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Plasmonic Nanoparticle-Based Digital Cytometry to Quantify MUC16 Binding on the Surface of Leukocytes in Ovarian Cancer

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssensors.0c00567>.

Figure S1, schematic concept of the three-dimensional dark-field microscopy imaging setup; Figure S2, dynamic light scattering measurement of anti-CA125 and anti-Biotin antibody-conjugated 80 nm spherical gold plasmonic nanoparticles (PNPs); Figure S3, dynamic light scattering measurement and UV—visible absorption spectra of anti-CA125 and anti-Biotin antibody-conjugated PNPs treated with various concentrations of CA125 antigen; Figure S4, color quantization for monomer, dimer, and trimer based on the red/green intensity ratios (R/G ratios); Figure S5, mathematical model for evaluating the systemic error of the color quantization method for bound PNPs in MUC16 binding on the surface of the cell; Figure S6, PNP-based digital cytometric assay on ovarian cancer cells (OVCAR3) with and without centrifugation; Figure S7, optimization of incubation conditions for the ratio of treated PNPs to cells in the PNP-based digital cytometric assay on ovarian cancer cells (OVCAR3); Figure S8, cell membrane mask generated by a deep convolutional neural network (U-Net) to exclude unbound PNPs nearby the cells in the enumeration of bound PNPs on the surface of cells; Figure S9, longitudinal study of bound MUC16/CA125 on the surface of EOC patient's PBMCs over a 17 month period at 1 month intervals; Figure S10, evaluation of the specific binding ability of anti-CA125 PNPs toward its targets on the patient's and healthy subject's PBMCs with various PBMC to PNP ratios; Figure S11, dark-field microscopy image montages of anti-CA125 PNPs bound to individual PBMCs in samples from five healthy donors and five serous invasive EOC patients; Figure S12, flow cytometric analysis for the evaluation of bound MUC16 on the surface of PBMCs from five healthy donors and five serous invasive ovarian cancer patients; Figure S13, scanning electron microscopy images of OVCAR3 clone treated with antibody-conjugated PNPs; and Table S1, ages and the CA125 levels in the serum of healthy donors and ovarian cancer patients (PDF)

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Abstract

Although levels of the circulating ovarian cancer marker (CA125) can distinguish ovarian masses that are likely to be malignant and correlate with severity of disease, serum CA125 has not proved useful in general population screening. Recently, cell culture studies have indicated that MUC16 may bind to the Siglec-9 receptor on natural killer (NK) cells where it down-regulates the cytotoxicity of NK cells, allowing ovarian cancer cells to evade immune surveillance. We present evidence that the presence of MUC16 can be locally visualized and imaged on the surface of peripheral blood mononuclear cells (PBMCs) in ovarian cancer via a novel “digital” cytometry technique that incorporates: (i) OC125 monoclonal antibody-conjugated gold nanoparticles as optical nanoprobe, (ii) a high contrast dark-field microscopy system to detect PBMC-bound gold nanoparticles, and (iii) a computational algorithm for automatic counting of these nanoparticles to estimate the quantity of surface-bound MUC16. The quantitative detection of our technique was successfully demonstrated by discriminating clones of the ovarian cancer cell line, OVCAR3, based on low, intermediate, and high expression levels of MUC16. Additionally, PBMC surface-bound MUC16 was tracked in an ovarian cancer patient over a 17 month period; the results suggest that the binding of MUC16 on the surface of immune cells may play an early indicator for recurrent metastasis 6 months before computational tomography-based clinical diagnosis. We also demonstrate that the levels of surface-bound MUC16 on PBMCs from five ovarian cancer patients were greater than those from five healthy controls.

Graphical Abstract

Keywords

ovarian cancer; MUC16/CA125; leukocytes; plasmonic gold nanoparticle; dark-field microscopy; digital cytometry; computational analysis; longitudinal study

With an estimated new 22,240 cases and 14,070 deaths in 2018, ovarian cancer is the most deadly of the gynecological malignancies in the United States.¹ Among several types of ovarian cancers, epithelial ovarian carcinoma (EOC) is the most common and lethal form, often spreading beyond the ovaries to the peritoneal cavity and its organs.^{2–4} Ovarian cancer does not have specific symptoms in its early stages, leading most ovarian cancers to be diagnosed at advanced stages that are associated with poor prognosis and high case-fatality ratios.^{3,5} In contrast, when ovarian cancers are diagnosed early at the localized stage (stage I), up to 90% of ovarian cancer patients can be cured by surgery in combination with chemotherapy.⁶

Cancer antigen 125 (CA125) was first identified in serum in the early 1980s and found to have utility in monitoring women with EOC.⁷ Known to arise from a high-molecular-weight glycoprotein, it was not identified as a repeating epitope of the human mucin protein MUC16 until 2001.⁸ Multiple studies have demonstrated the clinical utility of serum CA125 measurements as a tool to monitor response to therapy and predict survival after diagnosis of EOC.⁹ While there has been great interest in serum CA125 for early detection, multiple studies failed to find support for its use. In particular, two large randomized clinical trials examining serum CA125, with and without transvaginal sonography, did not see a clear benefit in screening for ovarian cancer.^{10,11} The recent UKCTOCS study in 2016, the largest of its kind, unfortunately also arrived at the same conclusion.¹² Nevertheless, due to the lack of better alternatives, patients who are at high risk of EOC are still screened via CA125 serum measurements or sonography. These methods are not reliable as elevated levels of serum CA125 appear in only 50% of women with early-stage disease. Despite unsuccessful attempts to incorporate CA125 serum measurements into early detection regimens,¹³ the CA125 marker still has potential as a monitoring tool for diagnosed EOC patients. Given the utility of the CA125 antigen in monitoring diagnosed EOC, there is a need to better understand the physiology of CA125, such as its interaction with the immune system, to seek next steps in its broader use in the screening or treatment of EOC.

MUC16 is a high-molecular-weight mucin that is overexpressed on the surface of ovarian tumors and shed into the surrounding interstitial fluid.^{14,15} Shed MUC16 is found in peritoneal fluid and in the serum fraction of peripheral blood.^{14,16} Recent studies have also suggested interactions between shed MUC16 and immune cells. They indicate that the cytolytic function of human natural killer cells (NK cells) is inhibited by low levels of MUC16 cell surface binding, resulting in suppression of the innate immune response and acceleration of EOC metastasis.^{17–19} This observation suggests a potential route of immune evasion for ovarian tumors and prompts interest in the binding of MUC16 to the surface of classes of circulating immune cells where it may positively or negatively affect tumor progress in patients. The role of cell surface-bound and shed forms of MUC16 in tumor pathogenesis is now an active area of research. Flow cytometry is one of the most commonly used

Flow cytometry is one of the most commonly used techniques to investigate biomolecules on the surface of cells,^{20,21} and numerous EOC studies have employed flow cytometry to investigate biomarkers and predict prognosis after chemotherapy.^{22,23} Despite the many advantages of flow cytometry, the sensitivity of this technique is insufficient to detect and quantify extremely low-abundance (1–100) cell surface biomolecules.²⁴ This limitation arises from a combination of factors including cellular autofluorescence, interference and attenuation of fluorescence emission due to the nucleus and organelles, and cross-talk between fluorescent labels. Although several studies have proposed to utilize plasmonic gold nanoparticles to enhance sensitivity and to avoid photobleaching and spectral overlap of fluorescent labels in multiparametric flow cytometry,^{25–27} these studies mainly investigated cell surface receptors at a density of 20,000 to 100,000 per cell (e.g., CD4 and CD8 receptors). Here, the challenge was to detect low levels of receptor-bound antigens/ligands on a single cell. New techniques were thus required to overcome this sensitivity limitation and achieve sensitive and accurate quantification of low-abundance cancer antigens captured on the surface of immune cells, particularly the surface-bound MUC16 in ovarian cancer research.

Here, we report a plasmonic nanoparticle (PNP)-based digital cytometry method capable of detecting and quantifying the low abundance binding of MUC16 on the surface of human peripheral mononuclear cells (PBMCs). To create this molecular imaging tool for EOC, anti-CA125 antibody-conjugated 80 nm gold nanospheres were employed as a target-specific optical probe and detected via a customized dark-field microscopy (DFM) system. The extremely bright spectral scattering properties of plasmonic nanoparticles combined with dark-field imaging allow for the detection of individual bound PNPs with high sensitivity and signal-to-noise ratios, overcoming the challenges associated with fluorescence labeling. For quantification of MUC16 on the surface of PBMCs, images containing individual PNPs bound to MUC16 were counted and quantified by employing an automated deep-learning digital counting algorithm. To demonstrate the feasibility and reliability of this PNP-based digital cytometry method as a molecular detection toolkit for HOC, three clones of the ovarian cancer cell line OVCAR3, expressing varying levels of MUC16, were investigated and their different MUC16 expression levels were successfully discriminated. To translate this method to clinically relevant samples, a retrospective longitudinal study of an EOC patient over a 17 month period was carried out and found dynamic changes in MUC16

binding to PBMCs that varied independently of serum CA125. The digital cytometry assay was able to detect an apparent increase in MUC16 shedding 3–4 months before the serum CA125 assay. Additionally, we discovered that PBMCs from EOC patients showed strong surface MUC16 binding (10–100 events) in contrast to observations in PBMCs acquired from a healthy control group (0–10 events). Interestingly, no clear correlation was observed between serum CA125 and MUC16 bound to PBMCs. These findings demonstrate the ability of this new nanoparticle-based digital cytometry method to study a potentially important new compartment for MUC16/CA125 in the context of EOC.

RESULTS

Design of the PNP-Based Digital Cytometry

The PNP-based digital cytometry platform integrates three complementary technologies to quantify minute, single-molecule level changes in the binding of biomolecules on the surface of cells: (i) target-specific antibody-conjugated 80 nm plasmonic gold nanospheres with high monodispersity and stability under biological conditions, (ii) a customized dark-field microscopy (DFM) imaging system to detect targeted PNPs on the surface of cells without interference from background contributions such as autofluorescence, and (iii) a computational approach for accurately enumerating the number of bound PNPs on the surface of each cell. Metallic nanoparticles have a unique light interaction property known as surface plasmon resonance, which results in strong light scattering at specific wavelengths dictated by the nanoparticle size and composition. This scattering intensity is orders of magnitude brighter than a comparable fluorophore or autofluorescence and is free from photobleaching. Dark-field microscopy, a light-scattering sensitive imaging modality, enables image acquisition of individual PNPs in three dimensions for particle enumeration. When paired with image analysis tools, PNP binding to cell surfaces can be quantified at the single-molecule level.

Figure 1 outlines the PNP digital cytometry procedure to quantify cell surface-bound MUC16/CA125. Anti-CA125 antibody-conjugated 80 nm spherical gold PNPs (anti-CA125 PNPs) are incubated with isolated PBMCs. Stringent washing steps are used to remove unbound or nonspecifically bound anti-CA125 PNPs to achieve high signal-to-background ratios. The PBMCs are then imaged by custom DFM to acquire three-dimensional depth stacks (Figure S1 in the Supporting Information). Following data acquisition, each individual immune cell within a field of view is selected via a semiautomated image analysis tool that subsequently crops the region of interest (ROI) centered around each cell into high-resolution z-stack images. These images are then fed into an automated computational digital counting algorithm.

Binding events show distinct colors under dark-field microscopy due to the localized surface plasmon resonance scattering of PNPs.²⁸ Specifically, individual 80 nm gold nanospheres appear as green (peak at 560 nm) under DFM due to their specific plasmonic resonance. If two individual PNPs are located close enough together such that their surface plasmons couple, the plasmonic resonance band is redshifted, resulting in a yellow-orange dot (dimer, peak at 610 nm). In the same way, three closely located PNPs can plasmonically couple, resulting in the appearance of a red dot (trimer, peak at 670 nm). From these phenomena, we

are able to quantify closely located PNPs beyond the optical diffraction limit by conventional DFM.²⁹ By applying this color quantization method to plasmonically coupled PNPs, the number of targeted PNPs is determined even though the distance between individual particles may be smaller than the optical diffraction limit of a conventional microscope.^{30–34} Based on this strategy, the computational algorithm was developed to automatically count and quantify individual colored “dots” associated with targeted PNPs on the surface of PBMCs in DFM images.

Characterization of Antibody-Conjugated PNPs.

To evaluate the monodispersity of antibody-conjugated PNPs, anti-CA125 PNPs were measured by DFM. As shown in Figure 2a, over 80% of PNPs appeared as green dots that indicate the presence of a monomer, a single 80 nm gold nanosphere.³⁵ Occasionally, yellow-orange (dimer) and red (trimer) dots were also observed. Dynamic light scattering (DLS) carried out on the sample showed a hydrodynamic diameter of 110.3 ± 0.3 nm (polydispersity index, PDI = 0.112), a value that included the 15 nm-thick coating of antibodies conjugated to the 80 nm PNP core (Figure S2 in the Supporting Information).

The sensitivity of anti-CA125 PNP binding was validated by testing with increasing concentrations of isolated human CA125 antigen at 0, 250, 500, 1000, 2500, and 5000 U/mL. As a negative control, anti-Biotin antibody-conjugated 80 nm gold nanospheres (anti-Biotin PNPs) were utilized. As shown in Figure 2b, the hydrodynamic size of anti-CA125 PNPs increased with the concentration of CA125, while the size of anti-Biotin PNPs was constant. This finding indicates the specific capture of CA125 by anti-CA125 PNPs with little to no nonspecific binding. Note that at values greater than 2500 U/mL CA125 antigen, a plateau in the DLS size data was observed, indicating that the antibody binding sites on individual anti-CA125 PNPs became saturated with the CA125 ligand. Interestingly, the aggregation of anti-CA125 PNPs was never observed via DLS analysis and UV-visible absorption spectra even at high concentrations of CA125 antigen (Figure S3 in the Supporting Information). Such stability is attributed to the fact that anti-CA125 antibodies attached to the PNPs are monoclonal antibodies (OC125) that could capture only a single epitope of the commercial CA125 antigen.

Lyophilization was used to preserve the sensitivity and stability of the antibody-conjugated PNPs for storage.³⁶ To confirm the specific binding ability of anti-CA125 PNPs after lyophilization, the anti-CA125 PNPs at 1 month and 1 year post-lyophilization were reconstituted and reacted with CA125 antigen. As shown in Figure 2b, the reconstituted anti-CA125 PNPs were still able to specifically capture the CA125 antigens with the same sensitivity as fresh anti-CA125 PNPs prior to storage. Notably, even 1 year after lyophilization, the specific binding ability and sensitivity of anti-CA125 PNPs were preserved. This indicates that lyophilization is a suitable method to preserve the stability of antibody-conjugated nanoparticles.

Color Quantization Method for Plasmonic-Coupled PNPs.

As mentioned above, under DFM with 63 $\times$ (0.75 NA) objective lens, the number of bound PNPs on the cells can be enumerated via their distinct colors,³⁷ as monomers, dimers, and

trimers of 80 nm gold nanoparticles are smaller than the limit of optical resolution for the microscope (approximately 350 nm). In other words, monomers, dimers, and trimers with dimensions up to ~350 nm will all appear as airy disks, the point spread function (PSF), but with discretely different colors. In order to validate this color quantization method in our system, an anti-CA125 PNP sample was prepared under dry conditions and measured by a DFM equipped with a hyperspectral CCD camera (Nuance, PerkinElmer, Inc., USA). Anti-CA125 PNPs were randomly dispersed and aggregated to present as green, yellow-orange, and red dots within the field of view (Figure 3a). To acquire plasmonic resonant light scattering spectra, 15 PNPs each of green, yellow-orange, and red were chosen and used to generate averaged extinction spectra. As expected, the plasmonic resonance bands were centered at 560, 610, and 670 nm for green, yellow-orange, and red dots, respectively (Figure 3b). Of note, the trimers of PNPs can have different configurations including triangular, linear, and bent shapes with various angles. As a consequence, the plasmonic resonance peaks could be spread out from 650 nm to over 800 nm.^{38–40} As all these different configurations of the PNP trimers are indistinguishable with DFM in this study, we utilized the red/green channel intensity ratio (R/G ratio) thresholds of RGB color image to categorize green, yellow-orange, and red dots instead of the specific plasmonic resonance peaks. In other words, the monomer, dimer, and trimer of PNPs were distinguished by the R/G ratio thresholds, which were determined by DFM RGB images of dried anti-CA125 antibody-conjugated PNPs and the corresponding hyperspectral microscopy images. Based on these results, the R/G ratios of the RGB images were categorized into 0–1 for green, 1–2 for yellow-orange, and over 2 for red (see Figure S4 in the Supporting Information). Furthermore, since MUC16 is reported to be a long linear glycoprotein with 60 tandem repeats of the CA125 binding site along its length,¹⁵ the bound PNP on the MUC16 is expected to predominantly form a linear array. In such case, only one linear array of the PNP trimer can fit within one 350 nm PSF and a larger than the trimer cluster of PNPs will be shown as elongated spatially discrete spots. A mathematical modeling was also carried out to evaluate the error of the color quantization method for assigning the red PSF as a trimer; it suggests that less than 10% error of undercounting will be introduced in the quantification of PNP binding on the surface-bound MUC16 on the cell (see Figure S5 in the Supporting Information). PBMCs were also measured using the hyperspectral imaging system to assess the Mie scattering spectrum of individual cells. Importantly, the highest intensity of the PBMC's light scattering spectrum was centered at 470 nm, distinctly separate from the plasmonic resonance of PNPs. To isolate the plasmonic resonances and suppress the blue scattering from cells, a 530 nm longpass filter (530LP RapidEdge, Omega Optical, VT, USA) was used in subsequent DFM imaging experiments.

PNP-Based Digital Cytometry on Ovarian Cancer Cell (OVCAR3) Clones.

To demonstrate the feasibility of PNP-based digital cytometry for quantifying MUC16 on the surface of cells, three clones of the ovarian cancer cell line OVCAR3 were investigated. The OVCAR3 cell line is known to have a high baseline MUC16 expression level,⁴¹ but the cell line is not homogeneous. Three clones from the parent OVCAR3 line were isolated into low, intermediate, and high MUC16 expression levels. Subsequent subculturing of these isolated samples and analysis by flow cytometry revealed stable levels of MUC16 expression: low MUC16 in AB9 line (mean fluorescence intensity, MFI = 1430),

intermediate MUC16 in BC9 (MFI = 1993), and high MUC16 in AG8 (MFI = 2571, Figure 4a). First, highly MUC16 expressed OVCAR3 cells were treated with various ratios of cell to anti-CA125 PNPs (1:300, 1:500, 1:2000, 1:3000, and 1:4000) to find the optimal ratio of cells to PNPs that can provide a sufficient amount of anti-CA125 PNPs to target the entire surface MUC16/CA125 on a single cell while minimizing the excess amount of unbound anti-CA125 PNPs. As shown in Figure S7 in the Supporting Information, an anti-CA125 PNP binding plateau over the 1:2000 of the cell to PNP ratio indicates that the 1:2000 ratio of cell/PNPs is optimal for providing enough number of PNPs to detect the full amount of MUC16/CA125 on the single cell's surface while minimizing the excess amount of unbound PNPs. Based on these results, three OVCAR3 clones were treated with anti-CA125 PNPs (as a positive control) and anti-Biotin PNPs (as a negative control) at a 1:2000 ratio of cell/PNPs. After treatment with antibody-conjugated PNPs, OVCAR3 cells were imaged as described above with DFM to detect bound PNPs. More than 150 cells were then selected via semiautomated image inspection to generate high-resolution regions of interest (ROIs) for analysis. The digital counting algorithm then automatically enumerated the targeted PNPs. A sham-treated, no-PNP control arm (cell only) was included for both the anti-CA125 and anti-Biotin experiments.

To quantify the antibody-conjugated PNP binding, histograms resulting from the digital counting algorithm were fitted using a Poisson distribution algorithm. In a Poisson distribution, the random variable is a count of the number of occurrences of an event in a given unit of time, distance, area, or volume. The main assumption in building a Poisson distribution is that events are discrete, occurring independently, and the probability that an event occurs in a given length of time does not change through time. This distribution well describes the PNP-based assay and the method of counting particles as the individual nanoparticle binding is a discrete event and cannot be negative unlike the analog continuous intensity measurements acquired in traditional fluorescence-based cytometry. That is, instead of detecting a signal level, this approach uses a digital cytometric approach where each binding event is counted uniquely. Given a random distribution of relatively rare antigens on the surface of cells, the probability of an antibody-conjugated PNP diffusing randomly through space encountering an antigen can be approximated as a Poisson distribution.

Figure 4b,c shows the characterization of each of OVCAR3 clones via the digital nanoparticle cytometric assay. When only cells of each clone, without any treatment with PNPs, were imaged and analyzed, the method showed only a small number of false positives, leading to counting histograms largely confined to a single spike at zero counts. PNP counts were also observed to be confined near zero for the anti-Biotin negative control, confirming the lack of nonspecific binding of the nanoparticle assay. Running the assay with anti-CA125 PNPs revealed distinctly different levels of surface nanoparticle binding across the three clones, as evidenced in the counting histograms. When fitted with a Poisson distribution, the AB9 clones showed low anti-CA125 PNP binding ($\sim 8 \pm 2$ PNPs/cell, mean $\pm$ standard deviation (SD) for three replicates) slightly higher than the nonspecific binding level. In contrast, the BC9 and AG8 showed substantially higher anti-CA125 PNP binding on their surface, $\sim 35 \pm 1$ and $\sim 39 \pm 1$ PNPs/cell (mean $\pm$ SD, $n = 3$), respectively. The observed digital binding histograms for the surface MUC16 expressions of OVCAR3 clones

were similar to those observed in the flow cytometry results (Figure 4a). The difference between anti-CA125 PNPs binding on BC9 and AG8 cells was not statistically significant (two sample t-test with Bonferroni's correction, $p > 0.05$) from three experimental replicates.

Longitudinal Study of MUC16/CA125 on the Surface of EOC Patients' PBMCs.

Serum CA125 is currently used clinically to monitor EOC patients following initial treatment; an increase in serum CA125 levels post-treatment has been found to be associated with disease recurrence and has utility in planning therapeutic interventions. To investigate the use of the PNP digital cytometry method in post-treatment monitoring, a retrospective longitudinal study was performed with quantitative evaluation of MUC16/CA125 binding to the surface of EOC patient's PBMCs (Figure S9 in the Supporting Information). Samples were collected over a 17 month period at 1 month intervals, starting at the time of the patient's first recurrence in the fall of 2016. Blood from the subject was immediately processed via Ficoll-Paque gradient centrifugation to isolate PBMCs, which were then frozen for storage. On the day of the assay, the previously frozen PBMCs were thawed and treated with anti-CA125 PNPs at a 1:2000 ratio of cells to PNPs. This optimal ratio of cells to PNPs was determined experimentally using PBMCs from an EOC patient and a healthy donor (Figure S10 in the Supporting Information). Conventional serum CA125 tests were run as part of routine clinical practice on identical blood samples collected at each timepoint. It is worth noting that one data point in the longitudinal study was not considered for analysis as a medical intervention, platelet transfusion, was performed on February 2017 that is thought to have altered the patient's physiological condition.

Figure 5 plots both the measured serum CA125 level and the mean value of the bound anti-CA125 PNPs per cell from Poisson distribution fitting along with 95% confidence interval. The Poisson means of the bound anti-CA125 PNPs per cell showed a similar trend to serum CA125 from the start of monitoring over a period of 10 months during which time the patient responded to Taxol/carboplatin chemotherapy. In August 2017, the patient switched to maintenance treatment with the anti-VEGF agent, bevacizumab. Interestingly, since that time, the Poisson mean of the bound anti-CA125 PNPs per cell was observed to depart from the serum CA125 trend and increase rapidly, while serum CA125 levels remained constant over a 6 month period. The reason for this divergence is not clear; however, it should be noted that the PBMC-bound MUC16 levels do not necessarily have to correlate with CA125 levels in serum since the CA125 serum assay investigates the amount of CA125 epitope (shredded MUC16) within the serum while the PNP-based digital cytometry is focused on the surface bound MUC16 and its fragments. In addition, the surface binding of the MUC16 mechanism could be different with the mechanism of shedding CA125 into the serum. Interestingly, in February 2018, 6 months after the PBMC-bound MUC16 level elevated in the PNP-based digital cytometric assay, the patient was found to have recurrent metastatic disease via computed tomography (CT). It was hypothesized that this increase in PBMC-bound MUC16 could be indicative of the increased MUC16 shedding associated with recurrence. This finding suggests that PBMC-bound PNP measurements could play an early warning role in routine monitoring.

PNP-Based Digital Cytometry of Human PBMCs Acquired from Healthy Subjects and EOC Patients.

To validate the feasibility of PNP-based digital cytometry approach as a molecular diagnostic tool in a clinically relevant context, PBMCs from five healthy subjects and five preoperative EOC patients without neoadjuvant chemotherapy were evaluated to quantify immune cell surface-bound MUC16. All EOC patients were diagnosed with serous invasive ovarian cancer, and their CA125 levels in serum were greater than the clinical threshold of 35 U/mL. Healthy control PBMC samples were acquired from age-matched healthy donors (approximately 60-year-old females) with serum CA125 levels all under 35 U/mL (Table S1). As prior studies noted increased binding of MUC16 to at least one subset of leukocytes,^{17,18} it was hypothesized that EOC patients would have overall elevated levels of anti-CA125 PNP binding when compared to healthy controls.

To first establish a clinical baseline binding of anti-CA125 PNPs to PBMCs, healthy control samples were measured by the nanoparticle digital cytometry assay to create binding histograms. Fitting the resulting histograms with a Poisson distribution revealed an average baseline binding level of ~5 anti-CA125 PNPs/cell. This is not necessarily a surprising result as all individuals have MUC16/CA125 fragments in their serum, which can bind to circulating PBMCs. In contrast, four of the five EOC patients were found to have higher anti-CA125 PNP surface binding than the healthy controls. Specifically, as shown in Figure 6, the majority of PBMCs (~80%) from healthy controls showed fewer than 5 anti-CA125 PNP binding per cell. In case of EOC patients, more than 40% of PBMCs showed greater than 5 anti-CA125 PNP binding, with the sole exception of patient 3 (Figure 6b and Figure S11 in the Supporting Information). On the other hand, any significant differences of the surface-bound MUC16/CA125 level on the PBMCs between EOC patients and healthy donors were not observed in the conventional flow cytometric analysis (Figure S12 in the Supporting Information). Interestingly, the binding of anti-CA125 PNPs was not consistent across patients, nor was the PNP binding found to correlate with each patient's own serum CA125 level. For example, while patient 3 had a high (~800 U/mL) serum CA125 level, the observed PNP binding was the lowest of the patient group and not significantly dissimilar from that of healthy controls. In contrast, patient 5 had low serum CA125 levels and extremely high anti-CA125 PNP binding. This divergence between free CA125 in serum and PBMC surface-bound CA125 may be of clinical interest as it reveals a previously unknown binding compartment of MUC16/CA125 potentially related to immune system response.

DISCUSSION

We have developed a highly sensitive and reliable plasmonic nanoparticle (PNP)-based digital cytometric assay to quantify and monitor ultralow abundances of cell surface-bound MUC16/CA125. This assay combines the sensitivity of plasmonic nanoparticles with dark-field microscopy and computational analysis to digitally count nanoparticle binding events on the surface of individual cells. The binding specificity of the anti-CA125 PNPs was demonstrated alongside a lyophilization protocol that enables long-term storage of assay reagents. The ability of the assay to accurately measure MUC16 on the surface of cells was validated using OVCAR3 clones previously characterized via flow cytometry. Additionally,

the specific binding of anti-CA125 PNPs on the surface of OVCAR3 clones and PBMCs from an ovarian cancer patient was also validated by scanning electron microscopy (SEM, Figure S13 in the Supporting Information).

The longitudinal patient study demonstrated that PBMC surface-bound MUC16 is a new and largely independent compartment of CA125. PNP binding trended with serum CA125 levels under one therapeutic condition and then rapidly diverged once the therapeutic regimen was changed. Although the reason for this difference is not yet understood, it is worth mentioning that if cell-bound CA125 is in a simple equilibrium with free CA125 in serum, the CA125 level in serum and cell-bound CA125 would be expected to rise and fall together. However, the longitudinal study clearly shows that there is not necessarily a straightforward equilibrium between the serum and cell-bound compartments. While the observed divergence could have arisen from the change in treatment regimen or disease recurrence, it is also possible that the early elevation of cell-bound CA125 is caused by the presence of CA125 at locations other than peripheral blood, such as in ascites or in the lymph system. Generally, leukocytes not only exit the peripheral blood stream to hone in on tumor sites (and sites of inflammation, more generally) but also traffic back through the lymphatic system to the peripheral blood.⁴² One hypothesis is that PBMCs pick up MUC16/CA125 at the tumor site or at additional new metastatic tumor sites where the local shed CA125 concentration is high. If confirmed, this would imply that MUC16 binding to the surface of PBMCs could indeed be a sensitive leading indicator of increased tumor mass.

While the results in Figure 6 showed that serum CA125 and surface-bound CA125 may not correlate from subject-to-subject, cell surface binding of anti-CA125 PNPs was found to be significantly different between EOC patients and age-matched healthy donors. Four out of five patient samples showed high binding of anti-CA125 PNPs to PBMCs that was seemingly independent of the patient serum CA125 level. Interestingly, the PNP binding levels across patients were highly variable, potentially arising from several separate factors. EOC patients recruited to the study had noticeable symptoms and were diagnosed via pathology and CT, but it is not known how long patients had harbored the disease prior to diagnosis and subsequent blood draw, meaning that the progression of disease is likely quite different between recruited subjects. In addition, the status of each subject's immune system and its interaction with the tumor is not known, representing an unknown factor in understanding MUC16/CA125 binding to immune cells. It should be noted that the assay here examined all PBMCs; it is hypothesized that MUC16/CA125 binding may occur to only a subset of these cells. As the balance between leukocytes is known to evolve throughout the course of EOC and treatment,^{43,44} the divergence between serum CA125 and PNP binding levels may be connected to a loss of specific cell populations, for example, NK cells or B cells. Longitudinal monitoring of individuals prior to diagnosis would be ideal for establishing per individual baseline binding levels, as would identification of specific PBMC subtypes.

This study enumerated the levels of MUC16/CA125 bound to the surface of all PBMCs, but there is interest in MUC16 binding to unique lineages and subtypes of leukocytes, including B cells, T cells, and monocytes. While image analysis was used to enumerate the number of bound PNPs per cell, it was not possible to distinguish between PBMC types based on size

and appearance of cells alone. Given the different roles and phenotypes of leukocyte subclasses, it would not be surprising if the surface-bound fraction of MUC16 differs between cell types. Except for NK cells, the remaining subtypes of PBMCs (T cells, B cells, and monocytes) have not been fully investigated yet with respect to shed surface-bound MUC16 binding. If there are differences in MUC16 binding among PBMC subtypes, it could help explain the divergence between the observed cell-bound MUC16/CA125 and serum CA125 levels. For example, if a patient was lacking in a specific type or set of leukocytes, the observed binding to PBMCs overall would be impacted. We are currently developing a more advanced form of the PNP digital cytometry assay focused on detecting the PBMC cell type via lineage markers and employing an automated *XYZ* stage in DFM or microfluidic chip-based three-dimensional DFM imaging tool for automating of DFM imaging acquisition that will substantially improve the throughput of the PNP-based digital cytometry. A next-step study beyond the scope of this paper will explore the role of bound CA125 on specific PBMC subsets in predating metastatic disease and prognosis. It should be noted that, while focused here on MUC16, the method developed could be also readily translated to detect and quantify a number of different low-abundance cell surface proteins and can be extended for monitoring patient response to immunotherapy regimens.

EXPERIMENTAL SECTION

Preparation of Peripheral Blood Mononuclear Cells (PBMCs).

Blood samples from patients suspected of ovarian cancer were collected prior to therapy under a protocol (16–255) approved by the Dana Farber Harvard Cancer Center Institutional Review Board (IRB). Normal healthy control specimens were collected from age-matched healthy women (2018P001677) under a protocol approved by the Partners Healthcare IRB. The patient participating the longitudinal study knew of the project and requested that her blood be monitored during her therapy. She signed a consent acknowledging the voluntary nature of her participation and that results of the assay could not be used to guide her therapy.

Upon receiving the fresh blood samples, PBMCs were immediately isolated by Ficoll density gradient centrifugation, according to its standard protocol (Ficoll-Paque PLUS, GE healthcare, Chicago, IL, USA). The isolated PBMCs were then washed with phosphate-buffered saline (PBS, pH 7.4) without Ca^{2+} and Mg^{2+} followed by dispersion in an appropriate volume of complete cell culture media (cRPMI consisting of RPMI-1640, 10% fetal bovine serum (FBS), and 2% penicillin/streptomycin (5000 U/mL, Pen/Strep)). Subsequently, the PBMC population in the suspension was determined by counting the number of cells with a hemocytometer. The PBMCs were then centrifuged and redispersed in appropriate volumes of 95% cRPMI and 5% DMSO to achieve 1×10^7 cells/mL cell population. The prepared PBMCs were immediately transferred to each cryovial for a single experiment and placed in a -80°C freezer for storage until analysis.

Preparation and Characterization of Antibody-Conjugated Plasmonic Nanoparticles (PNPs).

The 80 nm gold nanospheres conjugated with monoclonal anti-CA125 antibody (OC125) or monoclonal anti-Biotin antibody were purchased from BBI Solutions (Cardiff, UK). To maintain the specific binding abilities of antibody-conjugated PNPs, they were lyophilized and stored in a desiccator at 4 °C. As a target-specific optical probe, the lyophilized antibody-conjugated PNPs were reconstituted by adding 200 μL of distilled water prior to the experiment. The final concentration of the reconstituted antibody-conjugated PNPs was determined by measuring the extinction peak at 550 nm. To evaluate the specific binding ability of the reconstituted antibody-conjugated PNPs toward its antigens, 2 μL of anti-CA125 antibody-conjugated PNPs (anti-CA125 PNPs, positive control) or anti-Biotin antibody-conjugated PNPs (anti-Biotin PNPs, negative control) was reacted with 48 μL of various concentrations of CA125 antigen in PBS without Ca^{2+} and Mg^{2+} , respectively: 0, 250, 500, 1000, 2500, and 5000 U/mL. After a 1 h incubation in a 37 °C water bath, the mixture solutions were diluted in 450 μL of PBS without Ca^{2+} and Mg^{2+} and then measured by dynamic light scattering (Zetasizer Nano Z, Malvern Panalytical Ltd., Royston, UK).

PNP-Based Digital Cytometric Assay Protocol.

Frozen PBMCs were thawed and immediately transferred into prewarmed 9 mL of cRPMI. The PBMCs were centrifuged and redispersed in 6 mL of prewarmed cRPMI. Afterward, the PBMC suspension in cRPMI was kept in a 37 °C water bath for 1 h. After stabilizing the PBMCs, they were centrifuged and redispersed in appropriate volume of PBS with Ca^{2+} and Mg^{2+} to achieve 1×10^7 cells/mL cell population. Next, the reconstituted antibody-conjugated PNPs were added into the PBMC suspension in PBS with Ca^{2+} and Mg^{2+} at a given cell-to-PNP ratio. The mixture solution was then vortexed for 45 s followed by incubation for 1 h with continuous mixing (750 rpm) at room temperature. After a 1 h incubation, the resulting solution was centrifuged and washed with PBS with Ca^{2+} and Mg^{2+} two times (1400 rpm for 3 min at room temperature) to remove unbound and nonspecifically bound PNPs as well as aggregated PNPs that could occur during incubation (Figure S6 in the Supporting Information). The PBMCs treated with antibody-conjugated PNPs were then redispersed in appropriate volume of PBS with Ca^{2+} and Mg^{2+} to achieve 1×10^7 cells/mL. A 8 μL volume of PBMC suspension was dropped on a glass slide and was covered by a coverslip; the coverslip was then mounted onto the glass slide with black nail polish (Ted Pella, Inc., Redding, CA, USA).

Dark-field Microscopy Imaging Setup.

Three-dimensional dark-field imaging was performed using a commercial inverted dark-field microscope (Axiovert 200 M, Zeiss, Jena, Germany) coupled with a color CCD camera (8051C-CL, Thorlabs Inc., Newton, NJ, USA). The system employs an oil immersion ultradark-field condenser (NA of 1.2–1.4, Zeiss), a 63 $\times$ objective (0.75 NA LD Plan-Neofluar, Zeiss) mounted on a piezoelectric objective scanner (P-725, PI, Hannover, Germany), and a 530 nm longpass filter (530LP RapidEdge, Omega Optical, VT, USA) for z-stack acquisition. All three-dimensional DFM images were acquired with a 28.5 μm depth z-stack with a 0.7125 μm step size with custom-written image acquisition software that

synchronizes camera acquisition and piezoelectric position. Each focal plane was imaged with a 10 ms acquisition time. The image dimensions are 0.1 $\mu\text{m}/\text{pixel}$ in the X and Y directions and 0.75 $\mu\text{m}/\text{pixel}$ in the Z direction. Illumination was provided by an integrated 100 W halogen source.

Digital PNP Counting Algorithm.

Bound PNPs in each cell 3D image stack were counted via a custom-built 3D blob detection routine that consists of the following steps.⁴⁵ First, for each 2D image, bright points were found using the Laplacian of Gaussian blob detection algorithm. While more accurate blob detection methods exist, they tend to fail when the image appearance does not match the ideal blob model, being such the case of bright PNPs due to the appearance of airy disks or nearby PNPs. All bright point detection locations are joined into 3D clusters by the density-based spatial clustering of applications with noise clustering method.⁴⁶ Clusters that have a small number of detection hits, which are close to the edges of the image, or whose associated image intensity is lower than a threshold are eliminated. Finally, those detection hits in the central layer of the cluster are reported as the location of a PNP cluster and its color represents the number of PNPs (1 if it is green, 2 if it is yellow-orange, and 3 if it is red). For each cell, the total number of PNPs weighted by their color is reported. Moreover, in order to exclude unbound PNPs nearby the cells in the quantification of PNP binding on the surface cells, the mask of the cell membrane is automatically generated by a trained U-Net⁴⁷ (Figure S8 in the Supporting Information). A margin of two micrometers added to the mask of cell membrane. PNPs outside of such region are disregarded in digital counting since they are considered to be unbound PNPs that were not removed on the wash steps. The U-Net operates in the maximum intensity projection of the 3D color z-stack centered at the cell of interest and thus finds the maximum contour of the cells. An expert manually generated the masks on the maximum intensity projections of 1000 cells, a subset of the images analyzed in this paper. Training is performed on a graphics processing unit (GPU), using the adaptive momentum (ADAM) optimizer.⁴⁸ Standard data augmentation techniques (translations, rotations, and zoom) are applied to the training dataset. Once the U-Net is trained, it is used in all the cell images acquired to automatically estimate the regions of the image where bound PNPs might be present.

Cell Culture of Ovarian Cancer Cell (OVCAR3) Clones.

The epithelial ovarian cancer cell line (OVCAR3) clones were maintained in a humidified 5.0% CO_2 , 37 °C incubator in complete cell culture media (cRPMI, consisting of 500 mL of RPMI 1640, 20% FBS, 10 mL of 5000 U/mL Pen/Strep, and 5 mL of 100 mM sodium pyruvate). Prior to the experiment, the OVCAR3 clones were cultured and washed with PBS without Ca^{2+} and Mg^{2+} three times. Cells were plated in 96-well plates using limiting dilution conditions. The cell solutions were diluted so that each well received one single ovarian cancer cell clone. Microscopic visualization of each well confirmed that the well contained one single OVCAR3 clone. The wells that contained more than one OVCAR3 cells were discarded. The wells with one OVCAR3 cell were allowed to continue, and the single cell clones were expanded through serial passaging. When sufficient numbers of each clone were obtained, the cells were monitored by flow cytometry to monitor MUC16 levels on the surface. Clones that contained normal, intermediate, and no levels of MUC16 on the

OVCAR3 clones were established and monitored over several months to demonstrate that the MUC16 levels in the clones did not differ. These OVCAR3 clones were established and used in the experiments outlined in this study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Schematic procedure of plasmonic nanoparticle-based digital cytometry.

Figure 2. Characterization of the anti-CA125 antibody-conjugated plasmonic nanoparticles (anti-CA125 PNP). (a) Dark-field microscopy image of anti-CA125 PNP and (b) evaluation of specific binding ability of anti-CA125 PNP to CA125 antigens before and after lyophilization. The anti-Biotin antibody-conjugated PNP (anti-Biotin PNP) were utilized as a negative control. The size of samples was measured by dynamic light scattering (DLS).

Figure 3. Color quantization of plasmonic-coupled PNPs under dark-field microscopy condition. (a) Dark-field microscopy image of dried anti-CA125 PNPs and (b) plasmonic resonant light scattering spectra of green, yellow-orange, and red dots as well as native PBMCs from hyperspectral images. Under DFM conditions, gold PNPs are shown in distinct colors based on their plasmonic resonance light scattering properties: green for monomers of an 80 nm gold nanosphere, yellow-orange for dimers, and red for trimers.

Figure 4.

PNP-based digital cytometry results of MUC16 (having CA125 epitopes) expression on ovarian cancer (OVCAR3) clone cells having low (AB9), intermediate (BC9), and high (AG8) MUC16 expression levels. (a) Flow cytometry results of the MUC16 expression level on the OVCAR3 clones. The three OVCAR3 clones were treated with monoclonal anti-CA125 antibodies labeled with Alexa Fluor 488 fluorophores (AF488-OC125). Flow cytometric analysis was performed for cells from each of OVCAR3 clones without the AF488-OC125 treatment, as a negative control (gray), and for each of AF488-OC125 treated OVCAR3 clones (red). Data is representative of three independent experiments. (b) Histograms of the number of bound antibody-conjugated PNPs per cell for cells without PNPs, cells treated with anti-Biotin PNPs, and cells treated with anti-CA125 PNPs. (c) Statistical quantification of the CA125 biomarker on the surface of individual single cells with Poisson distribution by computational automatic PNP counting algorithm. Mean $\pm$ standard deviation (SD, $n = 3$ replicates). A pairwise t-test with Bonferroni's correction was performed on the mean of the bound anti-CA125 PNPs per cells in the OVCAR3 clones. *** $p < 0.001$; NS, not statistically significant ($p > 0.05$).

Figure 5.

Longitudinal study of CA125 levels on the surface of EOC patient's PBMCs. (a) Dark-field microscopy image montages and (b) histograms of anti-CA125 PNPs bound to individual PBMCs in samples from representative months (Jan 2017, Aug 2017, and Jan 2018). (c) Statistical quantification (lambda of Poisson distribution fitting) of the number of anti-CA125 PNPs binding on the surface of EOC patient's PBMCs with 95% confidence intervals (red) in comparison with CA125 levels in serum (black) as a function of time. The scale bars are 10 μm .

Figure 6.

Evaluation of anti-CA125 PNPs binding on the surface of PBMCs from five healthy donors (H1, H2, H3, H4, and H5) and five serous invasive EOC patients (P1, P2, P3, P4, and P5). All PBMCs were treated with a 1:2000 ratio of PBMCs to anti-CA125 PNPs. (a) Histogram of anti-CA125 PNPs bound to individual PBMCs and (b) percentage of PBMCs bound over 5 anti-CA125 PNPs/cell. The clinically relevant healthy control baseline of anti-CA125 PNP binding (ca. 5 PNPs/cell) was determined by averaging the lambda of Poisson distribution fitting in the number of bound anti-CA125 PNPs on five healthy donors.