,^{21–28} the sensing performance has been greatly limited by conventional imaging analysis and manual particle counting methods. When integrating nanoplasmonic digital imaging with microfluidic immunoassays, the inability to perform rapid and accurate digital signal processing for a large amount of image data has thereby become a major challenge for sensitive high-throughput multiplex detection of cytokines in clinical settings.

Herein, we developed a nanoplasmonic digital immunoassay by integrating a machine learning assisted nanoplasmonic imaging method with a microfluidic immunoassay platform that overcomes major constraints for cytokine profiling in real patient samples (Scheme 1). The immunoassay exploits a one-step sandwich microarray format utilizing anticytokine capture antibody (CAb) arrays as the primary capture layer, and Ag

nanocubes (AgNCs) conjugated with paired detection antibodies (DABs) as the signal transducers for massive parallel detection of multiple cytokines in a small sample volume. The formed sandwich immunocomplex can be imaged individually under a dark-field microscope due to the strong plasmonic scattering of the AgNCs. The generated highly sensitive and selective digital signals can be readily counted by a customized machine-learning-based image processing method. By establishing the correlation between digital signal count and the cytokine concentration, our immunoassay allows simultaneous detection of six cytokines across a wide dynamic range of 1–10,000 pg mL^{−1} with a limit of detection down to subpg mL^{−1}. The integrated microfluidic platform enables high-throughput analysis of 8 different samples per chip with a total of 288 tests using only 3 μ L serum samples. Using ELISA as a benchmark method, the immunoassay was validated for practical application in serum cytokine measurement with excellent accuracy and reliability and was successfully applied for profiling cytokine storms in COVID-19 patients.

RESULTS AND DISCUSSION

Design and Principle of Machine-Learning-Assisted Microfluidic Nanoplasmonic Digital Immunoassay.

Scheme 1 illustrates the sensing principle of the machine-learning-assisted microfluidic nanoplasmonic digital immunoassay for multiplex cytokine detection. Interleukin-1 beta (IL-1 β), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-10 (IL-10), tumor-necrosis-factor alpha (TNF- α), and interferon-gamma (IFN- γ) are selected as the target cytokines due to their abnormal expression levels in the serum of COVID-19 patients.^{1,3,4} The proposed immunoassay consists of three main components: a multicapture-antibody microarray chip for the microfluidic immunoassay (**Scheme 1a**), detection-antibody-conjugated 100 nm AgNCs (AgNC-Dab conjugates) for nanoplasmonic digital imaging (**Scheme 1b**), and a machine-learning-based image processing method for digital signal counting (**Scheme 1c**). The antibody microarray chip contains 8 parallel microfluidic sample channels (300 nL sample volume for each channel), which lie perpendicular to the 6 meandering capture antibody stripes with 6 turns on a glass substrate. This chip is thus designed to allow a 6-plex cytokine detection with 6 segments of parallel collocating antibody arrays in each channel and a total array of 288 square immunosensing areas (200 $\mu\text{m} \times 200 \mu\text{m}$) for the entire chip. It should be noted that a reference stripe of pristine 100 nm AgNCs is added in parallel to the capture antibody stripes for easy localization of the antibody microarrays under dark-field imaging and provision of reference signals. The microfluidic immunoassay is carried out using a rapid one-step sandwich format. The AgNC-Dab conjugates with the six cytokine detection antibodies are mixed with the target samples and then introduced into the microfluidic immunoassay system, forming six types of sandwiched immunocomplexes (*i.e.*, capture antibody/cytokine/detection-antibody-AgNC) on their corresponding capture antibody arrays (**Scheme 1a**). The 100 nm AgNCs are selected as the labeling reagent to provide the light-scattering signal for nanoplasmonic digital imaging (**Scheme 1b**) owing to the following four advantages: (i) they can be readily produced with ultrahigh purity and uniformity, ensuring good reproducibility of the immunoassay;^{35,36} (ii) they show excellent stability in assay buffers during the functionalization, reaction, and imaging processes, offering high robustness for the immunoassay;^{22,37} (iii) they can be readily modified with functional groups like -COOH through Ag-thiol bonding for antibody conjugations with tunable configurations;³⁷ (iv) most importantly, they could provide extremely intense light-scattering signals under dark-field imaging with superior signal-to-noise ratio, enabling record high sensitivity for the nanoplasmonic cytokine immunoassay.³³ The image of every capture antibody array (288 images in total) is taken using an electron-multiplying charge-coupled device (EMCCD) camera under a dark-field microscope, and the number of the captured AgNCs on the surface of each antibody array is counted using the machine-learning-based image processing method, which can be converted to the concentrations of target cytokines accordingly (**Scheme 1c**). The machine-learning-based image processing method is a customized MATLAB code developed using convolutional neural network (CNN) algorithm. The CNN method can process the dark-field image data automatically, distinguish the detection signals (*i.e.*, the scattering spots of AgNCs) from background noise precisely, and provide rapid analysis of the particle number of AgNCs with high statistical accuracy and throughput. Such an

efficient image processing method dramatically reduces the time required for data analysis and thus enables the practical application of the nanoplasmonic digital imaging technology for cytokine profiling.

Fabrication and Characterization of Microfluidic Nanoplasmonic Digital Immunoassay.

The multicapture-antibody microarray chip was fabricated using a microfluidic flow-patterning technique.⁹ Briefly, an amino-modified glass substrate was patterned with 6 different meandering capture antibody stripes using a polydimethylsiloxane (PDMS) mask layer *via* a glutaraldehyde-mediated conjugation protocol.³⁸ Subsequently, a sample-flow PDMS layer was attached onto the glass substrate perpendicularly to the 6 capture antibody stripes, resulting in a micromosaic immunoassay for the multicapture-antibody microarray chip (see **Methods** for details). **Figure 1** panels a and b show the atomic force microscopy (AFM) topographic images of the surfaces of the glass substrate before and after the antibody microarray fabrication. It can be observed that the surface of the glass substrate became rougher after the immobilization of capture antibodies, suggesting the presence of antibodies on the microarray surface. We also acquired the AFM phase images of the same region of the antibody microarray to further verify the surface functionalization. As shown in **Figure 1c,d**, the texture of the antibody microarray surface presented a uniformly distributed needle-like texture with a higher phase angle compared to that of a clean glass substrate, due to the much softer feature of antibodies compared with the glass.^{39,40} These results confirm the successful immobilization of uniformly distributed antibodies on the surface of the glass substrate for the multicapture-antibody microarray chip.

We then synthesized 100 nm AgNCs using a seed-mediated growth method (see **Methods** for details)³⁵ and functionalized them with the six different detection antibodies to produce the AgNC-Dab conjugates as the signal transducers. As shown by the transmission electron microscopy (TEM) images of the 100 nm AgNCs obtained from a batch of the standard synthesis (**Figure 1e** and **Figure S1** in **Supporting Information**), the AgNCs displayed a uniform size distribution and well-defined cubic shapes. The edge length of the AgNCs synthesized from this batch was measured to be 100.3 ± 4.8 nm by analysis of 300 randomly selected nanoparticles. Meanwhile, the edge length of the AgNCs synthesized from three different batches were analyzed to be 100.6 ± 5.4 nm (**Figures S1 and S2** in **Supporting Information**). The coefficient of variation of the edge length for the interbatch measurement ($n = 3$) was calculated to be as low as 5.4%, indicating that the AgNCs can be readily reproduced with high uniformity. In addition, the amount (4 mL at 0.4 nM) of 100 nm AgNCs obtained per batch of the standard synthesis could allow up to 5333 microfluidic nanoplasmonic digital immunoassays (3 μL of 0.1 nM per assay) as proposed ($(4 \text{ mL} \times 0.4 \text{ nM}) / (3 \mu\text{L} \times 0.1 \text{ nM}) = 5333$). The synthesis process could be scaled up by 10 times to produce a much larger quantity of the AgNCs per batch without compromising the sample quality (**Figure S3** in **Supporting Information**), making it possible for large-scale production of the digital immunoassay. The 100 nm AgNCs were deposited on a glass substrate for characterization under dark-field imaging, and their representative dark-field images and scattering spectra were taken and recorded as shown in **Figure 1f,g**, respectively. The AgNCs exhibited extremely strong light scattering with a major plasmonic scattering peak at ~ 568 nm. The scattering cross-section was calculated to be as high as $1.04 \times 10^5 \text{ nm}^2$ according to the finite element method (FEM) simulation (**Figure S4** in **Supporting Information**),

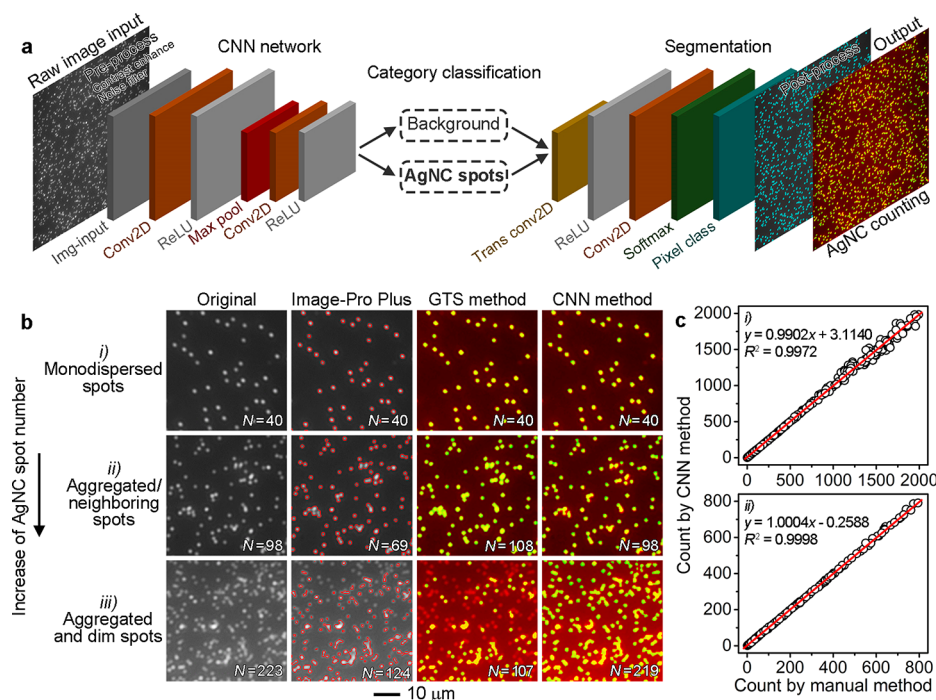


Figure 2. Evaluation of CNN method for counting particle numbers of AgNCs. (a) Schematics showing the algorithm architecture of the CNN method, where Img-input is the image input layer, Conv2D is the convolution 2D layer, ReLU is the rectified linear unit layer, Max pool is the max pooling 2D layer, Trans conv2D is the transposed convolution 2D layer, Softmax is the softmax layer, and Pixel class is the pixel classification layer. (b) Three representative images obtained from our digital immunoassay with AgNC spot number ranging from low to high and their corresponding image processing results obtained by Image-Pro Plus, GTS, and CNN methods. Note that (i) when the AgNC spot number was low, the spots were monodispersed on the image; (ii) with the increase of the AgNC spot number, some of the spots became aggregated; and (iii) at very high number of AgNCs, some of the spots became dim in addition to aggregated. (c) Correlation analysis between the CNN method and standard Image-Pro-Plus-assisted manual counting method in counting AgNC number in the ranges of 0–2,000 (i) and 0–800 (ii) from 288 test images.

which is the highest among various nanoscale optical materials with dimensions smaller than 100 nm.⁴¹ More importantly, each of the scattering spots under dark-field imaging (Figure 1f) represented one isolated AgNC, confirmed by the scanning electron microscopy (SEM) image of the same region (insets in Figure 1f). The AgNCs displayed similar scattering intensity and resonance spectra, indicating the successful synthesis of high-quality AgNCs with extremely intense and highly uniform single-particle scattering characteristics. Furthermore, the synthesized AgNCs can be stored in DI water at room temperature for at least 18 months without significant changes to the size, shape, and the plasmonic properties (Figure S5 in Supporting Information), revealing the high storage stability of our AgNCs. Following that, the as-synthesized AgNCs were modified with HS-PEG₃₄₀₀-COOH on their surfaces and then conjugated with anticytokine detection antibodies *via* EDC/NHS chemistry (see Methods for details).^{38,42} To verify the successful conjugation of the AgNC-DAb, we performed dynamic light scattering (DLS) to measure the hydrodynamic sizes of the AgNCs before and after the antibody functionalization, showing an evident increase from 136.7 to 157.9 nm (Figure 1h). The size increase suggests the presence of antibodies on the surface of the AgNCs as the mean size of common immunoglobulin antibodies is ~ 11 nm.⁴³ It is worth noting that the light-scattering behavior of AgNCs was very well preserved after the conjugation of detection antibodies (Figure S6 in Supporting Information) and can be stored for more than 6 months (Figure S7 in Supporting Information), devising the AgNC-DAb conjugates as the labeling reagent for the nano-

plasmonic immunoassay. Here, the 100 nm Ag nanocubes were intentionally employed in our sandwich nanoplasmonic digital biosensor instead of Au nanorods, which have been widely utilized in many previous reported label-free nanoplasmonic digital biosensors.^{24–29} This is mainly because of the completely two different sensing principles between these nanoplasmonic digital biosensors. Label-free nanoplasmonic biosensors rely primarily on the refractive index change surrounding the plasmonic nanomaterials before and after target binding. Au nanorods with an anisotropic shape exhibit a localized surface plasmon resonance (LSPR) that is highly sensitive to the local refractive index and thus are more suitable for label-free sensing. While our sandwich nanoplasmonic digital biosensor requires strong single-particle scattering signals from the captured plasmonic nanomaterials after the formation of the sandwich immune complexes. The 100 nm Ag nanocubes with a symmetric geometry and much larger scattering cross-section provide highly stable and intense scattering signals, rendering them the ideal plasmonic nanomaterials for high-quality single-particle imaging in our sandwich nanoplasmonic digital sensing.

To demonstrate the feasibility of the as-proposed microfluidic nanoplasmonic digital immunoassay, we evaluated the platform in response to a mixed cytokine solution containing IL-1 β , IL-2, IL-6, IL-10, TNF- α , and IFN- γ at a concentration of 100 pg mL⁻¹. The cytokine solution was mixed with the AgNC-DAb conjugates and then injected into the microfluidic sample channel for 60 min incubation. The images of the multiantibody arrays before and after the assay were obtained using a dark-field microscope. The antibody arrays showed a very clean back-

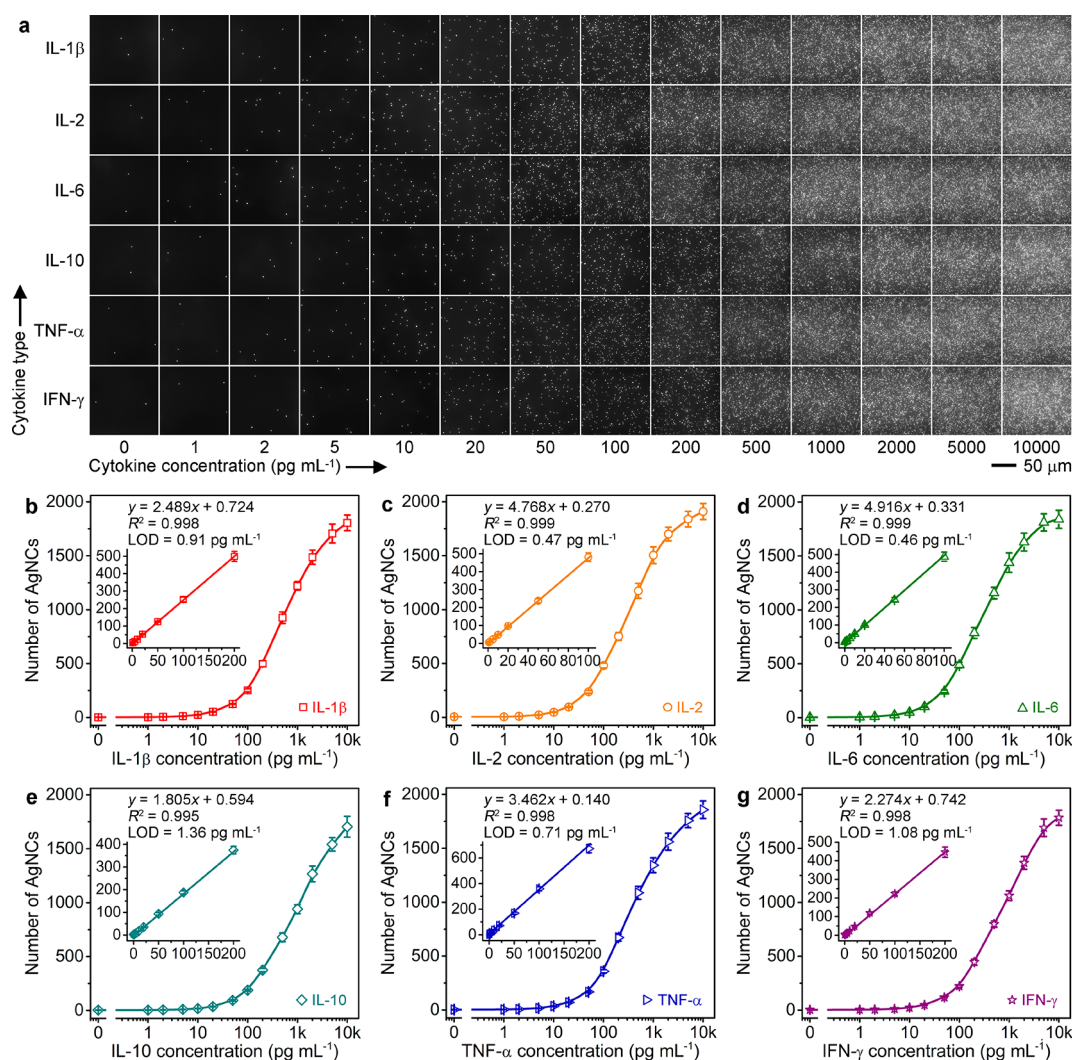


Figure 3. Detection of multicytokine standards using the machine-learning-assisted microfluidic nanoplasmonic digital immunoassay. (a) Dark-field images of antibody microarrays taken from the detection of multicytokine standards containing IL-1 β , IL-2, IL-6, IL-10, TNF- α , and IFN- γ with various concentrations in the range of 0–10,000 pg mL⁻¹. (b–g) Calibration curves of IL-1 β (b), IL-2 (c), IL-6 (d), IL-10 (e), TNF- α (f), and IFN- γ (g) generated by plotting the particle number of AgNCs against corresponding cytokine concentration. Insets show the corresponding linear regions of the calibration curves. Note that six images (six replicated sensing areas in one channel) were used for each concentration of each cytokine to establish the calibration curves.

ground with no scattering signals detected before sample loading (Figure 1i(i)). After the detection, numerous bright spots were observed on the surface of the multiantibody microarrays (Figure 1i(ii)), which originated from the single-particle light-scattering of the AgNCs due to the sandwich immunocomplex formation. The number of the scattering spots (*i.e.*, particle numbers of AgNCs) on the antibody microarrays of IL-1 β , IL-2, IL-6, IL-10, TNF- α , and IFN- γ were counted to be 251.0 ± 10.8 , 482.0 ± 19.1 , 490.2 ± 15.7 , 189.0 ± 7.3 , 361.8 ± 14.7 , and 221.2 ± 7.7 ($n = 6$), respectively (Figure 1j(ii)). In contrast, the spot numbers on the microarrays for the detection of blank cytokine solution (0 pg mL⁻¹) were counted to be 2.0 ± 0.9 , 2.8 ± 0.8 , 2.5 ± 0.5 , 2.0 ± 0.6 , 2.3 ± 0.8 , and 2.2 ± 0.4 ($n = 6$), respectively (Figure 1i(iii),j(iii)), suggesting negligible nonspecific signals for the immunoassay.

Machine-Learning-Based Image Processing Method for Nanoplasmonic Digital Counting. To achieve rapid and high-throughput cytokine detection for real sample measurement, the nanoplasmonic digital immunoassay ultimately requires a simple, accurate, and ultrafast image processing

method that can automatically identify and count the captured AgNCs on the 288 multiantibody microarrays per chip. More importantly, the signal counting process needs to clearly distinguish the detection signals (*i.e.*, the scattering spots of AgNCs) from false signals/background noise (including aggregated/neighboring spots) and acquire the desired readout in a large scattering intensity variance (including bright and dim spots). Manual counting of the particle number of AgNCs on each image by human eyes is cumbersome and time-consuming, apparently impractical for high-throughput imaging analysis. Commercial software (*e.g.*, ImageJ and Image-Pro Plus) and conventional image processing methods (*e.g.*, global thresholding and segmentation (GTS)) are limited mainly by poor accuracy in recognizing the aggregated/neighboring and dim spots on the dark-field images, yielding a low signal-to-noise ratio in the immunoassay. To address this issue, we developed a machine-learning-based image processing method using convolutional neural network (CNN) visualization for particle counting (named “CNN method”).^{44–46} Figure 2a shows the algorithm architecture of the CNN method. It involves dark-

field image data read-in/preprocessing (including noise filtering and contrast enhancement), detection signal/background image segmentation by pretrained CNN, postprocessing, and result output. CNN as the key component was pretrained to classify and segment image pixels by labels of the detection and background signals (see [Methods](#) for details). [Figure 2b](#) shows three representative images acquired by our digital immunoassay with AgNC spot numbers ranging from low to high levels, and the corresponding image processing results obtained by Image-Pro Plus, GTS, and CNN methods. As indicated by [Figure 2b](#), all the three methods could accurately count the detection signals for the monodispersed bright spots at a low AgNC count. However, Image-Pro Plus and GTS methods fall short in precise image labeling and accurate signal counting for the aggregated/neighborly and dim spots compared to the CNN method, especially at high AgNC counts. To further verify the accuracy of our CNN method, we analyzed 288 test images containing different particle numbers of AgNCs in the range of 0–2,000. It should be noted that these images were different from those used for CNN training. The obtained counting results were compared with those analyzed by Image-Pro-Plus-assisted manual counting as the benchmark. As shown in [Figure 2c\(i\)](#), the AgNC counts from the two methods display an excellent linear regression with a slope of 0.9902, a small intercept of 3.1140, and a coefficient of determination of $R^2 = 0.9972$. It is worth pointing out that the accuracy of our CNN-based method could be further improved with the AgNC number in the range of 0–800 (Slope = 1.0004, Intercept = −0.2588, and $R^2 = 0.9998$; [Figure 2c\(ii\)](#)), which was later determined to be the linear range of detection for our digital immunoassay. Moreover, the CNN image processing shows excellent accuracy and consistency for analysis of images acquired from different experiments performed on different days ([Figure S8](#)), indicating the high robustness of our CNN method. Notably, it only took ~30 s to process a total of 288 images obtained from one immunoassay chip and produce the corresponding detection results (CPU: AMD Ryzen 5 1600 Six-Core Processor) using the customized MATLAB code. In contrast, the Image-Pro-Plus-assisted manual counting method requires up to ~6,000 times as long (~50 h) to accomplish the same task with much more complicated procedures.

In addition, as seen from the dark-field images in [Figure 2b](#) and many other dark-field images in this study (especially those with high densities of AgNC scattering spots), the spots in certain regions were much bigger and brighter than other “normal” spots (spots in average), and some of them were in irregular shapes in comparison to those with rounded shapes. These phenomena could be ascribed to the known Abbe diffraction limit, where a diffuse spot can be formed when two objects are in close proximity (distance shorter than ~200 nm).^{47,48} Therefore, those bigger, brighter, and noncircular spots might consist of multiple AgNC particles, resulting in underestimation of the AgNC counting in the images. With the advancement in super-resolution nanoplasmonic imaging³⁰ and enhanced CNN algorithm for in-depth information on spot brightness and size, we believe the CNN image processing method could break the fundamental limit and become an enabling method for simple, rapid, accurate, robust, and high-throughput digital counting in nanoplasmonic digital immunoassay and many other imaging analysis applications.

Analytical Performance of Machine-Learning-Assisted Microfluidic Nanoplasmonic Digital Immunoassay. Prior to evaluation of the analytical performance of the

immunoassay, the experimental procedures were optimized to inject a concentration of 0.1 nM for AgNC-DAb conjugates and incubate with target analytes for 60 min for thorough antigen–antibody reaction ([Figure S9](#) in [Supporting Information](#)). The evaluation was then carried out using the following standard detection procedures: (i) the multiantibody microarray chip was incubated with a mixture containing multicytokine standards (or samples) and AgNC-DAb conjugates at room temperature for 60 min; (ii) after the incubation and washing, the images of multiantibody microarrays were recorded using a dark-field microscope and then analyzed by the CNN-based image-processing algorithm (see [Methods](#) for details). We evaluated the analytical performance including the sensitivity, sensing dynamic range, reproducibility, specificity, and cross-reactivity for the nanoplasmonic digital immunoassay.

We first determined the sensitivity and dynamic range of the immunoassay by analyzing multicytokine standards containing IL-1 β , IL-2, IL-6, IL-10, TNF- α , and IFN- γ at different concentrations. [Figure 3a](#) shows the dark-field images of the final multiantibody microarrays for detection of multicytokine standards ranging in concentration from 0 to 10,000 pg mL^{−1}. It can be seen that for each type of cytokine, the number of scattering spots of AgNCs on the multiantibody microarray increased as the cytokine concentration increased. By plotting the particle number of AgNCs as the detection signal against the cytokine concentration, calibration curves for the six target cytokines were established ([Figure 3b–g](#)). We can clearly and distinctly differentiate positive detection signals for all six cytokines in the concentration range of 1–10,000 pg mL^{−1} (4 orders of magnitude), suggesting a wide response range of the immunoassay for multiplex cytokine detection. As indicated by the insets of [Figure 3b–g](#), high-quality linear relationships were observed in the ranges of 1–200 pg mL^{−1} for IL-1 β ($R^2 = 0.998$), 1–100 pg mL^{−1} for IL-2 ($R^2 = 0.999$), 1–100 pg mL^{−1} for IL-6 ($R^2 = 0.999$), 1–200 pg mL^{−1} for IL-10 ($R^2 = 0.995$), 1–200 pg mL^{−1} for TNF- α ($R^2 = 0.998$), and 1–200 pg mL^{−1} for IFN- γ ($R^2 = 0.998$). Here, different slopes of the calibration linear curves (insets in [Figure 3b–g](#), 2.489 mL pg^{−1} for IL-1 β , 4.768 mL pg^{−1} for IL-2, 4.916 mL pg^{−1} for IL-6, 1.805 mL pg^{−1} for IL-10, 3.462 mL pg^{−1} for TNF- α , and 2.274 mL pg^{−1} for IFN- γ) were obtained that could be explained by the difference in cytokine-antibody binding affinities and the cytokines' molar masses. The higher cytokine-antibody affinities could allow more binding for the immune sandwich formation, leading to more AgNC scattering spots on the sensing arrays and thus steeper slopes in the calibration linear curves, while for cytokines with lower molar masses, larger binding possibilities driven by the higher molar concentrations (under the same mass concentration) are anticipated, resulting in more AgNC counting and corresponding slope difference. By comparing the slopes of the calibration curves (Slope_{IL-6} > Slope_{IL-2} > Slope_{TNF- α} > Slope_{IL-1 β} > Slope_{IFN- γ} > Slope_{IL-10}) and the molar masses of the cytokines (M_{IL-2} (15.5 kDa) < $M_{IFN- $\gamma$$ (16.7 kDa) < $M_{IL-1 $\beta$$ (17.3 kDa) < $M_{TNF- α (17.4 kDa) < M_{IL-10} (18.6 kDa) < M_{IL-6} (20.9 kDa)), we did not observe a strong correlation between these two factors, indicating the cytokine molar mass effect on the calibration slope values could be ruled out.$

The limits of detection (LODs) for the six cytokines were determined to be 0.91 pg mL^{−1} (IL-1 β), 0.47 pg mL^{−1} (IL-2), 0.46 pg mL^{−1} (IL-6), 1.36 pg mL^{−1} (IL-10), 0.71 pg mL^{−1} (TNF- α), and 1.08 pg mL^{−1} (IFN- γ) based on the $3\sigma/k_{\text{slope}}$, where σ and k_{slope} are the standard deviation of background signal acquired from a blank control ($n = 6$) and the regression

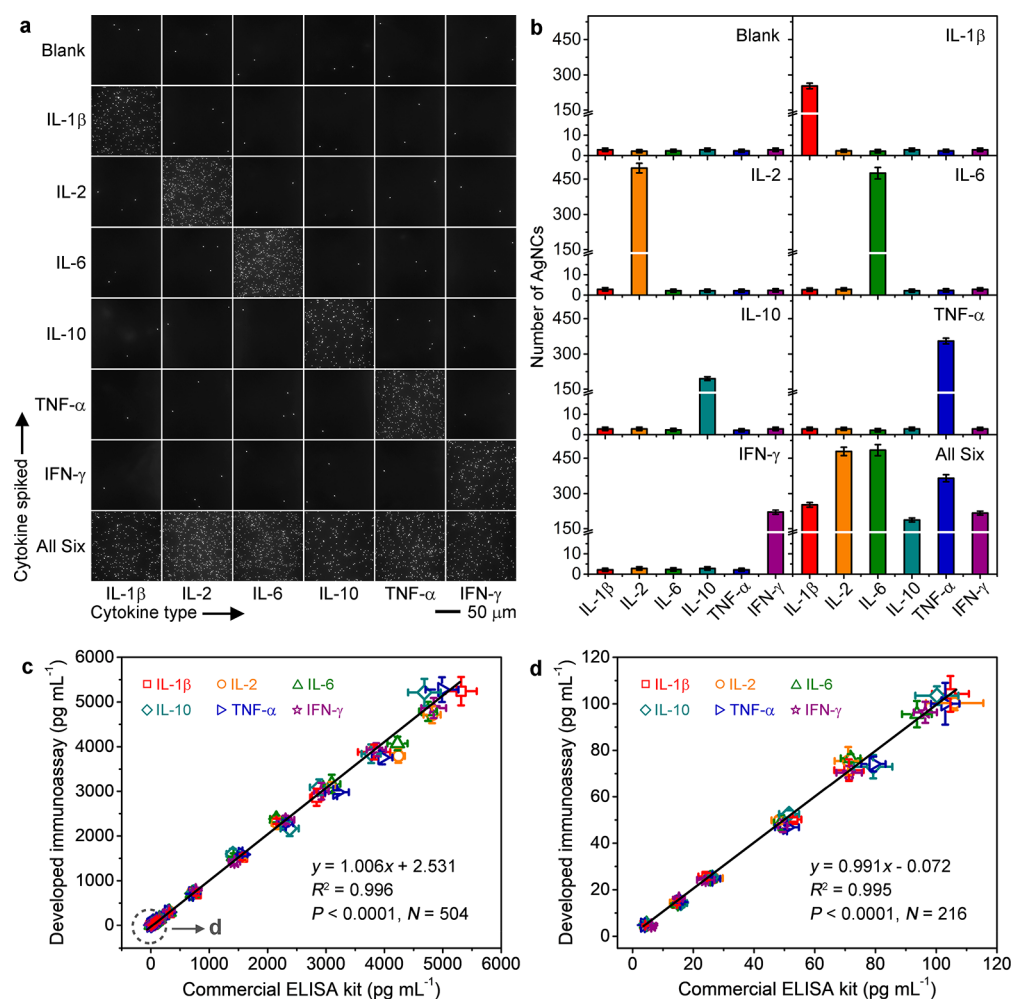


Figure 4. Detection of multicytokines spiked in negative human serum matrix using the machine-learning-assisted microfluidic nanoplasmonic digital immunoassay. (a) Dark-field images of antibody microarrays taken from the detection of human serum matrix spiked with different mixtures of cytokines. The concentration of each cytokine spiked in the human serum matrix is 100 pg mL $^{-1}$. (b) Bar graph showing the intensity of the detection signal (i.e., particle number of AgNCs) obtained from (a). (c, d) Correlation analysis between the developed machine-learning-assisted microfluidic nanoplasmonic digital immunoassay and commercial ELISA kit in quantifying cytokines from human serum matrix spiked with different concentrations of cytokines in the ranges of 5–5,000 pg mL $^{-1}$ (c) and 5–100 pg mL $^{-1}$ (d).

slope of each linear curve, respectively. The determined sensing dynamic ranges for the target cytokines by our multiplex immunoassay are one order magnitude wider than the standard single-plex cytokine ELISA method (dynamic ranges: 5–5,000 pg mL $^{-1}$ for all six cytokines, Figure S10 in Supporting Information). The achieved LODs down to the sub pg mL $^{-1}$ represent the highest class of sensitivity in nanoplasmonic biosensors for multiplex cytokine detection without signal amplification. Since the serum cytokine concentrations in COVID-19 severe patients span across the dynamic ranges of 1–10,000 pg mL $^{-1}$ including 0.5–130 pg mL $^{-1}$ for IL-1 β , 1–18 pg mL $^{-1}$ for IL-2, 1–10,000 pg mL $^{-1}$ for IL-6, 1–20 pg mL $^{-1}$ for IL-10, 1–1,000 pg mL $^{-1}$ for TNF- α , and 4–80 pg mL $^{-1}$ for IFN- γ ,^{1,49} our immunoassay provides a well suitable sensing dynamic range and LODs for profiling the serum cytokines in COVID-19 patients.

To highlight the advantages of our CNN method on digital counting in the immunoassay, we compared the sensing dynamic ranges and LODs of the immunoassay using the CNN method and the Image-Pro Plus method. Figure S11 shows the calibration curves of the immunoassay for the six target cytokines obtained from the Image-Pro Plus method. The

sensing dynamic ranges of the immunoassay using Image-Pro Plus method were determined to be 1–5,000 pg mL $^{-1}$ for IL-1 β , 1–1,000 pg mL $^{-1}$ for IL-2, 1–2,000 pg mL $^{-1}$ for IL-6, 1–5,000 pg mL $^{-1}$ for IL-10, 1–2,000 pg mL $^{-1}$ for TNF- α , and 1–5,000 pg mL $^{-1}$ for IFN- γ , which were 2–10-fold narrower than those using the CNN method. The calculated LODs of the immunoassays by Image-Pro Plus method (1.01 pg mL $^{-1}$ for IL-1 β , 0.51 pg mL $^{-1}$ for IL-2, 0.50 pg mL $^{-1}$ for IL-6, 1.44 pg mL $^{-1}$ for IL-10, 0.83 pg mL $^{-1}$ for TNF- α , and 1.16 pg mL $^{-1}$ for IFN- γ) were also compromised as the Image-Pro Plus method cannot provide accurate counting on the aggregated/neighbor-ing and dim spots, leading to the underestimation and miscounting of the AgNCs, especially at high AgNC counts. These results further demonstrate the advantages of our CNN method in accurate and robust digital counting that enhances the analytical performance of the nanoplasmonic digital immunoassay.

The reproducibility of our digital immunoassay was studied by replicate determinations on three different multicytokine standards with low, medium, and high concentrations (10, 100, and 1,000 pg mL $^{-1}$) using the same and different batches of multiantibody microarray chips and AgNC-Dab conjugates.

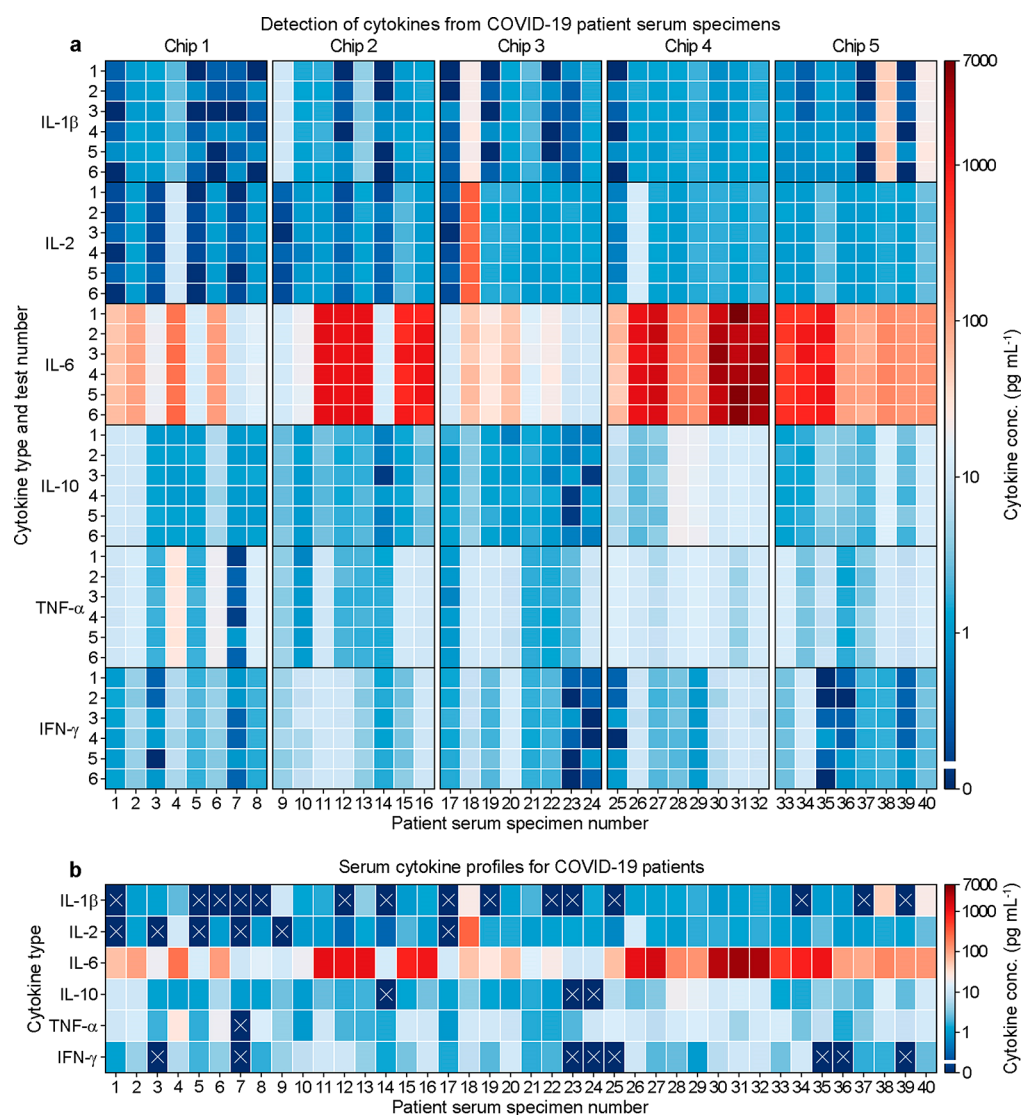


Figure 5. Application of the machine-learning-assisted microfluidic nanoplasmonic digital immunoassay for profiling the serum cytokines from COVID-19 patients. (a) Heatmap showing the detection results of cytokines from 40 COVID-19 patient serum samples. Each of the six cytokines in the specimens was detected simultaneously in six repeats with a total of 1440 tests over 5 microfluidic immunochips. (b) Heatmap showing the summary of the serum cytokine profiles obtained from 40 COVID-19 patient serum specimens. The cytokine levels were based on the average value of six replicate tests. Scales indicate value of cytokine concentration. Symbol “X” as “not applicable” represents that the cytokine concentration is lower than the LOD of our immunoassay.

The evaluation was based on the calculation of intra- and interbatch coefficients of variation (CVs, $n = 6$). Experimental results show that the intra- and interbatch CVs using the same and different batches of the chips and conjugates were in the ranges of 3.27–8.87% and 5.14–10.8%, respectively, for all six cytokines at 10, 100, and 1,000 pg mL⁻¹. The low CVs suggest excellent reproducibility and repeatability of the nanoplasmonic digital immunoassay for large scale production toward clinical usage.

From a clinical perspective, another key issue for practical application of a multiplex cytokine immunoassay is the specificity and cross-reactivity, considering that human serum is an inherently complex, multicomponent mixture. To evaluate the specificity and cross-reactivity of our multiplex cytokine immunoassay, we conducted three control tests as follows: (i) a negative human serum sample without target cytokines (*i.e.*, heat-inactivated and sterile-filtered human serum); (ii) positive serum samples containing only single analyte (negative human

serum spiked with IL-1 β , IL-2, IL-6, IL-10, TNF- α , or IFN- γ at a concentration of 100 pg mL⁻¹); (iii) positive serum samples containing all six target cytokines (negative human serum spiked with all the six cytokines at a concentration of 100 pg mL⁻¹). In all three control tests, only with the presence of target cytokines in the serum samples can we observe a noticeable number of AgNCs on the corresponding microarrays (Figure 4a,b). The measured particle numbers of AgNCs were further converted to analyte concentrations according to the calibration curves in Figure 3. As seen in Figure S12 in Supporting Information, the back-calculated cytokine concentrations correlated very well with their corresponding values (100 pg mL⁻¹), showing negligible cross-reactivity of the nanoplasmonic immunoassay for multiplex detection of six cytokines. The minimal cross-reactivity and background noise further confirm that other biocomponents coexisting in the human serum did not interfere with the immune sandwich formation, suggesting high

specificity of the multiplex immunoassay for target cytokine determination.

It should be emphasized that such excellent analytical performance is attributed to the seamless integration of the microfluidic immunoassay platform, the nanoplasmonic digital imaging technology, and the machine-learning-based image processing method. The synergistic combination of the three techniques into an integrated biosensor allows highly sensitive, accurate cytokine profiling in a high-throughput, multiplex manner, showing significant advantages over many existing cytokine biosensing techniques developed toward clinical applications.⁵⁰

Analysis of Human Serum Samples and Method Validation. Wide acceptance of an immunoassay requires its full validation in real sample detection scenarios. To demonstrate the potential practical application of our immunoassay in clinical diagnosis, we utilized our immunoassay to analyze 16 cytokine-spiked human serum samples and validated the results with the existing gold-standard method, *i.e.*, ELISA. These human serum samples were prepared by spiking a negative human serum sample with a mixture of all the six cytokines at 16 different concentrations ranging across the entire dynamic range of our immunoassay. Cytokines in each sample were quantified based on the calibration curves shown in Figure 3, and the obtained results were compared with references measured by commercially available cytokine ELISA kits. The comparison was performed using linear regression analysis between the two methods (Figure 4c,d). A strong positive correlation ($R^2 = 0.996$) was found between the measured cytokine concentrations by the developed immunoassay and the reference ELISA with a slope of 1.006 and an intercept of 2.531 (Figure 4c). It is worth mentioning that in a lower cytokine concentration range ($5\text{--}100\text{ pg mL}^{-1}$), the measured cytokine concentrations by the developed immunoassay still showed excellent agreement with those by ELISA (Slope = 0.991, Intercept = -0.072 , and $R^2 = 0.995$; Figure 4d). These results further demonstrated the high accuracy and reliability of our immunoassay in analyzing complex human serum samples even at low cytokine concentrations, implying its great potential in cytokine storm profiling in COVID-19 patients.

Application in Serum Cytokine Profiles and Cytokine Storm Monitoring for COVID-19 Patients. Finally, we applied the developed immunoassay for measuring serum cytokines in COVID-19 patients and utilized the cytokine profiles to assist the screening of high-risk patients with cytokine storms. To demonstrate the clinical usage of the nanoplasmonic digital immunoassay, we conducted simultaneous detection of 40 human serum specimens of COVID-19 severe patients collected from the University of Michigan Hospital (patients or their authorized representatives provided informed consent for the use of biospecimens, as approved by the Institutional Review Board of the University of Michigan (HUM000179668)). These COVID-19 patients were determined to be critically ill as they were experiencing respiratory failure symptoms and receiving hospitalization in the intensive care unit (ICU) for either mechanical ventilation or extracorporeal membrane oxygenation (ECMO). Within only 5 h, we completed six replicate detections of the six target cytokines in the 40 human serum specimens by performing a total of 1440 tests on 5 microfluidic immunochips. The detection results obtained from the 1440 tests were shown in Figure 5a, manifesting the simplicity, speed, high multiplex, and high-throughput features of our immunoassay in performing such massively parallel testing. The

concentrations of the six cytokines in these COVID-19 patient serum samples were calculated by averaging the detection results of the six replicate tests, and their serum cytokine profiles were summarized in Figure 5b accordingly. The serum cytokine profiles for the COVID-19 patients show a highly diverse range of $0.57\text{--}5,497.25\text{ pg mL}^{-1}$ with a few abnormally elevated cytokine secretions. Specifically, the serum sample Nos. 1–25 were collected from the COVID-19 patients without any treatments, showing serum cytokine concentrations of $1.32\text{--}42.23\text{ pg mL}^{-1}$ for IL-1 β , $0.57\text{--}514.27\text{ pg mL}^{-1}$ for IL-2, $10.71\text{--}1,732.71\text{ pg mL}^{-1}$ for IL-6, $1.43\text{--}11.95\text{ pg mL}^{-1}$ for IL-10, $1.11\text{--}48.92\text{ pg mL}^{-1}$ for TNF- α , and $1.73\text{--}16.60\text{ pg mL}^{-1}$ for IFN- γ . According to the previously reported average cytokine levels in healthy donors (IL-1 β , $\sim 0.4\text{ pg mL}^{-1}$; IL-2, $\sim 1.1\text{ pg mL}^{-1}$; IL-6, $\sim 1.7\text{ pg mL}^{-1}$; IL-10, $\sim 1.7\text{ pg mL}^{-1}$; TNF- α , $\sim 7.4\text{ pg mL}^{-1}$; IFN- γ , $\sim 0.5\text{ pg mL}^{-1}$),^{1,49} we observed that all 25 serum samples from the COVID-19 severe patients contained abnormally high levels of IL-6, while 52%, 60%, 64%, 52%, and 80% of the patients showed elevated secretion of IL-1 β , IL-2, IL-10, TNF- α , and IFN- γ , respectively. The observed elevated expressions of pro-inflammatory cytokines (*e.g.*, IL-6, IL-1 β , IL-2, TNF- α , and IFN- γ) and anti-inflammatory cytokines (*e.g.*, IL-10) in these COVID-19 patients were broadly consistent with recent studies.^{1,4,49,51,52} The elevated serum cytokine levels indicate that the majority of these COVID-19 patients were likely experiencing cytokine storms,^{1,4,49} who would be at high risk for developing acute respiratory distress and require guided therapies to alleviate this inflammatory state and improve prognosis.^{51,52} The serum sample Nos. 26–35 and Nos. 36–40 were collected from the COVID-19 patients with treatments of tocilizumab and a selective cytopheretic device, respectively. Significantly higher cytokine expressions (*e.g.*, IL-6 ($206.60\text{--}5,497.25\text{ pg mL}^{-1}$)) were observed especially in patients right after receiving the immunomodulatory treatment of tocilizumab (sample Nos. 26–35). The elevated serum IL-6 could be mainly attributed to the initial binding of tocilizumab to the IL-6 receptor that inhibits the receptor-mediated clearance, suggesting the importance of dynamic observation of cytokine profiles in understanding the patient response to the immunomodulatory treatment.^{53,54} The measured diverse serum cytokine profiles (from sub pg mL^{-1} to several ng mL^{-1}) in different patients further demonstrated the high sensitivity, accuracy, multiplexity, and wide dynamic range of our immunoassay in complicated serum cytokine analysis. Such an enabling technology would allow precise and timely monitoring of inflammatory response to reveal the cytokine storm features and immune status in COVID-19 patients, which could facilitate the therapeutic stratification and guide clinicians to treat COVID-19 patients more effectively.

CONCLUSIONS

In conclusion, we have demonstrated a high-performance machine-learning-assisted microfluidic nanoplasmonic digital immunoassay that enables high-throughput detection of multiple immune biomarkers in a rapid, sensitive, selective, accurate, and easy-to-implement manner. The key to the success of our strategy can be primarily ascribed to the systematic combination of the high-throughput and multiplex microfluidic immunoassay platform, ultrasensitive nanoplasmonic digital imaging technology, and rapid and accurate machine-learning-based image processing method. Owing to the synergistic effects of the advantages of these technologies, our one-step immunoassay allows simultaneous detection of six cytokines in sextuplicate of

8 different samples (3 μL each) per chip, with a record high detection limit of sub pg mL^{-1} , a wide linear dynamic range of 4 orders of magnitude, and a total assay time under 100 min. The clinical application of such a high-performance immunoassay has been successfully demonstrated in analysis of cytokine profiles using serum samples from COVID-19 patients, showing high accuracy and reliability in comparison to the commercial gold-standard ELISA. We believe that the presented immunoassay is a promising approach to allow continuous characterization of cytokine storms and thus provides timely and reliable information to optimize care for COVID-19 patients. The developed immunoassay can be readily expanded to a highly multiplex (>20) cytokine detection platform for comprehensive immune status analysis of patients in a clinical setting. Through integration with hand-held imaging systems and/or paper testing strips, the nanoplasmonic digital immunoassay can be further exploited as a point-of-care (POC) detection platform for cytokines and provide clinical significance in disease early diagnosis, management, and prevention.

METHODS

Fabrication of Multi-Capture-Antibody Microarray Chip. The multicapture-antibody microarray chip was fabricated using a microfluidic patterning technique⁹ through a glutaraldehyde-mediated conjugation protocol.³⁸ Briefly, a cleaned glass substrate was first functionalized with (3-aminopropyl)triethoxysilane (APTES) by a concentrated vapor-phase deposition method⁵⁵ to generate an amino-terminated silane monolayer on the glass surface. Subsequently, the amino-silanized glass substrate was temporarily bonded with an antibody-patterning PDMS mask layer containing six parallel meandering microfluidic channels. The amino-silanized glass surfaces in the six microfluidic channels were modified with six different types of anticytokine capture antibodies using glutaraldehyde-mediated conjugation chemistry.³⁸ Finally, the antibody-patterning microfluidic PDMS mask layer was replaced with a sample-flow PDMS mask layer with 8 microfluidic channels. The microfluidic channels of the sample-flow PDMS layer were placed perpendicularly to the capture antibody stripes. This fabrication process generated 288 sensing arrays (200 $\mu\text{m} \times 200 \mu\text{m}$) on the surface of the chip, including 8 sample channels and 6 segments of 6 collocating parallel multiplex antibody arrays in each channel (detailed fabrication procedures can be found in [Supporting Information](#)).

Synthesis of the 100 nm AgNCs. The AgNCs of ~ 100 nm in edge length were synthesized using a reported seed-mediated growth procedure with minor modifications,³⁵ where AgNCs of ~ 42 nm in edge length served as the seeds. Initially, the ~ 42 nm AgNC seeds were prepared by the reduction of AgNO_3 with ethylene glycol (EG) in the presence of poly(vinylpyrrolidone) (PVP) using a one-pot synthesis method³⁶ and stored in 4.0 mL EG (~ 8.1 nM in particle concentration, detailed preparation procedures can be found in [Supporting Information](#)). Then, 3.0 mL of EG was added into a glass vial and preheated to 150 $^\circ\text{C}$ under magnetic stirring. After 25 min of preheating, 0.9 mL of 20 mg mL^{-1} PVP solution in EG, 0.2 mL of the ~ 42 nm AgNC seeds in EG, and 0.6 mL of 36 mg mL^{-1} AgNO_3 solution in EG were pipetted into the vial sequentially. The ~ 100 nm AgNCs were obtained by cooling the reaction solution with an ice-water bath after the reaction had proceeded for ~ 90 min with the major extinction peak of the reaction solution reaching ~ 585 nm.⁵⁶ After being washed once with acetone and then ultrapure water *via* centrifugation, the ~ 100 nm AgNCs were stored in 4.0 mL of ultrapure water for future use (~ 0.4 nM in particle concentration).

Preparation of Anti-Cytokine Detection Antibody Conjugated 100 nm AgNCs (AgNC-DAB Conjugates). The six AgNC-DAB conjugates were prepared by conjugating 100 nm AgNCs with six types of anticytokine detection antibodies using HS-PEG₃₄₀₀-COOH and EDC/NHS as the linker and the coupling agents, respectively.^{38,42} In brief, 400 μL of 10 mg mL^{-1} HS-PEG₃₄₀₀-COOH aqueous solution

was mixed with 100 μL of the as-synthesized 100 nm AgNCs (0.4 nM), followed by incubation at room temperature for 5 h under gentle shaking. The mixture was then centrifuged at 1,000 rpm for 10 min, and the obtained precipitates (*i.e.*, AgNC-S-PEG₃₄₀₀-COOH) were washed twice with ultrapure water and dispersed in 300 μL of 10 mM phosphate-buffered saline (PBS, pH 7.4). Subsequently, 100 μL of 25 mM EDC aqueous solution and 100 μL of 50 mM NHS aqueous solution were simultaneously added into the AgNC-S-PEG₃₄₀₀-COOH suspension. After incubation at room temperature for 20 min under gentle shaking, the nanoparticles (*i.e.*, EDC/NHS-activated AgNC-S-PEG₃₄₀₀-COOH) were washed three times with ultrapure water and dispersed in 400 μL of PBS. 100 μL of anticytokine detection antibody solution (at a concentration of 200 $\mu\text{g mL}^{-1}$) was added into the nanoparticle suspension and incubated at 4 $^\circ\text{C}$ overnight with gentle shaking. Thereafter, 200 μL of 10% BSA in PBS was added into the nanoparticle suspension and incubated at room temperature for 60 min. After centrifugation, the products (*i.e.*, AgNC-DAB conjugates) were dispersed in 100 μL of PBS containing 0.05% Tween 20, 1% BSA, and 0.02% NaN_3 and were finally stored at 4 $^\circ\text{C}$ for future use (0.4 nM).

Training of the Convolutional Neural Network (CNN). The architecture of the CNN contains a downsampling process from a 512×512 dark-field AgNC image for category classification (nanoparticles as class "1", background as class "0") and an upsampling process for nanoparticle segmentation. The downsampling process consists of 6 layers, including an image input layer, two convolution 2D layers (Conv2D, 6 filters, kernel of 3×3), two rectified linear unit layers (ReLU), and one max-pooling layer (stride of 2). The upsampling process consists of 5 layers, including one transposed convolution 2D layer (Trans Conv2D), one ReLU layer, one Conv2D layer, one softmax layer, and one pixel classification layer which contains class weight balance.

The training of the CNN involves data set labeling, class weight balance, network training, and result evaluation. [Figure S13](#) shows the schematic of the data annotation workflow for training data set labeling. For the training data set, we selected 252 representative dark-field images that covered a wide range of cytokine concentrations from assay blank to 0 pg mL^{-1} to 10,000 pg mL^{-1} . To enhance the data set labeling speed, we wrote a thresholding algorithm that computed a global threshold T from a grayscale image, using Otsu's method.⁵⁷ Using this global threshold together with the standard image erosions and dilations, we labeled the recognized AgNC spot pixel as class "1" and the background pixel as class "0" to generate a binary image mask called "Labeled image". Note that this labeled image was still preliminary and failed to detect the AgNC spots that had large intensity variance or that were aggregated. We then used several correction algorithms to manually add or remove the falsely recognized area. We used a 5×5 pixel unit size for normalizing the labeling of all the recognized AgNC spots including individual, aggregated, bright, and dim spots. In addition to the average individual spots, this normalization labeling process would not only help the neural network to recognize the very deem spots but also be able to separate the aggregated spots with strong intensity comparing to the global thresholding method (Otsu's method). The human corrected "Labeled image" was later used to train the neural network. In addition, we considered the class weight balance using the inverse frequency weighting method which gives more weight to less frequently appearing classes (AgNC class). The class weight was defined as

$$\text{class weight} = \frac{N_{\text{image total pixels}}}{N_{\text{class pixels}}}$$

where $N_{\text{image total pixels}}$ is the number of total image pixels of $512 \times 512 = 262,144$, and the $N_{\text{class pixels}}$ is the number of pixels for each class. This class weighting strategy was added into the neural network training process to enhance the pixel identification accuracy because the number of AgNC class pixels was significantly smaller than the number of background pixels. For the network training options, we used the stochastic gradient descent with momentum (SGDM) algorithm to minimize the loss function and set the total epoch number to be 200 with 20 samples for each mini-batch. We then selected four candidate

networks with training accuracy above 98% and further evaluated their performance using 126 well-selected test images and then finalized the currently used CNN. Finally, the pretrained CNN algorithm was integrated into the developed CNN-based image processing method and applied to recognize the entire area of AgNC spots in an image. The AgNC spot number (*i.e.*, particle number) in the image as the final output result was simply calculated by area-size sorting, which is equal to the recognized AgNC spot area divided by the 5×5 pixel unit size.

Standard Procedure for the Detection of Multiple Cytokines Using Machine Learning-Assisted Microfluidic Nanoplasmonic Digital Immunoassay. Prior to detection, multicytokine standards with various concentrations (ranging from 0 to 10,000 pg mL⁻¹) were prepared by dissolving IL-1 β , IL-2, IL-6, IL-10, TNF- α , and IFN- γ together in PBS containing 0.05% Tween 20 and 1% BSA (dilution buffer), and a mixed AgNC-DAB conjugate solution (0.1 nM) was prepared by dissolving the same amounts of the as-prepared six types of AgNC-DAB conjugates together in dilution buffer. In a standard detection procedure, 3 μ L of the multicytokine standards or human serum samples was mixed with 3 μ L of the AgNC-DAB conjugate mixture (0.1 nM) and loaded into the multiantibody microarray chip. After incubation at room temperature for 60 min, the channels were washed with PBST. The images of the antibody arrays in each channel were semiautomatically taken using a dark-field LSPR microscope for ~ 25 min to complete the acquisition of the 288 images (please see the [Supporting Information](#) for the detailed microscope configuration). Note that 100 nm AgNC reference arrays in each channel were used to locate the regions of antibody arrays. The number of AgNCs on each image was recorded as the detection signal using the CNN-based image processing method and took ~ 30 s to process the 288 images. A view area of $138 \mu\text{m} \times 138 \mu\text{m}$ (center part) from the $200 \mu\text{m} \times 200 \mu\text{m}$ antibody arrays was captured as the detection results for analysis to ensure a high quality of obtained single-particle nanoplasmonic scattering images and to avoid the background scattering signals and interference from the PDMS layer along the edge of the microfluidic channel.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsnano.1c06623>.

Detailed experimental description; TEM images, dark-field image, and simulated scattering cross-section spectrum of 100 nm AgNCs; dark-field images, SEM images, and scattering spectrum of 100 nm AgNC-DAB conjugates; correlation analyses between the CNN method and Image-Pro-Plus-assisted manual counting method; optimization of experimental conditions; calibration curves of cytokines using commercial ELISA kits; calibration curves of cytokines for the developed immunoassay using Image-Pro Plus method for AgNC number counting; quantification of cytokine concentrations in human serum samples; data annotation workflow for labeling training images; and 3D models and photographs of the antibody-patterning PDMS mask layer and sample-flow PDMS mask layer ([PDF](#))

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Author Contributions

Z. Gao conducted all the experiments and wrote the manuscript. Y. Song contributed to the development of machine-learning-based image processing method. T. Y. Hsiao assisted in all the experiments. J. He assisted in the preparation of microfluidic chips. C. Wang performed the SEM characterization. J. Shen and S. Dai performed the AFM characterization. A. MacLachlan assisted in the manuscript writing. B. H. Singer provided guidance for detection of human serum specimens. K. Kurabayashi and P. Chen provided constructive guidance for the overall design and direction of the experiments and edited the manuscript. All authors contributed to the data analysis and reviewed the manuscript.

Notes

The authors declare no competing financial interest.

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