Spectral Image Contrast Based Flow Digital Nanoplasmon-metry For Ultrasensitive Antibody Detection

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Research

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Abstract

Background: Gold nanoparticles (AuNPs) have been widely used as local surface plasmon resonance (LSPR) immunoassay for biomolecule sensings. It is primarily based on two conventional methods - absorption spectra and colorimetric. While the low figure of merit (FoM) of the LSPR and high-concentration AuNPs requirement restrict their limit of detection (LOD), which is around ng to μg per mL in antibody detection if there is no other signal or analytes amplification. By now, the improvement of sensitivity has bogged down for years. It also reveals a great challenge of the current LSPR immunoassay in biosensing - pushing the boundary of the current LOD.

Results: In this work, we developed a spectral image contrast-based flow digital nanoplasmon-metry(FDNM) to push the LOD boundary. Comparing the scattering image brightness of AuNPs in two neighboring wavelength bands near the LSPR peak, the signal of peak shift is extremely amplified and quickly detected. Introducing the digital analysis, the FDNM provides an ultra-high signal-to-noise ratio and less sample volume requirement. Compared to conventional analog LSPR immunoassay, FDNM has a LOD down to 1 pg mL^{-1} within only a 15-minute detection time and $500\text{ }\mu\text{L}$ sample volume. Antibody against spike proteins of SARS-CoV-2 in artificial saliva that contained various proteins was also conducted to validate the detection of FDNM in complicated samples. Of the detection, FDNM shows significant discrimination with a LOD of 10 pg mL^{-1} and a broad dynamic detecting range of five orders of magnitude.

Conclusion: Together with the quick readout time and simple operation, this work outstandingly demonstrated the high sensitivity and selectivity of the developed FDNM in rapid antibody detection. The spectral image contrast and digital analysis further provide a new generation LSPR immunoassay of AuNPs.

Background

Local surface plasmon resonance (LSPR) is a particular SPR triggered by electromagnetic illumination. A coherent oscillation of free electrons occurs on the surface of plasmonic nanoparticles (NPs), such as gold and silver nanoparticles (Au, Ag NPs).[1, 2] Given that, LSPR of Au and AgNPs shows a resonance peak in the visible wavelength regime, and a noticeable light scattering or absorption is presented.[3] Also, the peak resonance wavelength is related to the surface refractive index on the plasmonic NPs. It indicates once analytes attaching onto the surface, the LSPR peak would shift (typically is redshift). Thus, it can be used as a label-free ruler to quantify the analytes. Owing to these unique properties, plasmonic NPs have been widely used for biomolecular sensings such as antigens and antibodies. They are also well-known as the LSPR immunoassay for various disease detections.[4–7]

There are kinds of LSPR immunoassays, primarily based on the two conventional methods, the UV-visible absorption spectrum (UV-Vis),[8–10] and colorimetric detection, including naked-eyed and lateral flow assays (LFAs).[11–14] The UV-Vis employs the absorption spectra to evaluate the biomolecule binding

according to the LSPR peak shift of the monodispersed NPs. However, the shift is tiny and the limit of detection (LOD) is restricted mainly by the low figure of merit (FoM) of the LSPR sensor. On the other hand, naked-eyed and LFAs detect the analytes based on the intense color change induced by the aggregation of NPs either in the liquid phase or on test papers. Its simplicity, high user-friendliness, and low cost make it the most prevalent assay for the point-of-care test. But at the same time, a significant color change indicates the requirement of a great number of NPs. Considering the number of analytes loading on each NPs, it results in an inferior LOD (ng to μg per mL) to the UV-Vis if there is no other signal or analytes amplification. By now, the improvement of sensitivity has bogged down for years, and it also reveals a great challenge of current LSPR immunoassay in biosensing - push the boundary of the current LOD.[15]

To break the deadlock, we proposed a spectral image contrast-based flow digital nanoplasmon-metry to improve sensitivity. The idea of spectral image contrast comes from precise measurement of the change of central position using two-segments-based sensors. In the spatial distribution, the movement of the central position of an optical spot can be well determined by a one-dimensional position-sensitive sensor. In the spectral distribution, the resonant peak wavelength can also be determined by using two bandpass filters near the resonant peak. As diagramed in *Figure 1 (a)*, the spectral contrast (SC) is the scatter intensity ratio in two segmental wavelengths bands (band A and B) neighboring the LSPR peak.[16] In the beginning, the scatter LSPR peak of the AuNPs is close to the border between the two segmental wavelength bands. Their integral areas in scatter spectra are approximated. When presented as the scatter images, the images passed these two bandpass filters would show equal brightness, as shown as the p1 in *Figure 1 (b)*. As the analytes are binding on, the LSPR peak redshifts and thus presents a much brighter scatter in band A than B, as shown as the p2 and p3 in *Figure 1 (b)*. Based on the brightness change in scattering images, we derive the spectral image contrast, $\gamma = (I_A - I_B) / (I_A + I_B)$, from the SC. The difference between I_A and I_B (the $(I_A - I_B)$) indicates the analogously spectral contrast related to the LSPR peak shift. The normalization to the sum of I_A and I_B (the $(I_A + I_B)$) can efficiently eliminate the intensity fluctuation from the light scattering. As diagramed in *Figure 1 (a-b)*, the γ would be close to 0 at the beginning (p1), and the $\gamma = (I_A - I_B) / (I_A + I_B)$ would increase as analytes binding on (p2 and p3). Overall, spectral image contrast weights the LSPR shift to enhance the variation and provides a quick spectral evaluation in LSPR immunoassay. The spectral contrast method can also be combined with a CCD to fast read LSPR shifts of single gold nanoparticles in the fluidic channel. This method offers outstanding peak wavelength linearity, high wavelength resolution, fast response time, and a simple optical system.

Moreover, in the traditional LSPR immunoassay, the signal is an averaged output of an assembly of AuNPs. Thus, some positive-detected could be submerged in the average signal, and then it's hard to cross over the threshold for a minimum detectable signal (as shown in *Figure 1 (c)*). Recently, to improve sensitivity, digital analysis has become a trend of biomolecule detection.[17–19] The idea of digital detection is to dividually examine the ultra-small detection units such as each single AuNPs, and the output becomes the frequency of the positive-detected. Based on the spectral image contrast, the digital LSPR image (D-LSPRI) analysis of AuNPs was also developed, as depicted in *Figure 1 (c)*. The digital

detection takes advantage of the binary decision to provide a much higher signal-to-noise (S/N) ratio and lower LOD. Furthermore, the one-by-one examination could also achieve a significant statistic while only need less sample volume. The introduction of a microfluidic channel further facilitates the sampling and digital LSPRI analysis.

To verify the capability, we compared the LOD of antibody detection to the other two conventional LSPR immunoassay methods—UV-Vis and naked-eyed assay. Within the integration of LSPR immunoassay, D-LSPRI, and microfluidic system, the FDNM performs a LOD of 1 pg mL^{-1} of the anti-BSA, four to seven orders of magnitude lower than UV-Vis and naked-eyed assay. Antibody against the spike protein of SARS-CoV-2 in artificial saliva that added various human proteins and antibodies were also used to validate the sensitivity and specificity in the complicated sample. The result shows a LOD of 10 pg mL^{-1} and a six-order-of-magnitude broad dynamic range. The apparent data discrimination from 0 pg mL^{-1} further demonstrates the outstanding sensitivity of the FDNM in complicated samples. Though the LOD is revised in the complicated sample, the developed FDNM still presents the same grade of LOD (10 pg mL^{-1}) as ELISA while being label-free, much more accessible, and having a quicker detection time (< 15 minutes).

Results And Discussions

Setup of the Flow Digital Nanoplasmon-metry

Based on the concept of the single AuNPs examination described above, flow digital nanoplasmon-metry that integrated a microfluidic chip and a darkfield illumination system was developed, as illustrated in *Figure 2 (a)*. First, the microfluidic chip leads a streamed AuNPs into the dark-field illumination system. It was made of stacking layers of glasses, acrylic junctions, and double-sided tapes. The glasses were first treated with surface hydrophobic modification by the vapor-phase deposition to prevent the non-specific bindings from flowing AuNPs. Briefly, glasses were first put in a chamber together with one mL of trichloro(1H,1H,2H,2H-perfluorooctyl)silane (PTOCTS), and the chamber was then vacuumed and kept at around 0.5 atm for 30 minutes. Glasses were then baked at 120°C for one hour to facilitate the covalent binding between PTOCTS and glasses. The contact angles measurement results shown in *Figure S1* indicate a hydrophobic surface of the glasses. Glasses were subsequently sealed and tubed, and the microchannel nature was defined by two layers of double-sided tapes (3M, thickness = $60 \mu\text{m}$), as shown in *Figure 2 (b)*.

The dark-field illumination system can quickly record the LSPR information of each AuNPs flowed in the microfluidic chip. It was based on the Olympus upright microscopy. A 60x (NA = 0.7) air type objective and a dark-field condenser (Cyto Viva) with a 20-Watts halide light source were used in this work. AuNPs were drawn into the microchannel described above in the following experiments, and the scattering light of AuNPs would be excited by the dark-field illumination. The scattering would then pass through a 530-nm short-pass dichroic mirror that the cutoff is located at the peak of the LSPR scattering of the bare AuNPs, and be separated into two channels with two color bands (green and yellow). Light in each

channel would consequently pass through two bandpass filters to further extract significant bands of LSPR of AuNPs. The two used filters have un-overlapped neighboring filter bands, and the transmitted wavelength bands were 540 ± 10 nm (filter A) and 520 ± 10 nm (filter B). The LSPR spectra of AuNPs were transferred into intensity information by using a dichroic mirror and two bandpass filters and were quickly recorded as images by a scientific complementary metal-oxide-semiconductor (sCMOS) camera. *Figure 2 (c)* also shows the real scattering images of bare AuNPs in the split parallel channels A and B, and the bright spots indicate single AuNPs. In the data collection, AuNPs were first tracked in channel A (yellow circled) and colocalized in channel B (green circled), as shown in *Figure 2 (d)*. Meanwhile, the intensities of AuNPs in the parallel channels were also recorded for the following digital LSPRI analysis.

Digital-LSPR-Image Analysis

Figure 3 (a) shows the intensity scatter plot of the bare AuNPs, and the BSA modified AuNPs ($\text{BSA}_{100\%}\text{@AuNPs}$) before and after the anti-BSA conjugation. Each dataset contains 15000 data points, and the detecting time is less than 15 minutes, and it shows a linear distribution between the I_A and I_B . There are three component factors, I_A , I_B , and the location in the plot. Though the location of the data points is slightly inclining to the weaker I_B while more robust I_A after the BSA modification and the conjugation of anti-BSA, it is still too complicated to evaluate the dataset from this coordinate. Therefore, we introduced the principal component analysis (PCA) to extract and process the required digital LSPR information.[20] Based on the distribution on the intensity scatter plot, we use an orthogonal transformation to convert a set of possibly correlated high dimensional data (the location, I_A , and I_B) into a set of linearly uncorrelated variables, which are the counts (N) and the spectral contrasts $\gamma = (I_A - I_B)/(I_A + I_B)$ here, as shown in *Figure 3*. By defining a new orthogonal coordinate system, the LSPR shifts of AuNPs are optimally described in a digital dataset. The difference between I_A and I_B (the $(I_A - I_B)$) indicates the analogously spectral contrast related to the LSPR peak shift.[16] The normalization to the sum of I_A and I_B (the $(I_A + I_B)$) efficiently eliminates the intensity fluctuation from the light scattering. The counts show the significant LSPR shift statistic distribution and help us study the variation of the dataset trends more straightforward.

Figure 3 (b) shows the γ value distributions versus the AuNPs counts, which exhibits a Gaussian distribution. We can find that with the increasing surface-attached biomolecules (bare, BSA modified, and then anti-BSA conjugated), the γ values of most data points increased, and thus the distribution shifted to the higher γ . This result is consistent with the principle of LSPR immunoassay that the protein binding induces the redshift of LSPR in resonant spectra, as depicted in *Figure 1 (a)*. However, there is still a slight difference between the conventional and digital LSPR analyses. In the traditional LSPR analysis, the readout is the average shift of the LSPR peak. As for the digital LSPR analysis, the readout is the frequency of the positive-detected, and it signifies more biomolecule binding induces more the AuNPs that cross over the LSPR threshold. Thus, the threshold set plays a critical role in digital analysis. In this work, we set a cutoff at the upper 95 % confidence interval (CI) of the $\text{BSA}_{100\%}\text{@AuNPs}$ as the binary

threshold to define the positive-detected. The upper 95 % CI connotes that without the analytes (negative control, N. C.), 97.5 % of the dataset lies behind the cutoff and presents as negative-detected. In contrast, the rest 2.5 % of the dataset is considered as the background (N_0). The readout would be the relative positive-detected counts (N/N_0) increment follows the analytes attached, as shown in the inset of *Figure 3 (b)*.

Influences of the Probe Protein to PEGylation Ratio on AuNPs to the Analysis

As described before, the readout in the digital analysis is the positive-detected number of the immunoagents, the AuNPs. To increase the sensitivity, it needs to increase the positive-detected number of AuNPs within limited analytes as more as possible. Therefore, the probe proteins modified onto the AuNPs play a crucial role in the sensitivity of the digital analysis. Generally, when the probe protein ratio rises, each AuNP could provide more binding sites to the analytes. However, it might result in an uneven division of the limited analytes in the high probe ratio condition. Some AuNPs capture more analytes, while the others can only get fewer or even none from the rest. In the digital LSPR immunoassay, a minimum analyte on the AuNPs is necessary for crossing over the threshold. The uneven distribution would result in the drop of the positive-detected counts. On the other hand, the very low probe protein ratio would result in insufficient binding sites on the AuNPs. They are barely detectable even if the binding sites are fully occupied with the analytes. There is a trade-off of the probe protein ratio on AuNPs to maximize the counts of digital detection and optimize the sensitivity.

In the experiment, we used the PEG-(NH₂)₂ to adjust the probe protein ratio on AuNPs. The PEG-(NH₂)₂ is a flexible linear polymer, which can be immobilized onto AuNPs *via* strong physisorption as same as the probe proteins.[21, 22] Meanwhile, the addition of PEG-(NH₂)₂ can facilitate the stability of AuNPs in a complicated matrix.[23] Three ratios of BSA to PEG-(NH₂)₂, 1:0, 1:4, and 1:9 in wt% were modified on AuNPs (marked as BSA_{100%}@AuNPs, BSA_{20%}@AuNPs, and BSA_{10%}@AuNPs, respectively), and *Figure S2* shows their UV-Vis absorption spectra. The BSA/PEG-modified AuNPs interacted with different concentrations of anti-BSA solution. The anti-BSA binding sensitivities and dynamic range were compared. *Figure S3* shows the I_A versus I_B intensity scatter plots and *Figure 4 (a-c)* are their corresponding γ distributions before and after conjugations of the anti-BSA with various concentrations. We can find that the γ -distributions vary with the surface modifications on AuNPs. The rising ratio of the PEG-(NH₂)₂ leads to the descending cutoff (upper 95 % CI of N. C.). It is attributed to that the addition of PEG-(NH₂)₂ reduces the equivalent surface molecule weight and refractive index on AuNPs, which is consistent with the redshift results of the UV-Vis absorption peak. The result demonstrates the reliability of the developed digital method in detecting the LSPR shift with different surface molecule-absorptions on AuNPs.

The detection sensitivities were evaluated by detecting different concentrations of anti-BSA from 0 pg mL⁻¹ to 1 µg mL⁻¹. From the results displayed in *Figure 4 (d)*, the use of BSA_{100%}@AuNPs does not appear to have evident signal discrimination in detecting anti-BSA below 100 ng mL⁻¹. It is because of the uneven division of the limited analytes and consequently reduces the positive-detected counts. Once the anti-BSA molecules are enough (> 100 ng mL⁻¹), most AuNPs can receive sufficient analytes to cross the cutoff and result in a signal jump. For the BSA_{10%}@AuNPs, despite an increase in the signal in correlation with the low anti-BSA concentration, the higher concentration gives rise to the saturated binding on AuNPs. The saturation limits the detecting dynamic range. In comparison, BSA_{20%}@AuNPs performs excellent sensitivity and large dynamic range from 1 pg mL⁻¹ to 1 µg mL⁻¹ (> six orders of magnitude) without the saturation. It is noted that the data deviation in the repeated experiments is increased with the amount of PEG-(NH₂)₂. This result suggests the non-uniformity of the anchor protein distribution on AuNPs. Considering the overall performance of sensitivity, dynamic range, and standard deviation, the BSA_{20%}@AuNPs are used as the digital LSPR immuno-agents.

Comparison of Flow Digital Nanoplasmon-metry with Traditional LSPR Immunoassays

After the optimization, the flow digital nanoplasmon-metry (FDNM) performance was also compared with the traditional LSPR immunoassay, the UV-Vis absorption spectra, and naked-eye-based detection. First, it should be noted that with a limited analyte, a higher number of the immuno-agent would pose fewer analytes loading on each agent. As a consequence, it would be detrimental to the LOD. Therefore, to optimize the LOD of these three methods, each methods' minimal detectable AuNPs concentrations were tested, thereby maximizing their analytes-to-immuno-agent ratio. *Figure S4* displays the results of detecting various concentrations of 50-nm bare AuNPs. The minimal detectable concentration is 5 × 10⁹ NP mL⁻¹ for naked-eye and 5 × 10⁸ NP mL⁻¹ for the UV-Vis absorption spectra and FDNM.

In this comparison, the BSA_{20%}@AuNPs with the minimal detectable concentration were used as the immuno-agent for each method to detect various concentrations of anti-BSA. *Figure 5* shows the results, and the LOD of naked-eye, UV-Vis absorption and FDNM is 10 µg mL⁻¹, 10 ng mL⁻¹, and 1 pg mL⁻¹, respectively. Unlike UV-Vis absorption spectra and FDNM, the signal of naked-eye detection is mainly contributed from the aggregation of AuNPs and its consequent color change.[24, 25] It generally needs numerous analytes to knock off the stability and monodispersity of the colloidal AuNPs. Besides, the higher agent concentration demand also reduces the analyte-to-agent ratio, which further disadvantages the detection. Eventually, it would need a much higher concentration of analytes to trigger a visible signal output and results in a much higher LOD. As for the UV-Vis absorption spectra and FDNM, the basis of the signal output of both of them is contributed from the redshift of the LSPR peak. However, it is intriguing that even used the same concentration of immuno-agent, the LOD of UV-Vis absorption spectra is four orders of magnitude higher than the FDNM. We attributed it to the much lower signal-to-noise ratio within the analog measurement. The signal of the UV-Vis absorption spectra is the average LSPR peak shift,

which is an analog output that requires an internally even signal increase of every AuNPs. Therefore, bits of positive-detected signals might be submerged in the average. By contrast, the FDNM provides a digital detection that allows binary decisions for each AuNPs. The discrete counting enables the detection to pick out the individual positive-detected, thereby increasing the signal-to-noise ratio even under the same analyte-to-agent ratio with analog measurement. According to the results, *Figure 5 (d)* schematically diagrams the LODs and dynamic ranges that the analog and digital LSPR immunoassay can achieve. This data thoroughly demonstrates that digital detection benefits biomolecule detection with orders of magnitude lower LOD and broader dynamic detecting range, consistent with the previous studies.[18, 19]

Detection of the Antibody against SARS-CoV-2 Spike Protein

COVID-19, a highly infectious respiratory disease caused by a newly discovered virus-SARS-CoV-2, induces millions of deaths worldwide. However, with the processing of the vaccine, herd immunity has become the key to restart social and economic activity. To facilitate the restart, governments are considering the immune passport, which provides a certificate that people get the antibody to protect them against the virus and not contagious.[26–28] Thus, antibody detection against the SARS-CoV-2 plays a crucial role in the coming post-COVID-19 era. Nowadays, enzyme-linked immunosorbent assay (ELISA) is considered the gold standard in clinical antibody detection because of its high sensitivity (~ 10 pg mL⁻¹). However, the compromise between the limit of detection (LOD) and detection time (hours and even days) is always an issue that has been hampered for years, not to mention the onerous requirements caused by expensive instruments and specialized operators. These drawbacks push scientists to look for more convenient methods while keeping a similar or higher LOD grade.

In the pilot experiments above, we have demonstrated the FDNM's reliability, quick response (≤ 15 minutes), high sensitivity (LOD ~ 1 pg mL⁻¹), and broad dynamic ($>$ six orders of magnitude). Here, we further demonstrate the capability of the developed FDNM in antibody detection against the spike protein of SARS-CoV-2, and *Figure 6* shows the results. According to the upper 95 % CI of the SP per-modified 50-nm AuNPs (SP_{20%}@AuNPs), the cutoff was set, which is 0.255. Compared to the BSA_{20%}@AuNPs, the larger cutoff is attributed to the larger molecule weight (~ 101 kDa) of the SP, and it is consistent with the UV-Vis absorption spectra as displayed in *Figure S6*. Using the SP_{20%}@AuNPs as the immuno-agent, the shifts to larger γ values in the AuNPs count versus γ distribution can be observed as the mAb_{SP} increases (*Figure 6 (a)*). It results in an increasing AuNPs count number that crosses over the set cutoff. By contrast, the detection of anti-BSA, which is used as the specificity test, does not appear any noticeable shift at the concentration of 100 ng mL⁻¹ and under. While even it shows a jump at the concentration of 1 μ g mL⁻¹ as shown in *Figure 6 (c)*, the signals of mAb_{SP} with concentrations higher than 10 pg mL⁻¹ are still evidently distinguishable. However, this result also suggests that sensitivity might fluctuate due to the matrix influence of the LSPR immunoassay. To clarify the matrix influence, we further use artificial saliva with and without spiked human antibodies to demonstrate detection in complicated samples.

Studies have demonstrated that the SARS-CoV-2 entry human body is mainly via the nasopharynx and can stimulate the secretory antigen-specific antibody responses.[29–31] No matter in blood or saliva, selectivity in complicated biological media largely determines the accessibility of the launch of LSPR immunoassays. Based on the potential for the onsite sampling requirement of more accessibility and safety in the future, we chose the artificial saliva containing human serum albumin and multiple antibodies to mimic human saliva as the complicated sample.[32] The detection of increasing concentration of mAb_{SP} in the mimicking human saliva (saliva buffer, S. B.) was then conducted. *Figure 6 (d-e)* displays the result, and where the blank is the artificial saliva without adding any proteins. As the complicated sample, it can be found that the cutoff set according to the 95 % CI of N. C. is larger than in the pure sample (TE buffer, *Figure 6 (a-b)*). Plus, the difference of N/N₀ between the blank and N. C. is approximate to the 1 µg mL⁻¹ of anti-BSA in *Figure 6(c)*. This result is attributed to the matrix influence in the complicated sample. The matrix influence affects the LOD of FDNM. However, the approximate N/N₀ of the different additives (proteins) and base matrices also indicates limited impact. From *Figure 6 (d-e)*, though the cutoff was raised, there is still an evidently increasing AuNPs count number that crosses over the set cutoff as the mAb_{SP} is expanding. The apparent count discrimination from N. C. (0 pg mL⁻¹) demonstrates the outstanding capability of the FDNM in detecting antibodies against SARS-CoV-2 spike protein in saliva. It shows a LOD of 10 pg mL⁻¹ and a six-order-of-magnitude broad dynamic range at least. Though the LOD is revised in the complicated sample, the developed FDNM shows the same grade of LOD (10 pg mL⁻¹) as ELISA while being label-free, much more accessible, and having a quicker readout time.

Conclusion

Testing is considered a key to control the epidemic, and antibody is an essential index to evaluate the efficacy of the developed vaccine and the herd immunity in an area in the post-COVID-19 era. Doubtlessly, not only for the outbreak of COVID-19 but also the other faster, widespread, and more fatal new pandemics in this era. In this work, we developed a flow digital nanoplasmon-metry (FDNM) and successfully demonstrated the capability in antibody detection against the SARS-CoV-2 spike protein in pure and complicated samples. Integrating local surface plasmon resonance (LSPR) immunoassay, digital local surface plasmon resonance image(D-LSPRI), and the microfluidic system, FDNM has advantages of label-free, high sensitivity, quick readout, and high accessibility in sampling. By comparing the UV-Vis absorption spectra and the counts versus γ distribution of AuNPs modified with various ratios of BSA and PEG-(NH₂)₂, FDNM shows high reliability discriminating the surface RI on AuNPs. Furthermore, in the anti-BSA detection compared to the conventional analog LSPR immunoassay-the UV-Vis absorption spectra and naked-eye, the outstanding performance of high sensitivity and broad dynamic range of FDNM and the digital analysis was successfully demonstrated.

Given the simple, accessible, and non-invasive sampling properties, salivary tests are considered an attractive option in this pandemic era. However, the great challenge of salivary antibody tests is the two to three orders of magnitude lower concentration of the analytes than in serum. To overcome the

drawback, an ultrasensitive while simple detection is urgently demanded. In the experiments, the FDNM shows a much lower LOD in a complicated sample, which is 10 pg mL^{-1} , than most other analog LSPR-immunoassay as listed in *Table S2*. [33–45] The result also presents significant discrimination and dynamic detecting range in the complicated saliva samples. Taken together with its quick readout time (less than 15 minutes) and simple operation, it well demonstrated the capability of the FDNM in rapid detection against spike protein of SARS-CoV–2 in saliva. Not only for the immune certificate, but the FDNM can also be used to facilitate the evaluate the efficiency of the vaccine processing, which needs simple, accessible, sensitive, and frequent testing. It is of great worth both in science and society.

Materials And Methods

Materials

Poly(ethylene glycol) bis(amine) (PEG-(NH₂)₂, Mw 2000), tris-EDTA (TE) buffer (pH = 8), trichloro(1H,1H,2H,2H-perfluorooctyl)silane (PTOCTS), artificial saliva (pH = 6.8), human serum albumin (HSA), immunoglobulin A (IgA from human serum, Product No.: I2636, Lot. No.: 091M4758), immunoglobulin G (IgG from human serum, Product No. : I2511, Lot. No.: 081M4859), and immunoglobulin M (IgM from human serum, Product No. : I8260, Lot. No.: 108M4827V) were purchased from Sigma-Aldrich, Taiwan. It should be noted that the sampling dates of Human IgA, IgG, and IgM are all prior to 2019, the outbreak of COVID–19, and thus the samples contain no antibody against the SARS-CoV–2. The citrate-capped spherical gold nanoparticles (AuNPs) with a diameter of 50 nm were purchased from nanoComposix, U.S. SARS-CoV–2 spike protein (SP, Mw ~ 101.4 kDa), and corresponding antibody (mAb_{SP}) were purchased from Sino Biological Inc., China. Fetal bovine serum (FBS) was purchased from Invitrogen, Taiwan.

In human saliva, there are kinds of albumin and immunoglobulins. Thus, to simulate the human saliva as much as possible, according to the reference[32], HSA, IgA, IgG, and IgM were added into the artificial saliva with the final concentration $60 \text{ } \mu\text{g mL}^{-1}$, $140 \text{ } \mu\text{g mL}^{-1}$, $16 \text{ } \mu\text{g mL}^{-1}$, and $4.1 \text{ } \mu\text{g mL}^{-1}$ as the saliva buffer (SB). It was then stored at 4 °C for the following experiments.

Surface modification of spherical gold nanoparticles for mAb_{SP} detection

One milliliter of gold nanoparticles (AuNPs) with an initial particle concentration of $4 \times 10^{10} \text{ particles mL}^{-1}$ was first centrifuged at a condition of 5000 rpm and 10 minutes at 4 °C, and then the suspension was removed. The same volume of TE buffer, which contained $20 \text{ } \mu\text{g mL}^{-1}$ of SP and $80 \text{ } \mu\text{g mL}^{-1}$ of PEG-(NH₂)₂, was used to redisperse the AuNPs, and the mixture was kept at 4 °C. After 12 hours, the unbound protein and PEG-(NH₂)₂ were then removed by centrifugation (5000 rpm, 10 minutes at 4 °C). SARS-CoV–2 spike protein-coated AuNPs (SP_{20%}@AuNPs, 1 mL in microtube) were subsequently washed by

centrifugation again and then redispersed in the tris-EDTA buffer. The final concentration of SP_{20%}@AuNPs was adjusted to 5×10^{10} particles mL⁻¹ as the stock solution and stored at 4 °C for the following experiments.

Working process

The FDNM is carried out in a darkfield illumination system and the microfluidic channel. In the experiments, AuNPs per-modified with probe protein (BSA or SP) were first mixed with the sample (500 µL) with a final concentration of 5×10^8 NP mL⁻¹. The mixture was kept at 37 °C for 30 minutes to facilitate the conjugation of the probe protein on AuNPs to the target analytes (antibodies) in the sample. After that, the mixture was drawn into the microfluidic channel by a syringe pump, and the flow rate was kept at 5 µL per minute. The scattering images of the flowing AuNPs were recorded by the optical setup described before at a frame rate of 1 fps. The scattering intensity contrast of each AuNPs in imaging channels A and B was used as the digital-LSPR-image analysis for antibody detection. In each sample, 15000 AuNPs would be counted as a dataset, and the detecting time was less than 15 minutes. After the detection, the channel was flushed with Aqua regia and deionized water to remove the residue of AuNPs inside.

Declarations

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare no potential competing financial interest.

Authors' contributions

S. H. Wang and P. K. Wei designed the experiment. S. H. Wang, C. W. Kuo, and S. C. Lo performed the experiment with the assistance of W. K. Yeung and T. W. Chang. S. H. Wang, C. W. Kuo, S. C. Lo coded the analysis program and visualized the data. S. H. Wang and P. K. Wei supervised the study and completed the manuscript. All authors read and approved the final version.

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Figures

Figure 1

(a) Spectral contrast in LSPR immunoassay. (b) Schematic diagram of the spectral image contrasts in response to AuNPs conjugated with the different number of analyte antibodies. (c) Schematic diagram of the digital LSPR detection. The magenta dash lines indicate the threshold for the minimum detectable signal of each analysis.

Figure 2

(a) Configuration of the flow digital nanoplasmon-metry (DFNM) based on a darkfield illumination and split imaging system. (b) The nature of the lab-built microfluidic channel. (c) The scatter image of bare AuNPs in the split channel A and B and (d) were traced by the Matlab program.

Figure 3

(a) Intensity scatters plot of the bare AuNPs (black dots), and the BSA modified AuNPs (BSA100%@AuNPs) before (red dots) and after (blue dots) the anti-BSA conjugation. (b) AuNPs counts versus spectral contrasts $\gamma = (I_A - I_B)/(I_A + I_B)$ distribution extracted from (a).

Figure 4

AuNPs counts versus spectral contrasts $\gamma = (I_A - I_B)/(I_A + I_B)$ distributions of (a) BSA_{100%}@AuNPs, (b) BSA_{20%}@AuNPs, and (c) BSA_{10%}@AuNPs conjugated with different concentration of anti-BSA. (d) The $\Delta N/N_0$ signals in response to the various concentration of the anti-BSA that conjugated to BSA_{100%}@AuNPs, BSA_{20%}@AuNPs, and BSA_{10%}@AuNPs. The sample number of each condition is six, and the error bar is represented by $\pm$ SD.

Figure 5

Readout signals of (a) naked-eye, (b) UV-Vis absorption spectra, and (c) FDNM in response to the various concentration of the anti-BSA conjugated to BSA20%@AuNPs. The red lines indicate the readout signal of LOD, and the magenta rectangles indicate the detecting dynamic range in this pilot experiment. The sample number of each method is six, and $\pm$ SD represents the error bar. (d) The schematic plot summarizes the LOD and dynamic range that the analog (UV-Vis absorption) and digital (FDNM) based LSPR immunoassay can achieve in this work. The format of this schematic plot was based on reference [19].

Figure 6

AuNPs counts versus spectral contrasts γ distributions of SP_{20%}@AuNPs conjugated with different concentrations of (a) mAb_{SP} and (b) anti-BSA in TE buffer. (c) the digital readout signals $\Delta N/N_0$ in response to the mAb_{SP} and anti-BSA. The red lines indicate the readout signal of LOD. (d) AuNPs counts versus spectral contrasts γ distributions of SP_{20%}@AuNPs conjugated with different concentrations of mAb_{SP} in the artificial saliva (without any additive proteins, blank) and saliva buffer (S.B.) that contains HSA, IgA, IgG, and IgM. (e) the statistic $\Delta N/N_0$ in response to the mAb_{SP}. The sample of each condition is six, and the error bar is represented by $\pm$ SD.

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