Digital plasmonic nanobubble detection for rapid and ultrasensitive virus diagnostics

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Rapid and sensitive diagnostics of infectious diseases is an urgent and unmet need as evidenced by the COVID-19 pandemic. Here, we report a strategy, based on DiGitAl plasMONic nanobubble Detection (DIAMOND), to address this need. Plasmonic nanobubbles are transient vapor bubbles generated by laser heating of plasmonic nanoparticles (NPs) and allow single-NP detection. Using gold NPs as labels and an optofluidic setup, we demonstrate that DIAMOND achieves compartment-free digital counting and works on homogeneous immunoassays without separation and amplification steps. DIAMOND allows specific detection of respiratory syncytial virus spiked in nasal swab samples and achieves a detection limit of ~100 PFU/mL (equivalent to 1 RNA copy/µL), which is competitive with digital isothermal amplification for virus detection. Therefore, DIAMOND has the advantages including one-step and single-NP detection, direct sensing of intact viruses at room temperature, and no complex liquid handling, and is a platform technology for rapid and ultrasensitive diagnostics.
The ability to rapidly detect diseases with high precision is of paramount importance as evidenced by the current COVID-19 pandemic. Digital immunoassays have been a remarkable conceptual advance over the past two decades due to their capabilities of single-molecule detection and absolute quantification. They partition the analytes into microwells or emulsion droplets as small compartments for independent signal amplification and digital counting, leading to the sensitivity enhancement by up to 10^5-fold over the conventional immunoassays (i.e., enzyme-linked immunosorbent assay) and detects analytes without the need for separation, amplification, and physical compartments (such as droplets or microwells) at room temperature.

Results

DIAMOND allows single-nanoparticle (NP) detection and sizing. We first evaluated the ability of DIAMOND for single-NP detection. Suspensions of 75 nm AuNPs (as characterized in Figs. S2, S3, and Table S2) were injected into a 200 µm capillary by a syringe pump with a flow rate of 6 µL/min (Fig. S1a). The fluid was irradiated and detected synchronously by the aligned pump and probe lasers (Fig. S1b and S1c). We set a laser scanning speed of 1000 µm/s and flow speed of 2500 µm/s to avoid particles being counted twice. For simplicity, we converted the particle concentration into the expected average number (λ) of AuNPs per detection zone:

\[ \lambda = c \cdot V \]  

(1)

where \( c \) is particle concentration (NPs·mL^{-1}) and \( V \) is 16 µL. Figure 2a shows the representative DIAMOND results for the given \( \lambda \), where discrete PNB signals with fluctuated intensity could be observed when \( \lambda \leq 4 \). To explain this, we plotted the Poisson distributions (Fig. S4) based on the below equation:

\[ f(k; \lambda) = P(x = k) = \frac{\lambda^k e^{-\lambda}}{k!} \]  

(2)

where the \( k \) is the actual AuNP number per detection zone, and \( P \) is the Poisson probability. It is evident that the probability of zero AuNP (\( k = 0 \)) passing through the detection zone increases with decreasing \( \lambda \) and thus leads to discrete PNB signals. In particular, in the case of \( \lambda = 0.04 \), each PNB signal highlighted in Fig. 2a refers to a single AuNP being detected according to the Poisson distribution (Fig. S4d). This result confirms the capability of DIAMOND for single-NP detection. To correlate the PNB signals with AuNP concentration, we calculated the frequencies of positive PNB signals in each trace as “on” signals (\( f_{on} \)) and plotted it as a function of \( \lambda \) (Fig. 2b). A high correlation coefficient (\( R^2 = 0.998 \)) indicates the accurate AuNP quantification via \( f_{on} \) counting. Furthermore, we found a linear relationship (slope = 0.985, \( R^2 = 0.998 \), Fig. 2c) between the theoretical probability (\( P \)) predicted by Poisson statistics and the experimental \( f_{on} \) suggesting that DIAMOND is a calibration-free technique and allows absolute quantification.

Next, we used DIAMOND to detect AuNPs of different sizes. AuNP suspensions (15, 35, 50, and 75 nm) at the same concentration (Figs. S2, S3, and Table S2) were prepared for the test. Figure 2d shows the representative PNB signal traces. Due to a large number of AuNPs per detection zone (\( \lambda = 240 \)), consistent PNB signals were observed in each laser pulse. Figure 2e shows representative PNB signals taken from each case and suggests that larger NP size leads to larger PNB signals.

To quantify this relationship, we extracted the values of amplitude and area-under-the-curve (AUC) from each PNB signal (Fig. S5) and plotted their distribution profile. They partition the analytes into microwells or emulsion droplets as small compartments for independent signal amplification and digital counting, leading to the sensitivity enhancement by up to 10^5-fold over the conventional immunoassays (i.e., enzyme-linked immunosorbent assay) and detects analytes without the need for separation, amplification, and physical compartments (such as droplets or microwells) at room temperature.

DIAMOND allows characterizing heterogenous NP suspensions. We then evaluated the ability of DIAMOND to detect a heterogeneous population of NPs. The rationale is that the NP clusters or aggregates discussed later may act as bigger particles. We first tested detecting 75 nm AuNPs in the background of 15 nm NPs. Figure 3a shows the representative PNB signal traces taken from three sets of samples, including 15 and 75 nm AuNPs and a mixture of them. The large intensity variation of PNB signals observed in the mixture sample is due to the addition of 75 nm AuNPs. To sort those specific signals resulting from 75 nm AuNPs in the mixture, we developed a three-step analytical protocol. First, we extracted the amplitude and AUC values of each PNB signal as indices for quantification and plotted the corresponding results in bivariate (Fig. 3b). Individual scatters represent independent PNB events measurements. Second, we fitted normal distributions to the amplitude and AUC of 15 nm
AuNPs as a reference (Fig. 3b, case i) and calculated the threshold (T) using five times standard deviations (σ) above the mean (μ). As such, the two thresholds covered over 99.9% of the scatters. Last, we assigned the thresholds to the mixture sample for data sorting. The scatters above the thresholds refer to the positive or “on” PNB signals resulting from 75 nm AuNPs in the mixture (Fig. 3b, case ii). For direct comparison, we benchmarked it with f_{on} from 75 nm AuNPs alone (thresholds = 0, Fig. 3b, case iii) and the Poisson probability (Fig. 3c). The agreement between experimental results and theoretical prediction indicates that our method provides accurate and absolute quantification of large NPs from a strong background of small ones (i.e., 1 in 240). Importantly, the capability of DIAMOND to detect heterogeneity without an additional separation step may have potential implications as an analytical tool for NPs and requires further study.

**DIAMOND allows sensitivity enhancement for a homogeneous assay using NPs.** We further evaluated the feasibility of implementing DIAMOND for the homogeneous assay. Homogeneous assays are simple, one-step sensing methods that require minimal liquid handling and are promising for rapid detection. To validate our hypothesis in a model system, we used the AuNPs as probes to detect SiO_{2} beads (Fig. 4a). Figure 4b shows the transmission electron microscopy (TEM) image of the as-prepared product, where a core-satellites structure was formed with AuNPs fully covering the surface of SiO_{2} beads. Figure 4c, d
shows representative PNB signal traces and corresponding bivariate plot taken from serial assay solutions, respectively. Similarly, the thresholds \( (T = \mu + 5\sigma) \) of amplitude and AUC were calculated from the control group (highlighted in dashed lines) and assigned for counting “on” signals. The \( f_{on} \) was then plotted as a function of \( \lambda \) or particle concentration of SiO\(_2\) beads (Fig. 4e). A linear relationship \( (R^2 = 0.99) \) in the range of \( 0.0016–0.16 \) was observed and the limit of detection (LOD) was calculated to be \( 0.0028 \), equivalent to \( 1.75 \times 10^5 \) mL\(^{-1} \) or 290 aM (inset of Fig. 4e). Such detection limit is ~570-fold lower than the colorimetric detection (Fig. S6). It should be pointed out that the detection range of DIAMOND only covers 2 logs due to the limited counting number (i.e., 3000 pulses). Alternatively, we can use an analog method to analyze PNB signals (e.g., averaged AUC versus analyte concentration), which should provide an additional detection range beyond 100% \( f_{on} \). Furthermore, DIAMOND allows absolute quantification for homogeneous assays, as indicated by the linear correlation (slope = 1.007, \( R^2 = 0.999 \)) between the background-subtracted frequency \( (f'_{on}) \) and the Poisson probability (Fig. 4f).

**DIAMOND allows rapid and sensitive diagnosis of intact virus.** Finally, we applied DIAMOND to rapidly detect the respiratory syncytial virus (RSV). RSV is the major respiratory pathogen that accounts for up to 74,500 deaths in 2015 globally in children below age 5\(^{31}\). We chose Synagis (Palivizumab) as the detection antibody and conjugated it on the AuNPs through 3,3′-dithiobis(sulfosuccinimidyl propionate) as a cross-linker (Fig. S7). We used 15 nm AuNPs as labels because they can be prepared with uniform size and shape. The AuNP-Synagis probes specifically recognized the fusion proteins on the RSV surface at room temperature.
temperature and were ready for detection (Fig. 5a). Figure 5b shows the TEM images of AuNP probes targeting the RSV of different morphologies (highlighted in red). Figures S8 and 5c show the colorimetric detection of different respiratory viruses, including Parainfluenza viruses (PIV), Influenza viruses A (IVA), Human metapneumovirus (hMPV), and purified RSV. The results suggest the good detection specificity using AuNP probes and a LOD of 3.6 × 10^4 PFU/mL for RSV in colorimetric detection. For a direct comparison, we benchmarked the immunoassay results against the commercially available lateral flow immunoassay (LFIA) kit (BinaxNOW, Abbott), which has a LOD of 1.6 × 10^4 PFU/mL (Fig. S9). We then carried out DIAMOND for RSV detection and provided representative detection results in Fig. 5d. Following the same protocol discussed above, we set the thresholds based on the control group (Fig. 5d) and assigned them for f_on counting (Fig. 5e). In particular, a linear relationship (R^2 = 0.995) in the range of 10^2–10^4 PFU/mL was observed, and the LOD was calculated to be 108 PFU/mL (inset of Fig. 5e). Evidently, DIAMOND achieved a sensitivity enhancement of ~333-fold over the colorimetric result of homogeneous immunoassay and ~150-fold over the commercial LFIA.

To further demonstrate the potential clinical applications, we applied DIAMOND to detect RSV spiked in the nasal swab samples. It should be mentioned that when used for control virus detection (i.e., hMPV, PIV, and IVA, dispersed in borate buffer), the RSV-specific AuNP probes caused non-specific binding as suggested by the positive PNB signals (Figs. S11 and 6a, b). This phenomenon can be ascribed to that the control viruses were received in cell culture fluid that contains impurities like cell debris and thus leads to the non-specific aggregation. To address this issue, we used bovine serum albumin (BSA) to backfill the AuNP probes (inset of Figs. 6c and S12). The BSA has been frequently used in immunoassays as block reagent and can prevent the non-specific binding for improved detection specificity32. To evaluate the performance of BSA-backfilled AuNP probes, we incubated them with respiratory viruses spiked in nasal swab samples and subjected the complete assay solution to the DIAMOND test. The viral transport medium (VTM), hMPV, PIV, and IVA were used as negative controls, and all viral titers were kept the same as 10^5 PFU/mL. Figures S13 and 6c, d show the virus detection results using BSA-backfilled probes, where the positive PNB signals from the control viruses were n. s. (ii) Mixture, f_on=41.6% (iii) 75 nm, f_on=38.9%

Fig. 3 Identification of large AuNP in a heterogeneous AuNP solution by DIAMOND. a Representative PNB signals traces (100 pulses) for 15 and 75 nm AuNPs and their mixture. Schematics represent the sample information. b Bivariate plots of amplitude and AUC extracted from 3000 pulses for the three samples in (a). Cases (i–iii) refer to the suspensions of 15 nm, mixture, and 75 nm AuNPs, respectively. The dashed lines in cases (i) and (ii) indicate the same thresholds of amplitude (T_Amp) and AUC (T_AUC). The gray arrow in case (iii) highlights scatters at 0. c Bar plot of experimental frequencies (f_on) as determined in (b) for cases (ii) and (iii) and theoretical probability predicted by Poisson statistics. n.s. stands for no significant difference (p-value > 0.05). The error bars for experimental f_on indicate the standard deviations of three independent measurements.
Fig. 4 Detection of SiO2 beads in a homogeneous assay by DIAMOND. a Schematic of a homogeneous assay of SiO2 beads by AuNPs as a pair of targets and probes at room temperature (RT). Lower panel shows that when bead concentrations are insufficient to induce the color change, DIAMOND can detect the PNB signals. b TEM image of SiO2-AuNPs conjugates. Scale bar in inset is 200 nm. c Representative PNB signal traces (100 pulses) for the assay solutions. Schematics represent the assay information. d Bivariate plot of amplitude and AUC extracted from 3000 pulses for the assay solutions with different λ of SiO2 beads. Dashed lines indicate the positions of thresholds calculated from the control sample. e Quantification of SiO2 bead concentration and λ (SiO2) as a function of frequency counting (f_{on}). A logistic fitting is applied for the calibration curve. Inset shows the linear region of the calibration curve. The LOD was calculated as 3 standard deviations of the control dividing the slope of regression line. f Linear correlation between the background-subtracted frequency (f_{on}) and Poisson probability. Error bars in (e) and (f) indicate the standard deviations of three independent measurements.

significantly reduced, while the PNB signals from RSV can be easily distinguished from the control samples, yielding f_{on} = 100% that matches well with the theoretical probability. In contrast, the non-backfilled probes still cause non-specific aggregation upon detection (Fig S14). This result suggests that DIAMOND has a better detection specificity when utilizing BSA-backfilled probes. Similar to that of the purified viruses (Fig. 5), these probes enable sensitive detection of spiked RSV in nasal swab samples with a detection limit of 102 PFU/mL (Figs. S15 and 6c, f). Taken together, these data demonstrated that implementing DIAMOND on a homogeneous immunoassay allows sensitive analysis of viral samples in the human specimen matrix and supports the potential clinical applications.

To compare DIAMOND with other state-of-the-art methods, we performed measurements using digital loop-mediated isothermal amplification (dLAMP). dLAMP is a rapid molecule test and provides absolute quantification of nucleic acids, which has been used to quantify a variety of viruses.33-36 To perform the dLAMP, we used a commercially available fluorescent LAMP kit and microwell chips to detect genomic RNA from RSV (A2 strain). A set of primers (Table S3) were used according to a previous publication7. Figure S16a–e shows the fluorescence images of the dLAMP results after incubating at 65 °C for 30 min, where the fraction of positive wells (brighter) reduces as the concentration of target RNA decreases. A calibration curve (R^2 = 0.9998, Fig. S16f) was established as a function of the RNA inputs. The LOD was estimated to be 2 copies/µL using synthetic RNA (inset of Fig. S16f). We then used this method to detect RSV from the spiked samples. The RSV extracts were collected using a commercially available RNA extraction buffer and purified with a fluorescent LAMP kit. Figure S17a–d shows the dLAMP detection results for RSV (MT strain). A set of primers (Table S3) were used according to a previous publication7. We also used DIAMOND to detect RSV in nasal swab samples. The RSV extracts were collected using a fluorescent LAMP kit. Figure S17e–f shows the dLAMP detection results for RSV (MT strain). A set of primers (Table S3) were used according to a previous publication7. We also used DIAMOND to detect RSV in nasal swab samples. The RSV extracts were collected using a fluorescent LAMP kit.
Digital immunoassays create high standards as next-generation diagnostic platforms, such as calibration-free quantification and single-molecule detection of biomarkers for early diagnostics. The major barriers to its widespread use are the time-consuming protocols and laboratory infrastructures. Here we developed DIAMOND to overcome some of these bottlenecks. Specifically, DIAMOND uses a homogeneous immunoassay format and does not require additional sample washing, separation, and signal amplification steps. On the other hand, DIAMOND does not require chip preparation or on-chip reaction and can be performed with less assay time than the digital homogeneous assays. Also, DIAMOND can detect intact viruses without additional liquid handling (i.e., virus extraction, thermal incubation, and chip loading), and thus offer a simplified diagnostic approach at room temperature.

In the present study, we focused on developing and validating a versatile digital immunoassay. We envision several further improvements to this technology in order to bring it to a broad range of labs and practical applications. First, it is possible to design a small benchtop device for the plasmonic nanobubble (PNB) measurements that can be distributed to other labs. By replacing the research-grade picosecond pulse laser with a smaller nanosecond (ns) laser (e.g., Wedge-HB-532, RPMC), all components can be integrated into a benchtop device (15 × 15 × 6 inches, Fig. S18). Evaluation of PNB generation and detection by this ns laser shows robust results across a range of AuNP concentrations (Fig. S19). More importantly, the ns laser provides a repetition rate up to 2000 Hz for a much faster readout and thereby more efficient event counting. Second, further optimization of the optofluidic system can increase the sampling efficiency for the PNB measurements. In the present system, we used the readily available micro-capillary although the laser probes only a small portion of the sample (20–40% along and 5–10% orthogonal to the flow direction, respectively, Fig. S20). A low sampling efficiency leads to fewer events counted for a given sample volume and thus limits the dynamic range and sensitivity for the detection. A microfluidic flow-focusing system can readily solve this problem by creating a narrower flow path. Taking advantage of the high-throughput ns laser and focusing flow, we expect to increase the event counting (e.g., 1 million readings within 10 min, similar to flow cytometer). This will improve the detection range and sensitivity of DIAMOND (Fig. S21 and Table S4) because the digital counting performance essentially relies on the number of counted events. Last, it is worth exploring different modes of DIAMOND operation. Since the PNB generation is dependent on the laser fluence, we can perform the DIAMOND test in two modes by modulating the laser fluence above and below the PNB generation threshold, referred to as above- and below-threshold modes. Currently, we used the above-threshold mode with a high laser fluence at \( P_{\text{PNB}} = 100\% \) (blue arrow of Fig. S22), resulting in the generation of PNB signals from both the small NPs and larger clusters. Alternatively, we may adopt the below-threshold mode at a lower laser fluence (\( P_{\text{PNB}} = 0\% \), red arrow of Fig. S22) to only activate large clusters for PNB generation.

It is also possible to explore the feasibility of DIAMOND to detect smaller analytes than viral particles, such as protein...
b biomarkers. This, together with multiplexed detection, is important yet challenging for in vitro diagnosis\textsuperscript{10,18,41}. Due to the small size and limited binding sites of proteins, they can only absorb a few NPs on the surface. As a result, it may be challenging to reliably differentiate protein-specific PNB signals from background signals. Therefore, we can set up a diluted setting with a reliable differentiation of PNB signals from backfilling signals from zero, single, and multiple NPs passing through are unique\textsuperscript{10}. In this case, it is crucial to ask whether DIAMOND can recognize PNB signals from events when single and multiple NPs pass through together. As a first step, we have tested and validated a threshold-based gating method\textsuperscript{42} to determine the PNB signals generated from a specific number (k = 0, 1, 2...) of AuNPs (Fig. S23) using the case of λ = 0.4 shown in Fig. 2a as a model. In addition, multiplexed detection is also a critical aspect of a competitive diagnostic method. Future studies can focus on demonstrating the feasibility of multiplexing protein detection.

In summary, we have developed DIAMOND for virus detection via the AuNPs-based homogeneous immunoassay. DIAMOND allows single-NP detection and identifies large particles from a heterogeneous population. Such capability allows DIAMOND to be implemented on homogeneous assays without sample washing. Importantly, DIAMOND can specifically detect RSV among different respiratory viruses and achieve a detection limit of ~100 PFU/mL (or 1 RNA copy/µL). Compared with other digital assays, DIAMOND counts events in a compartment-free manner and utilizes a one-pot protocol for sample handling at room temperature. Our study provides a digital counting platform for rapid and ultrasensitive diagnostics of intact viruses at their early representation.

**Methods**

**Materials.** Tetrachloroauric(III) trihydrate (HAuCl₄·3H₂O, 16961-25-4, 99.9%), sodium citrate tribasic dihydrate (Na₃C₆H₅O₇·2H₂O, 6132-04-3, ≥99%), and hydroquinone (123-31-9, ≥99%), sodium chloride (NaCl, 7647-14-5, ≥99.5%) were purchased from Sigma-Aldrich. Sodium citrate tribasic dihydrate (Na₃C₆H₅O₇·2H₂O, 6132-04-3, ≥99%), sodium chloride (NaCl, 7647-14-5, ≥99.5%) were purchased from Sigma-Aldrich, 3,3-dithiobis (sulfosuccinimidyl propionate) (DTSSP, 21578, 50 mg) and borate buffer (1M, 28341) were purchased from Thermo Scientific. Sucrose ((57-50-1)), Magnesium sulfate hydrate (MgSO₄·7H₂O, 22189-08-8), HEPES (7365-45-9), Dulbecco's modification of Eagle's medium (DME) (Thermo Fisher Scientific), Dulbecco's modified Eagle's medium (DME) (Gibco 11050), and fetal bovine serum (FBS) (Gibco 10090) were purchased from Thermo Fisher Scientific.

**Notes.** The corresponding standard deviations of three independent measurements are shown in Fig. 5b, d, and f. Inset in (b), (d), and (f) show the schematic of AuNP-based probes used correspondingly. Inset in (f) shows the linear detection range and LOD. Error bars in (b), (d), and (f) indicate the standard deviations of three independent measurements.
Methods

**Nanoparticle synthesis and characterization.** Gold nanoparticle (AuNP) seeds were first synthesized using classical Plech Turkевич method with slight modifications. Briefly, 1 mL of HAuCl₄ aqueous solution (25 mM) was added to a clean 25 mL Erlenmeyer flask filled with 20 mL of pure water and allowed to boil on a hot plate with magnetic stirring. Subsequently, 1 mL of Na₂Ca₂H₂O (11.2 mM) aqueous solution was quickly injected into the boiling solution with a pipette. The solution was kept stirring and boiling for 10 min until its color turned red. After cooling the solution to room temperature, δH₂O was added to bring the volume to 100 mL. The products were stored in the dark at room temperature for future use. AuNPs with larger sizes were synthesized according to a seed-mediated growth method with slight modifications, where the above-mentioned AuNPs were employed as the seeds. In brief, the appropriate amount of pure water, HAuCl₄ precursor, Na₂Ca₂H₂O, and AuNP seeds were added orderly to a 250 mL flask under a gentle stirring at room temperature according to Table 2. Then the reducing aqueous solution containing a certain amount of hydroquinone was injected rapidly into the flask. The solution immediately switched color to purple and then to red in a few minutes. The reaction was left overnight at room temperature. Final products were stored in the dark at room temperature for future use. Note that all the particles’ concentrations in this study were determined based on the size-dependent empirical formula with a combination of UV-vis measurement and transmission electron microscope (TEM).

**Bioassay statements.** The research project was approved and performed strictly in adherence to CDC/NIH guidelines and the experimental protocols were approved by the University of Texas at Dallas Institutional Biosafety & Chemical Safety Committee and the University of Texas Southwestern Medical Center Biosafety Committee. Human Metapneumovirus (hMPV, CAT# 0810163FC) and Parainfluenza virus type 1 (PIV, CAT# 0810014CF) were purchased from ZeptoMetrix, and H1N1 influenza virus type A (IVA, CAT# IHA-003) was purchased from ProSpec-Tany TechnoGene Ltd. Human respiratory syncytial virus (RSV) A2 strain (CAT# VR-1540) was purchased from ATCC.

**Conjugation of AuNPs and Synagis as detection probes for RSV.** Conjugation of AuNPs and Synagis was adapted from a previous report. In brief, 5 mM DTSSP as a cross-linker was reacted with primary amines of Synagis at a molar ratio of 125:1 in 2 mM borate buffer first, then followed by an overnight membrane dialysis process to eliminate free DTSSP before concentrated using 100 kDa Amicon centrifugal filter. The resulted product (DTSSP-Syn) was then mixed with 15 nm AuNPs suspension in 2 mM borate buffer at a molar ratio of 500:1 and incubated overnight at 4 °C. Afterward, the products (15 nm AuNP probes) were washed three times with 2 mM borate buffer and re-dispersed in 2 mM borate buffer, and then kept at 4 °C for storage before further testing.

As for BSA backfilling, we utilized DTSSP as a covalent linker to modify BSA with a molar ratio of 125:1 in 2 mM borate buffer, and then incubated the 0.1% of DTSSP-BSA with 15 nm AuNPs probes on the ice bath for 1 h. The products (BSA-backed AuNPs probes) were washed three times using 2 mM borate buffer and re-dispersed in 2 mM borate buffer. The samples were kept at 4 °C for storage before further use.

**Large scale propagation of RSV A2 in HEp-2 cells.** RSV A2 strain was propagated in HEp-2 cells. HEp-2 cells were cultivated in EMEM/5% FBS and infected at a multiplicity of infection (MOI) of 0.01 to minimize the production of detectable interfering particles. Viral working stocks were prepared using 30–60% (w/v) non-continuous sucrose density centrifugation (Beckman Coulter SW-28 rotor, 28,000 rpm, 4 °C for 90 min). The virus-containing band at the 30–60% interface was collected (Fig. S23), distributed into aliquots, and stored at −80 °C.

**Detection of viruses using the AuNP-based probes.** Purified RSV and other closely related respiratory viruses such as hMPV, PIV, and IVA (used as received) were each extracted using the method of AuNPs-Synagis complexes (PS). Subsequently, the samples were sonicated and vortexed before mixing with 15 nm citrate-AuNPs suspension. The plaque-forming unit of viral working stocks was calculated based on the median tissue culture infectious dose (TCID₅₀) to quantify the RSV infectivity titer.

**Detection of virus in complex sample matrix using the BSA-backed AuNPs probes.** We used BD Universal Viral Transport Collection Kits to collect nasal samples from the noses of healthy adults (a group of 3 males, age range 30 ± 5). The collected samples were stored at −70 °C until use. The use of human nasal swab samples was approved by Institutional Review Board (IRB) at University of Texas at Dallas (ID: 20MR0993).

**Plasmmonic nanobubble generation and detection.** The plasmonic nanobubble (PNB) generation was conducted using ultraprot laser pulse (28 ps, 532 nm, Passum Instruments, Lomita, CA). The laser was focused into a thin capillary (VitroPhase, 300 μm) and irradiated by a pump laser. The laser fluence was adjusted by rotating the beam attenuator (ThorLabs, BVA05-532) and was measured using a laser power meter (FieldMaxII-TOP, Coherent). PNB signals were monitored by a continuous laser as probe beam (Red HeNe laser, 633 nm, R-30989, Newport) and its intensity was recorded with a photodetector (PFP510-FV, Thorlabs) and an oscilloscope (LeCroy WaveRunner204Xi-A).

For the 75 nm AuNPs detection, serial suspensions with concentrations equaling λ = 0.2, 0.1, 0.05, 0.025, and 0.01 mL/cm² were prepared and tested at a laser fluence of 10,000 mL/cm². All the measurements were recorded one minute per sample (3000 pulses at 50 Hz).

For the size differentiation assay, serial suspensions of AuNPs with 15, 35, 50, and 75 nm in diameter and 1.5 × 10¹⁰ mL⁻¹ (λ = 240) were concentration and tested under a laser fluence of 2000 mL/cm². For the mixture sample test, the particle concentrations of two AuNP suspensions were set the same as 1.5 × 10¹⁰ mL⁻¹ (λ = 240) and 3.125 × 10¹⁰ mL⁻¹ (λ = 0.5) for 15 and 75 nm AuNPs, respectively. The PNB detection was performed at a laser fluence of 7500 mL/cm². For the SiO₂ beads detection, the as-purified SiO₂ beads in ethanol were first centrifuged and washed with δH₂O twice and re-dispersed in 2 mM citrate-HCl buffer (pH = 3) with a particle concentration of 1 × 10¹⁰ mL⁻¹. The SiO₂ beads were sonicated and vortexed before mixing with 15 nm citrate-AuNPs suspension. The mixture was incubated for 30 min at room temperature before further tests. The PNB detection was performed at a laser fluence of 3000 mL/cm².

For the virus detection, the prepared assay solutions were directed to the DIAMOND test at a laser fluence of 3000 mL/cm².

**Digital LAMP (dLAMP) reaction.** For the synthetic RSV RNA detection, we mixed 10 µL of LAMP master mix, 2 µL of 10x primer mix (Table S3), 0.5 µL of fluororescent dye, and 7 µL of RNA template, and 1.5 µL of nucleic-free water at room temperature. We then loaded 15 µL of the mixture into the microwell array chip and subsequently sealed it with oil. The chip was incubated at 65 °C in a heating block for 30 min and subjected to fluorescent imaging using a slide scanner (Olympus VS 120) with the x2 objective.

For the RSV detection, 10 µL of RSV sample was mixed with 5 µL of extraction buffer at room temperature for 10 min and subjected to the purification following the manufacture’s instruction. The final product was dispensed in 40 µL elution buffer and stored in −20 °C before use. For the RNA detection, we mixed 10 µL of LAMP master mix, 2 µL of 10x primer mix (Table S3), 0.5 µL of fluorescent dye, and 7 µL of RNA extracts, and 1.5 µL of nucleic-free water at room temperature. We then loaded 15 µL of the mixture into the microwell array chip and subsequently sealed it with oil. The chip was incubated at 65 °C in a heating block for 30 min and subjected to fluorescent imaging using a slide scanner with the x2 objective.

**MATLAB for PNB detection.** The raw data of PNB signals were processed by a customized MATLAB script. The major functions of the script include pre-filteration, PNB signal recognition by signal-to-noisy-ratio (SNR), parameters extraction, threshold calculation, and frequency counting for “on” signals.

**MATLAB for fluorescent image analysis.** The fluorescent images of dLAMP detection were processed by a customized MATLAB script. The major functions of the script include image reading, determining the signal of microwells via pattern recognition, threshold calculation for each well, and frequency counting for “on” signals.
Characterization techniques and data analyses. The absorbance of samples in microtiter plates was read using microplate reader (Synergy 2, BioTek). The dynamic light scattering (DLS) was measured using Malvern ZetaSizer Nano ZS. Extinction spectra were obtained with a spectrophotometer (DU800, Beckman Coulter). The TEM images were taken using a JEOL JEM-2010 microscope operated at 120 kV. The pH values of buffer solutions were measured using a pH Meter (Accumet AP71). All data were collected in no less than triplicate and reported as mean and standard deviation for statistical analysis. A two-sample t-test assuming equal variance in Origin software was conducted to determine p values and statistical significance.

Poisson statistics. The theoretical prediction by Poisson statistics in our study was calculated based on Eq. (2). Specifically, the data in Fig. S4 was generated using Microsoft Excel Worksheet (Data-Data Analysis-Random Number Generation-Poisson).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Datasets supporting the findings of this work are available within the paper and its supplementary information files. A reporting summary file for this article is available. Additional information for research purposes is also available from the corresponding authors upon request. Source data are provided with this paper.

Code availability

The software used in this study is described in the "Methods" section. MATLAB codes for PNB signal analysis and dLAMP image analysis are available at https://doi.org/10.5281/zenodo.5708858. Additional information is available from the corresponding authors upon request.

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References

1. Wang, C., Horby, P. W., Hayden, F. G. & Gao, G. F. A novel coronavirus outbreak of global health concern. Lancet 395, 470–473 (2020).
2. Broughton, J. P. et al. CRISPR-Cas12-based detection of SARS-CoV-2. Nat. Biotechnol. 38, 870–874 (2020).
3. Wu, A. H., Fukushima, N., Puskas, R., Todd, J. & Goix, P. Development and preliminary clinical validation of a high sensitivity assay for cardiac troponin using a capillary flow (single molecule) fluorescence detector. Clin. Chem. 52, 2157–2159 (2006).
4. Rissin, D. M. et al. Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations. Nat. Biotechnol. 28, 595–599 (2010).
5. Wu, C., Garden, P. M. & Walt, D. R. Ultrasensitive detection of attomolar protein concentrations by droplet single molecule assays. J. Am. Chem. Soc. 142, 12314–12323 (2020).
6. Chen, H. et al. Quantitation of femtomolar-level protein biomarkers using a simple microbubble digital assay and bright-field smartphone imaging. Angew. Chem. Int. Ed. 58, 13922–13928 (2019).
7. Chang, L. et al. Single molecule enzyme-linked immunosorbent assays: theoretical considerations. J. Immunol. Methods 378, 102–115 (2012).
8. Wu, Y., Bennett, D., Tilley, R. D. & Gooding, J. J. How nanoparticles transform single molecule measurements into quantitative sensors. Adv. Mater. 32, e1904339 (2020).
9. Farka, Z. et al. Advances in optical single-molecule detection: en route to supersensitive bioaffinity assays. Angew. Chem. Int. Ed. 59, 10746–10773 (2020).
10. Yelleswarapu, V. et al. Mobile platform for rapid sub-picogram-per-millilitre, multiplexed, digital droplet detection of proteins. Proc. Natl Acad. Sci. USA 116, 4489–4495 (2019).
11. Siriram, M. et al. A rapid readout for many single plasmonic nanoparticles using dark-field microscopy and digital color analysis. Biosens. Bioelectron. 117, 530–536 (2018).
12. Sevenler, D., Daaboul, G. G., Elkin Kanik, F., Unlu, N. L. & Unlu, M. S. Digital microrays: single-molecule readout with interferometric detection of plasmonic nanorod labels. ACS Nano 12, 5880–5887 (2018).
13. Farka, Z., Mickett, M. J., Hlavacek, A., Skladal, P. & Gorris, H. H. Single molecule upconversion-linked immunosorbent assay with extended dynamic range for the sensitive detection of diagnostic biomarkers. Anal. Chem. 89, 11825–11830 (2017).
14. Liu, X. et al. Digital duplex homogeneous immunoassay by counting immunocomplex labeled with quantum dots. Anal. Chem. 93, 3089–3095 (2021).
15. de Albuquerque, C. D. L., Sobral-Filho, R. G., Poppi, R. J. & Brolo, A. G. Digital protocol for chemical analysis at ultralow concentrations by surface-enhanced Raman scattering. Anal. Chem. 90, 1248–12454 (2018).
16. Zhang, P. et al. Plasmonic scattering imaging of single proteins and binding kinetics. Nat. Methods 17, 1010–1017 (2020).
17. Akama, K. et al. Wash-and-amplification-free digital immunoassay based on single-particle motion analysis. ACS Nano 13, 13116–13126 (2019).
18. Akama, K. & Noji, H. Multiplexed homogeneous digital immunoassay based on single-particle motion analysis. Lab Chip 20, 2113–2121 (2020).
19. Visser, E. W. A., Yan, J., van, I. L. J. & Prins, M. W. J. Continuous biomarker monitoring by particle mobility sensing with single molecule resolution. Nat. Commun. 9, 2541 (2018).
20. Lukionova-Hleb, E. Y. et al. Plasmonic nanobubbles as transient vapor nanobubbles generated around plasmonic nanoparticles. ACS Nano 4, 2109–2123 (2010).
21. Lukionova-Hleb, E. Y. et al. On-demand intracellular amplification of chemoradiation with cancer-specific plasmonic nanobubbles. Nat. Med. 20, 778–784 (2014).
22. Lukionova-Hleb, E. Y. et al. Intraoperative diagnostics and elimination of residual microtumours with plasmonic nanobubbles. Nat. Nanotechnol. 11, 525–532 (2016).
23. Li, J. et al. Rock the nucleus: significantly enhanced nuclear membrane permeability and gene transfection by plasmonic nanobubble induced nanomechanical transduction. Chem. Commun. 54, 2479–2482 (2018).
24. Xiong, H. et al. Near-infrared light triggered-release in deep brain regions using ultra-photosensitive nanovesicles. Angew. Chem. Int. Ed. 59, 8608–8615 (2020).
25. Galzanche, E. I. & Zharov, V. P. Photocoustic flow cytometry. Methods 57, 280–296 (2012).
26. Bonar, M. M. & Tilton, J. C. High sensitivity detection and sorting of infectious human immunodeficiency virus (HIV-1) particles by flow virometry. Virology 305, 80–90 (2017).
27. Qin, Z. & Bischof, J. C. Thermophotoluminescent and biological responses of gold nanoparticle laser heating. Chem. Soc. Rev. 41, 1191–1217 (2012).
28. Qin, Z. et al. Quantitative comparison of photothermal heat generation between gold nanospheres and nanorods. Sci. Rep. 6, 29836 (2016).
29. Godakhindi, V. S. et al. Tuning the gold nanoparticle colorimetric assay by nanoparticle size, concentration, and size combinations for oligonucleotide detection. ACS Sens 2, 1627–1636 (2017).
30. Rolando, J. C. et al. Real-time, digital LAMP with commercial microfluidic chips reveals the interplay of efficiency, speed, and background amplification as a function of reaction temperature and time. Anal. Chem. 91, 1034–1042 (2018).
31. Tomita, N. et al. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat. Protoc. 3, 877–882 (2008).
32. Hu, Y. et al. Absolute quantification of H5-subtype avian influenza viruses using droplet digital loop-mediated isothermal amplification. Anal. Chem. 89, 745–750 (2017).
33. Mahony, J. et al. Development of a sensitive loop-mediated isothermal amplification assay that provides specimen-to-result diagnosis of respiratory syncytial virus infection in 30 min. J. Clin. Microbiol. 51, 2696–2701 (2013).
34. Mawatari, K., Ohashi, T., Ebata, T., Tokeshi, M. & Kitamori, T. Thermal lens detection device. Lab Chip 11, 2990–2993 (2011).
35. Cossarizza, A. et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies. Eur. J. Immunol. 47, 1584–1797 (2017).
36. Kim, S. H. et al. Large-scale femtoliter droplet array for digital counting of single biomolecules. Lab Chip 12, 4986–4991 (2012).
37. Wilson, D. H. et al. The Simoa HD-1 analyzer: a novel fully automated digital immunoassay analyzer with single-molecule sensitivity and multiplexing. J. Lab. Autom. 21, 533–547 (2016).
42. Verschoor, C. P., Lelic, A., Bramson, J. L. & Bowdish, D. M. An introduction to automated flow cytometry gating tools and their implementation. *Front. Immunol.* **6**, 380 (2015).
43. Kimling, J. et al. Turkevich method for gold nanoparticle synthesis revisited. *J. Phys. Chem. B* **110**, 15700–15707 (2006).
44. Driskell, J. D., Jones, C. A., Tompkins, S. M. & Tripp, R. A. One-step assay for detecting influenza virus using dynamic light scattering and gold nanoparticles. *Analyt. Chem.* **136**, 3083–3090 (2011).
45. Levitz, R. et al. Distinct patterns of innate immune activation by clinical isolates of respiratory syncytial virus. *PLoS ONE* **12**, e0184318 (2017).
46. Scotti, P. D. End-point dilution and plaque assay methods for titration of cricket paralysis virus in cultured *Drosophila* cells. *J. Gen. Virol.* **35**, 393–396 (1977).
47. Liu, Y., Ye, H., Xie, C. & Qin, Z. Digital plasmonic nanobubble detection for rapid and ultrasensitive virus diagnostics. *Zenodo* 10.5281/zenodo.5708857 (2021).

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Author contributions

Y.L. and H.Y. carried out the experiment and performed the data analysis and contributed equally to this work. Y.L., H.Y., and H.H. prepared the virus samples. Y.L., P.K., and C.X. developed the MATLAB script. Y.L., H.Y., and Z.Q. wrote the manuscript. Z.Q. and J.S.K. conceived the original idea and supervised the project. H.Y. helped supervise the project. All authors revised the manuscript and have given approval to the final version of the manuscript.

Competing interests

Y.L., Z.Q., H.Y., and J.S.K. are the inventors on a provisional patent related to this work filed by University of Texas at Dallas. Z.Q. and J.S.K. hold equity interest in Avsana Labs, Incorporated, which aims to commercialize the DIAMOND technology. The remaining authors declare no competing interests.

Additional information

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