ABSTRACT: Gold colloid changes its color when the internanoparticle distance changes. On the basis of analyte-induced aggregation or disaggregation behavior of gold nanoparticles (AuNPs), versatile colorimetric assays have been developed for measuring various kinds of analytes including proteins, DNA, small molecules, and ions. Traditional read-out signals, which are usually measured by a spectrometer or naked eyes, are based on the averaged extinction properties of a bulk solution containing billions of nanoparticles. Averaged extinction property of a large amount of nanoparticles diminished the contribution from rare events when the analyte concentration was low, thus resulting in limited detection sensitivity. Instead of measuring the averaged optical property from bulk colloid, in the present work, we proposed a digital counterpart of the colorimetric assay by imaging and counting individual AuNPs. This method quantified the analyte concentration with the number percentage of large-sized AuNPs aggregates, which were digitally counted with surface plasmon resonance microscopy (SPRM), a plasmonic imaging technique recently developed by us and other groups. SPRM was able to identify rare AuNPs aggregates despite their small population and greatly improved the detection sensitivity as demonstrated by two model systems based on analyte-induced aggregation and disaggregation, respectively. Furthermore, besides plasmonic AuNPs, SPRM is also suitable for imaging and counting nonplasmonic nanomaterials such as silica and metal oxide with poor extinction properties. It is thus anticipated that the present digitized assay holds a great potential for expanding the colorimetric assay to broad categories of nonplasmonic nanoparticles.

Gold nanoparticles (AuNPs) exhibit a large extinction coefficient due to localized surface plasmon resonance (LSPR), which is the origin of the color of gold colloids. Because LSPR is highly sensitive to the interparticle distance, gold colloids often change color in the presence of certain analytes via analyte-induced aggregation or disaggregation (dispersion), enabling fast and convenient colorimetric assays for the measurement of the analyte concentration. Since the pioneer work by Mirkin and co-workers, AuNPs-based colorimetric assays have been widely used to detect broad kinds of analytes including DNA, proteins, ions, and small molecules.

Such assays rely on the sensitive dependence of extinction property, i.e., the color, of gold colloids by changing the interparticle distance. In a typical AuNPs-based colorimetric assay, gold colloids consisting of well-dispersed gold nanoparticles were red colored due to a relatively large interparticle distance. The presence of analyte resulted in the formation of AuNPs aggregates because of the chemical or electrostatic interactions. Consequently, the reduced interparticle distance led to a color change from red to purple or blue, from which the concentration of analyte was determined. So far, optical extinction measured by a UV–visible spectrometer or naked eyes has been the major read-out signal in colorimetric assays. However, the optical extinction property reflects the ensemble average behavior of billions of individual nanoparticles in the detection volume. Ensemble measurements show a strong bias on rare events because the statistic average usually diminishes the contribution from a minor portion of nanoparticle populations. For example, when the analyte concentration is extremely low, very few aggregates are formed compared to the large population of original individual nanoparticles, which can hardly affect the averaged optical property. Therefore, optical extinction measurements usually exhibit limited capability to detect aggregates with low concentration, resulting in limited sensitivity.

Single nanoparticle counting techniques have been emerging rapidly and proven powerful in ultrasensitive detection of analytes. Several techniques, including dark-field imaging, plasmonic scattering, and single-particle mass spectroscopy, have been developed to count individual AuNPs, resulting in improved detection sensitivity of the
colorimetric assay. Despite their powerful capabilities, dark-field imaging and plasmonic scattering remain challenging for AuNPs with diameters below 20 nm, which are the most widely used sizes in AuNPs-based colorimetric assays. Laser illumination and flow channel have placed additional complexity in the sample preparation and handling.

Here, we propose a digitized counterpart of the AuNPs-based colorimetric assay by imaging and counting individual nanoparticles with surface plasmon resonance microscopy (SPRM), an optical imaging technique that was recently developed by us and other groups. The principle of this SPRM imaging system is illustrated in Scheme 1. A p-polarized incident light ($\lambda$ = 680 nm) is directed onto a gold-coated coverslip through an imaging technique that was recently developed by us and other groups. The principle of this SPRM imaging system is illustrated in Scheme 1.

Scheme 1. Schematic Illustration of the Digitized Colorimetric Assay by Imaging and Counting Individual AuNPs with SPRM

“SPRM is able to measure the size distribution of AuNPs in a digital way, in which the size of each nanoparticle is individually determined. Quantification of analyte concentration is achieved by analyzing the number percentage of large-sized AuNPs aggregates in the size distributions of dispersed sample (red histogram) and aggregated sample (purple histogram), respectively.

Instead of measuring the plasmonic property of AuNPs, SPRM measures the scattering of nano-objects through an existing surface plasmon wave, resulting in two important advantages. First, SPR signal is proven to be sensitive to the size of AuNPs and less sensitive to its morphology. Second, SPRM is suitable for imaging not only plasmonic nanoparticles (gold\textsuperscript{26} and silver\textsuperscript{29}) but also nonplasmonic nanoparticles ($\text{SiO}_2$,\textsuperscript{26} polystyrene,\textsuperscript{26} hydrogel,\textsuperscript{27} virus,\textsuperscript{31} bacterial,\textsuperscript{32} and so on). The present work thus paves the way toward universal “colorimetric” assays involving analyte-induced aggregation of both plasmonic and nonplasmonic nanomaterials.

**EXPERIMENTAL SECTION**

**Materials.** Chloroauric acid tetrahydrate (HAuCl$_4$·4H$_2$O) and tris(hydroxymethyl)aminomethane were purchased from Sinopharm Chemical Reagent Co., Ltd. Adenosine triphosphate (ATP), trisodium citrate, and poly-l-lysine (PLL) solution (0.1%, w/v in H$_2$O) were purchased from Sigma-Aldrich (Shanghai). Melamine (99%) was bought from Aladdin. The phosphate buffered saline (PBS, 1×) was obtained from Thermo Scientific. Other reagents were all of analytical reagent grade and used as received without further purification. The deionized water (DI water, 18.2 MΩ·cm resistivity) produced by Barnstead Smart2Pure3 UF (Thermo Fisher) was used throughout the study.

**Sample Preparation.** The SPR chips were BK7 glass coverslips coated with 2 nm of chromium and then 47 nm of gold. Each Au chip was rinsed with DI water and ethanol and blown dry under nitrogen before use. After the chip was further cleaned with a hydrogen flame to remove possible remaining contamination, 2% (w/v) in H$_2$O) PLL solution was dropped onto the chip surface for 3 h under room temperature. When the self-assembly of the PLL layer was fabricated, the chip was subsequently rinsed with DI water and 0.02× PBS solution. All the measurements of AuNPs aggregation were carried out at room temperature (25 °C) in a 0.02× PBS buffer solution.

**SPRM Setup and SPR Imaging.** The experiment of SPR imaging was performed on an inverted microscope (Olympus IX83) by using a high numerical aperture oil immersion objective 60× (NA 1.45). A red super luminescent light emitting diode (SLED) with wavelength of 680 nm (1 mW, QPhotonics) was used as the light source, and a polarizer was inserted on the optical path to generate p-polarized light so as to excite the surface plasmons at the Au substrate. The incident angle of such light was also adjustable and optimized for the purpose of delivering the largest surface plasmon response from AuNPs. The SPR chip was placed on the objective of the microscope using a high precision stage to index-matching liquid. The SPRM images were recorded by a CCD camera (AVT Pike F-032B from Allied Vision Technologies) at a frame rate of 3.0 frames per second. After injecting 0.02-fold PBS buffer (pH 7.4) on top of surface-modified SPR chip, the incident angle of the laser beam was adjusted to be near the SPR resonance angle at which the reflection recorded by the camera reached minimum intensity. Then, a given volume of AuNPs or mixed sample solution was drop into the liquid, and the raw SPRM images recorded from the camera were converted to 16-bit tiff format.
files with a Matlab program. Then, the intensity of each particle was determined by calculating the average intensity within a fixed small rectangle region-of-interest (ROI) selected to involve the particle. After a series of Fast Fourier Transformation (FFT) of individual ROI, Gaussian fittings of histograms of particle SPR intensities were carried out with Matlab to demonstrate intensity distribution of different aggregation cases.

**Detection of Melamine.** 100 μL of AuNPs suspension was first diluted 20 times with deionized water and used as a stock liquid for the detection of melamine. Different concentrations of melamine analyte solutions (50 μL) were added into the above colloidal AuNPs solution (350 μL). 80 μL of this mixed sample solution was dropped into the 20 μL 0.02× PBS buffer, and then, the aggregation of dispersed gold nanoparticles was measured with an SPR imaging system. As a comparison, the color change was also observed by the naked eyes and absorbance spectra photographs were taken with a Panasonic DMC-LX5 digital camera and recorded by a UV-vis spectrometer.

**Detection of ATP Based on Aptamer Target Binding.** In the ATP assay, all the DNA oligonucleotides used were synthesized from Sangon Biotechnology Inc. (Shanghai, China), and the sequences were as follows: ATP aptamer (a-ATP) (5′-ACC TTG GGG AGT ATT GCG GAG GAA GGT-3′) and the complementary oligonucleotide (c-ATP) (5′-ACC TTC CTC CGC AAT ACT CCC CCA GGT-3′). The assay procedure was performed mainly according to the literature. In brief, both the a-ATP (10 μM) and c-ATP (10 μM) solutions were mixed in 25 mM Tris-buﬀer containing 0.3 M NaCl (pH 8.2) for the hybridization. ATP of desirable concentrations was added into the solution and incubated in a 37 °C warm bath for 0.5 h. Subsequently, 2 μL of the above solution was injected into 100 μL of AuNPs, followed by treatment of 20 μL of 10 mM PB buﬀer (0.2 M NaCl, pH 7.4) for triggering the aggregation of AuNPs. Finally, 80 μL of such reaction sample was added into the 0.02× PBS for the SPR imaging measurement.

**RESULTS AND DISCUSSION**

**Imaging Individual Gold Nanoparticles with SPRM.** We first evaluated the capability of SPRM to image multiple AuNPs at the same time. 20 nm AuNPs were synthesized via a classical citrate-reduction method. The thorough characterization results were provided in Figure S1, including the extinction spectrum, dynamic light scattering (DLS), and transmission electron microscopy (TEM). In a typical experiment, as-synthesized gold colloids were first diluted 20 times with deionized water, and then, 80 μL of the diluted colloids were injected into an open-well chamber containing 20 μL of 0.02-fold phosphate buffered saline (PBS), which was placed on top of the gold-coated coverslip. The solution was thoroughly mixed for a couple of seconds to reach a final dilution factor of 25 and then remained undisturbed to enable the diffusion and adsorption of AuNPs onto the gold film.

Because the surface plasmon wave is a near-field electromagnetic wave, nano-objects that are closely attached to the gold film generate the largest SPRM signals. For this reason, we modified the gold film with positively charged poly-l-lysine (PLL), enabling the efficient electrostatic adsorption of negatively charged AuNPs onto the gold film. Once an individual nanoparticle hit and stayed at the surface of the gold film, a parabolic-shaped pattern would appear at the location of the collision due to the scattering of surface plasmon wave (Figure 1A). The whole dynamic process was continuously recorded by SPRM (Figure S2) and was provided as Movie S1, left panel. The camera frame rate was optimized to adjust the average number of collision events in the period of one frame, so that each of them could be separately identified.

It was found that all individual nanoparticles stroke the gold film surface and stayed there permanently. We attributed the driving force of the hit-n-stay to be Brownian motion and electrostatic interaction. After the collision, electrostatic force and van der Waals force kept the gold nanoparticle staying on the gold film. With the present surface modification of gold film, we did not observe any detachment event from the surface in a recording time of over 1 h.

In order to minimize the background drift during long-term recording, we introduced the differential SPRM images as shown in the center panel of Movie S1. Each differential SPRM image was produced by subtracting the previous frame of SPRM image from the present one. Due to the mathematic feature of first-order derivative, a hit-n-stay event of a single nanoparticle appeared as a blinking pattern in the differential SPRM movies (Movie S1, center panel). Additionally, using the differential SPRM images could also differentiate the moving NPs near the surface from the static NP at the surface. In a “hit and move” scenario (Movie S2), the SPRM patterns of moving NPs repeatedly appear, disappear, and reappear within a similar position of the view, indicating the constant movements. While in a “hit and stay” scenario (Movie S3), the SPRM pattern of a single nanoparticle appears only once in the video, because the static nanoparticle is invisible in the differential SPRM images.

**Imaging and Counting of Gold Nanoparticles.** We further demonstrated that SPRM was capable of imaging and counting gold nanoparticles. Differential SPRM images enabled us to distinguish two spatially overlapped AuNPs by the different times of arrival (see Figure S3). Thus, we did not need to move the field of observation when collecting AuNPs. About 500 collision events during 300 s were recorded. With a home-
developed image processing algorithm, the number and location of individual gold nanoparticles were determined by recognizing the parabolic-shaped patterns in the differential SPRM movies (Movie S1, right panel). The size was determined by analyzing the intensity of each pattern in a 2-D Fourier domain. Fourier transform greatly enhanced the accuracy of size determination by minimizing the fluctuation of selecting region of interests in the space domain. This method had been validated in our previous work and was described in detail in Figure S4. After collecting the SPRM signals of ~500 gold nanoparticles, a histogram could be created to demonstrate the size distribution as shown in Figure 1B.

In order to demonstrate the reproducibility of sizing, three independent experiments were sequentially performed to generate three histograms of the same gold colloids sample. They were found to be consistent with each other (Figure 1B). The obtained histograms exhibited similar Gaussian distribution sizes as that revealed in TEM results (Figure 1B), demonstrating the capability of SPRM to count and size as-synthesized 20 nm AuNPs.

It is clear that the size distribution of original AuNPs must be as narrow as possible in order to sensitively detect the analyte-induced aggregation. Thus, a filter membrane separation (cutoff diameter of 0.05 μm) was applied to the as-prepared gold colloids to avoid the false positive signal by removing any large-sized dust and unexpected aggregates formed during the synthesis and storage. The size distributions of original and filtered gold colloids were displayed in Figure 1C. It was found that the filtration step obviously reduced the population of large-sized AuNPs. This result further demonstrated the validity of SPRM to size AuNPs.

We subsequently demonstrated the capability of SPRM to size polydisperse gold colloids, which were produced by artificially mixing two colloids with average diameters (determined by TEM) of 20 and 40 nm (Figure S5), respectively. As shown in Figure 2A,B, pure samples of 20 and 40 nm gold colloids exhibited a well-defined single peak with average SPRM intensities of 0.5 and 2.8 × 10^4 (arbitrary unit), respectively. Previous results demonstrated that SPRM signal was proportional to the volume of nanoparticles. The measured SPRM signal ratio (∼6) was close to the volume ratio (∼8), which could be estimated from the cubic power of diameter ratio. The slight difference might be attributed to the deviation of AuNPs diameters measured by TEM. For the size distribution of mixed sample shown in Figure 2C, the histogram consisted of two distinguishable peaks with expected SPRM intensity values further validating the feasibility of SPRM in sizing mixed AuNPs with different diameters. We also demonstrated the capability of SPRM to count the relative number of AuNPs with different sizes. It was found that the number ratio of different sized NPs matched well with the artificial concentration ratio (Figure S6). Therefore, we believed there was no obvious size bias in our SPRM system when distinguishing the mixed populations.

Melamine-Induced Aggregation of Gold Nanoparticles. Melamine-induced aggregation and adenosine triphosphate (ATP)-induced disaggregation were selected to examine the performance of SPRM in determining analyte concentration by digitally imaging and counting AuNPs, respectively. The melamine- and ATP-induced aggregation of AuNPs under the experimental conditions was also confirmed by UV−vis, DLS, and TEM measurements, respectively (Figure S1).

Size distributions of diluted gold colloids in the presence of varied melamine concentrations were determined by SPRM as shown in Figure 3A−G. It was clear that the percentage of large-sized AuNPs gradually increased with the increasing melamine concentration, which was expected in an analyte-induced aggregation process. By choosing a threshold value as determined by the average SPRM value of over 200 individual nanoparticles plus three times the standard derivation (magenta-colored dash lines), the number percentages of aggregates exhibited a linear relationship with the logarithm of melamine concentration as shown in Figure 3H. The lowest detectable analyte concentration was 1 nM, which was 1 order of magnitude lower than the literature reports that measured the same analyte with traditional colorimetric assays, as well as other instrument-based detections, such as electrochemistry, chemiluminescence, fluorescence, and liquid chromatography.

The improved sensitivity was attributed to two factors. First, this digitized assay directly counted the number of gold aggregates with high accuracy, even though its population was extremely low. This method avoided common noise sources in the extinction measurements, for instance, the light intensity fluctuations in the optical system as well as moving mechanical parts like gratings. Second, since less than 1000 individuals were collected in a typical measurement, the concentration of AuNPs required in the present approach was orders of magnitude lower than that of the traditional assay, so that much higher molar ratio between analyte molecules and AuNPs could be achieved. As-synthesized gold colloids were usually diluted by

![Figure 2. Size distributions of AuNPs with TEM diameter of 20 nm (A) and 40 nm (B), respectively. (C) Size distribution of polydispersed AuNPs by mixing an equal amount of 20 and 40 nm AuNPs.](image-url)
approximately 25 times prior to use. Under such low AuNPs concentration, the extinction measurement with a common UV-vis spectrometer was difficult (Figure S7). In order to compensate for the reduced collision possibility between AuNPs with such a low concentration, the gold colloids were incubated in the presence of analytes at 37 °C to enhance the reaction efficiency.

ATP-Induced Disaggregation of Gold Nanoparticles. Analyte-induced disaggregation or dispersion was another commonly used strategy in the colorimetric assay. In such a case, preformed stable aggregates collapsed to generate AuNPs monomers again in the presence of analyte, accompanied with a color recovery from blue or purple to red. Surface modification of gold nanoparticles was always required to achieve an efficient disaggregation.

We chose the ATP-induced disaggregation of aptamer-functionalized AuNPs as a model system in the present work. The target-free ATP aptamer was first hybridized with its complementary DNA sequence to form a rigid duplex. In the presence of target ATP molecule, aptamers preferentially bound to ATP, leading to the disassembly of original duplex and the release of a single stranded DNA (ss-DNA). The released ss-DNA molecules thus adsorbed to AuNP, which significantly enhanced its resistance to salt-induced aggregation. Therefore, when incubated with the same amount of salt, gold colloids with higher ATP concentration tended to be more stable and kept the original size distribution.

Size distributions of gold colloids with different ATP concentrations were displayed in Figure 4A–H. It was found that, as expected, the number percentage of aggregates decreased with the logarithm of ATP concentrations in the range of 5 nM to 10 μM as shown in Figure 4I. The lowest detectable concentration of 5 nM was significantly lower than the previous reports using the colorimetric method for the detection of ATP.33,37,38 This sensitivity level was also comparable with other quantitative measurements of ATP.44−47

CONCLUSIONS

In this work, we demonstrated a SPRM technique that could investigate the size distribution of AuNPs by imaging and counting individual AuNPs. Such capability was subsequently utilized to determine the analyte concentration by taking advantage of analyte-induced AuNPs aggregation or dispersion. The quantification was based on measuring the number percentage of gold nanoparticle aggregates, leading to a digital read-out of aggregation status of AuNPs. The digital read-out was free of any fluctuations from the light source and mechanical system and thus exhibited greatly improved sensitivity in both model systems examined in the present work. On the other hand, because SPRM measures the influence of nanoparticles to a propagating surface plasmon wave excited on the surface of gold film, any nanoparticles with different refractive index from the buffer medium could be detected. SPRM is able to study the analyte-induced aggregation of many nonplasmonic nanoparticles with poor extinction properties, which can hardly be studied with the traditional colorimetric assays. The digital read-out thus holds a great potential to expand the applications of the colorimetric assay involving broad kinds of colorless nanoparticles.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b04244.

Experimental details, characterization of gold nanoparticles, Fourier transformation of SPRM images, mechanism of colorimetric detection of ATP, data analysis, and descriptions of movies. (PDF)

SPRM imaging of individual AuNPs and their intensities (AVI)
Differential SPRM images of AuNPs attach to the SPR chip surface for illustrating the “hit and move” collision event. (AVI)

Differential SPRM images of AuNPs attach to the SPR chip surface for illustrating the “hit and stay” collision event. (AVI)

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**Notes**
The authors declare no competing financial interest.

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