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A single-molecule flow platform for the quantification of biomolecules attached to single nanoparticles

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Abstract

We describe here a flow platform for quantifying the number of biomolecules on individual fluorescent nanoparticles. The platform combines line-confocal fluorescence detection with near nanoscale channels (1–2 μm in width and height) to achieve high single-molecule detection sensitivity and throughput. The number of biomolecules present on each nanoparticle was determined by deconvolving the fluorescence intensity distribution of single-nanoparticle-biomolecule complexes with the intensity distribution of single biomolecules. We demonstrate this approach by quantifying the number of streptavidins on individual semiconducting polymer dots (Pdots); streptavidin was rendered fluorescent using biotin-Alexa647. This flow platform has high throughput (hundreds to thousands of nanoparticles detected per second) and requires minute amounts of sample ($\sim 5 \mu\text{L}$ at a dilute concentration of 10 pM). This measurement method is an additional tool for characterizing synthetic or biological nanoparticles.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Author Contributions

SRJ, RH, WS, and DTC wrote manuscript. JY, CTK, YR, and XHZ produced polymer dots. SRJ, RH, WS, and YJ performed experiments and SRJ, RH, WS and YJ analyzed data. DTC supervised this project.

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Keywords

polymer dots; flow platform; single-molecule counting; high-throughput

INTRODUCTION

The past decades have witnessed the explosive development of nanoparticles, composed of a wide range of materials, such as gold nanoparticles,^[1] iron oxide nanoparticles,^[2] a variety of different polymer nanoparticles,^[3] and silica nanoparticles^[4]. These nanoparticles have been deployed in many biomedical applications, including immunofluorescence assays, cellular imaging, drug delivery, *in vivo* imaging, and therapy.^[1,3,5] Despite the diversity of these applications, one theme that runs through all of them is the need to functionalize the surfaces of the nanoparticles with the appropriate molecular recognition element, such as antibodies.^[6] Yet, currently no high-throughput flow method is readily available for quantifying both the average and distribution of the number of biomolecules conjugated to nanoparticles.

With bulk measurements, the average number of biomolecules per nanoparticle can be estimated by simply dividing the total number of biomolecules by the total number of nanoparticles.^[7] But the accuracy of bulk measurements is always in question because the total number of nanoparticles cannot be obtained accurately under many circumstances. For example, one common approach to determine the total number of nanoparticles is calculated from the total mass and the size of the nanoparticles present. However, size determination using dynamic light scattering can be inaccurate, and single-particle imaging techniques such as electron microscopy may work well for some particles, such as inorganic nanoparticles that do not shrink when dried, but not others, such as nanoparticles made from soft materials. Furthermore, many nanoparticles may not have spherical morphology, which can make size determination difficult.^[4,8]

Compared to bulk measurements, single-particle characterization and single-molecule counting methods can offer important advantages: they are usually more accurate than bulk estimates, but more importantly, they provide important information about the heterogeneity of the sample, that is, variabilities in the number of biomolecules conjugated to individual

nanoparticles. For example, using atomic force microscopy (AFM), individual quantum dots (Qdots) conjugated to streptavidin was imaged and the valence of each quantum dot was determined. Here, Qdot-streptavidin conjugates were incubated with biotinylated DNA, and AFM was used to count the number of DNA chains linked to each Qdot.^[8] As another example, our group previously developed a quantitative fluorescence microscopy method based on total-internal-reflection-fluorescence microscopy (TIRFM) to count the number of membrane proteins (tagged with fluorescent antibodies) present on the surface of single synaptic vesicles.^[9–11] While the imaging approaches provide valuable quantitative as well as visual information, they tend to be complex and tedious with low throughput. As a result, they are not suitable for routine characterization of nanoparticle-bioconjugates.

To address the lack of a high-throughput yet sensitive method for counting single molecules on nanoparticles, we here describe a flow platform that is capable of measuring the number of biomolecules attached to the surface of individual nanoparticles with single-molecule sensitivity while providing high-throughput (over hundreds of nanoparticles analyzed per second). This platform combines line-confocal fluorescence detection with near nanoscale channels (1–2 μm in width and height) to achieve high single-molecule detection sensitivity. As a proof-of-principle, we determined the number of streptavidin molecules conjugated to the surface of semiconducting polymer dots (Pdots), which is a new family of fluorescent nanoparticles that we and others have recently developed.^[3,12–14]

EXPERIMENTAL PROCEDURES

Line-confocal microscope Setup

The setup was modified from the instrument we described earlier.^[15] A Nikon TE2000 inverted microscope equipped with a 60x N.A. 1.3 objective was used. Two lasers (405 nm and 633 nm) were used for fluorescence illumination. Prior to entering the objective, each laser beam was shaped to form an elliptical beam with proper aspect ratio by using cylindrical optics. The same objective was used to collect the fluorescence signals. A rectangular pinhole (Melles Griot, Carlsbad, CA, Edmund Optics, Barrington, NJ, USA) was placed in the image plane. Dichroic mirrors were placed after the pinhole to split fluorescence to two separate avalanche photodiodes (APDs) (SPCM-AQR-14, Perkin-Elmer, Fremont, CA, USA). In front of each APD, a focusing lens, neutral density filter, and bandpass filter (HQ 455/50nm, HQ 675/50nm, Chroma, Rockingham, VT, USA) were inserted.

Streptavidin conjugation of Pdots

The detailed preparation procedure can be found in our previously published article.^[13] Briefly, we first prepared a solution containing 50 $\mu\text{g/mL}$ of conjugated polymer Poly(9,9-dioctylfluorenyl-2,7-diyl) end capped with dimethyl phenyl (PFO, MW 120000 Da, American Dye Source Inc., Quebec, Canada) and 16 $\mu\text{g/mL}$ of *Poly*(styrene-alt-maleic anhydride) (PSMA, Sigma–Aldrich, St. Louis, MO, USA) in tetrahydrofuran (THF). A 5-mL aliquot of the mixture was then quickly injected into 10 mL of water under vigorous sonication, and THF was removed by blowing nitrogen gas into the solution. In 4 mL of 20 nM THF-free Pdot solution, we added and mixed 80 μL of polyethylene glycol (5% w/v

PEG, MW 3350 Da), 80 μ L of HEPES buffer (1M, pH 7.3), 240 μ L (for calibration standard, 5 μ L was used) streptavidin (Invitrogen, Eugene, OR, USA) solution (1 mg/ml) and 80 μ L of freshly-prepared ethylcarbodiimide hydrochloride (EDC, Sigma–Aldrich, St. Louis, MO, USA) solution (5 mg/mL). The mixture was magnetically stirred for 4 hr at room temperature. The resulting Pdot-streptavidin conjugates were finally concentrated in a spin column (100K MW) and purified with a Bio-Rad Econo-Pac 10DG column (Hercules, CA, USA) filled with HR300 resin (Sigma-Aldrich, St. Louis, MO, USA). The hydrodynamic sizes of Pdots were measured with a dynamic light scattering (DLS) spectrometer (Malvern Zetasizer Nano ZS, Worcestershire, United Kingdom).

Preparation of Pdots with different sizes and density of surface functional groups and labeling of biotin-dye to streptavidin conjugated Pdots

Streptavidin conjugated to Pdots (Pdot-streptavidin) were first prepared using a nano-precipitation method reported earlier.^[13] Briefly, 4 mL of 50 ppm Pdots (molar concentrations for 26 nm and 34 nm Pdots were 9.0 nM and 4.0 nM, respectively), was mixed with HEPES buffer, 5% PEG, 10 mg/mL EDC and 1 mg/mL streptavidin. Then the mixture was magnetically stirred for 2.5–4 hours at room temperature in the dark. To identify singly labeled and fully labeled Pdot-streptavidin conjugates (Figure 4a), a series of experimental conditions with different molar ratios of Pdot:SA were set; the ratios were 1:0.5, 1:1, 1:10, 1:50, 1:100, and 1:200. Then biotin-Alexa647 was added to the conjugate solution to label Pdot-streptavidin conjugates with the far-red Alexa dye. 20 mM HEPES (pH 7.3), 0.1% PEG buffer solution containing 20 nM Pdot-streptavidin conjugates and 2 μ M biotin-Alexa647 (Cytodiagnostic, Ontario, Canada) were stirred at room temperature for overnight. Then the solution was purified with a Bio-Rad Econo-Pac 10DG column filled with HR300 resin (Sigma, St. Louis, MO, USA). The complexes were purified from excess free biotin-Alexa647 molecules in 10K MWCO Dialysis Cassettes overnight. The Pdot-streptavidin-biotin-Alexa647 complexes were measured on the platform and the median intensity of biotin-Alexa647 was analyzed. It should be noted that even the Pdots-streptavidin-biotin-Alexa647 complex was purified with size exclusion column and Dialysis Cassettes, a small amount of free biotin-Alexa647 molecules still remained in the complex solution. Figure 4a shows median intensity curve of molar ratios of the streptavidin and Pdot. When the median intensity reached a stable high plateau, that ratio was considered as the streptavidin number that fully labeled the surface of the Pdots. The green and blue triangles show the singly and fully labeled concentration ratio of streptavidin and Pdot, respectively.

Spectra of absorption and fluorescence

The absorption and fluorescence spectra were measured with a DU 720 scanning spectrophotometer (Beckman Coulter, Inc., CA) and a Fluorolog-3 fluorospectrometer (HORIBA JobinYvon, NJ, USA). Absorption and fluorescence spectra are shown in Figure S1.

Microfluidic sampling channels

Details of microfabrication have been described previously.^[15–16] Briefly, the microchannel system was designed in AutoCAD, then written onto a chrome blank to generate the

photomask (HTA Photomask, San Jose, CA, USA). The pattern on the photomask was transferred onto a silicon wafer using photolithography. The wafer was silanized in a desiccator containing tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (Sigma–Aldrich, St. Louis, MO, USA) before it was used as a master to replicate features in PDMS. Reservoirs were created by punching holes in PDMS at the ends of the straight channels. To form enclosed channels, the PDMS chip was bonded to a clean cover glass after oxidation of both the PDMS chip and the cover glass in an oxygen plasma.

Of note, we found that fluorescence intensity of biotin-Alexa647 in Pdot-streptavidin-biotin-Alexa647 complex's signal was variable in different experiment sets. The variabilities here included variabilities of the sample and the system. To minimize the sample variability, the same batch of Pdots were singly labeled and fully labeled with streptavidin using the same procedure at the same time. The system variabilities include excitation laser stability, relative channel position to laser focus line, and chip-to-chip variability. The optical path we used in this platform is dependent on a confocal geometry, which provided good signal-to-noise by rejecting out-of-plane light, but it was sensitive to the exact positioning of the objective relative to the channel in the z direction, the variation of which can be dependent on the chip. To minimize the chip-to-chip variability, we used the same chip for detection of singly labeled and fully labeled Pdot-streptavidin-biotin-Alexa647 complex. However, we must additionally ensure that the measured spike intensities did not vary from run-to-run or sample-to-sample. It was more difficult to meet in practice because spike intensities could be affected by exact z-position of the laser line in the channel, variabilities that existed from chip-to-chip (e.g. thickness of the coverslip that formed the floor of the channel and in contact with the objective), instrument drifts (e.g. laser or detector alignment), and operational conditions (e.g. flow rate).

To overcome these challenges, we employed an internal standard using the back reflected signal from gold nanoparticles. We chose gold nanoparticles because: 1) They were readily available in different sizes, which allowed us to choose the size that would best match the desired spike intensities in our detection channel; 2) Their back-reflected signal allowed the use with any laser excitation wavelength and thus color channel; 3) They were homogeneous in size and showed strong scattering, which minimized their detected intensity distribution and which facilitated calibration and improved quantification accuracy; 4) Their surface was readily modified, such as with PEG so that they did not self-aggregate or non-specifically attach to the channel surface; 5) They were robust and did not degrade or aggregate even during long-term (months) storage; and 6) They were inexpensive compared with other nanoparticles.^[17–21]

In our experiments, gold nanoparticles were mixed with streptavidin labeled Pdot-streptavidin-biotin-Alexa647 complex and introduced into the inlet reservoir of the chip. Because of the height difference (~ 5 mm) between the inlet and outlet reservoir, the complex solution would flow through the channel towards the outlet reservoir, and when the complex and gold colloid passed through the detection volume, signals were detected in the corresponding APD. The scattering intensities of the gold nanoparticles were analyzed by a MATLAB program we wrote (Figure S2). The gold nanoparticles' background and signal-

to-noise ratios were then analyzed and used as an internal standard, mostly for adjusting the z-position for each flow experiment.

Single-molecule colocalization microscopy

For imaging the colocalization of single Alexa647 molecules and Pdots, we used total-internal-reflection-fluorescence (TIRF) microscopy. The microscope used a 60× and 1.45 NA (numerical aperture) TIRF lens. The image was further magnified (by 1.5×) with an internal lens in the Nikon TE2000 microscope. The collected photons were imaged onto a sCMOS camera (Orca V6, Hamamatsu, Japan) using a 100 ms exposure time. Image J (NIH) was used for image analysis. To create a well to contain the sample solution we bonded a piece of coverslip glass to PDMS with a molded hole. Sample solution containing Pdots and dyes was diluted 100× prior to being pipetted into the well. Pdots and dyes were attached to the surface of the coverslip via non-specific absorption.

Data analysis and statistics

To remove false-positive events in the spike trace (Figure 1), we employed a threshold. The average intensity of the background and the standard deviation of the background was calculated, and the threshold was set as the average background plus 5 times the standard deviation. To calculate the intensity of each spike, the maximum intensity of each spike was located and added to the intensities of the adjacent left two points and right two points, then subtracting five times the background average (see Figure S3). When we analyzed the single-molecule intensity histogram, we did not observe any cut off at the low intensity range, indicating we had a low false negative rate and that we were not missing single molecules that passed through our probe volume. To determine statistical significance, we used Wilcoxon test with two tail and $\alpha = 0.05$ in Igor Pro 6.02 (Wavemetrics, USA). To deconvolve the intensity distribution of fully labeled Pdots using singly labeled Pdot intensity distribution, we applied an algorithm developed previously in our lab.^[9–11]

RESULTS AND DISCUSSION

Figure 1a schematically illustrates the single-molecule flow platform, which employed a line-confocal fluorescence detection we previously developed for the high-sensitivity and high-throughput detection of single-dye molecules in a near nanoscale channel (Figure 1a inset).^[15, 22] The microfluidic channel was designed to have a 2- μm wide and 75- μm long constriction. We chose a 2- μm wide channel because it was fairly easy to fabricate using SU-8 photoresist and soft-lithography, was not prone to clogging, did not create too much flow resistance, while offering good single-molecule sensitivity.

We chose a 75- μm length because it offered the right balance in resistance to achieve the desired linear flow velocity with simple gravity-driven flow.^[15] For sample injection and flow, two holes were punched with $\sim 1\text{--}2\text{ mm}$ diameter; Hole 1 was used for loading $\sim 5\text{ }\mu\text{L}$ of sample, and hole 2 was left empty as the waste outlet. Prior to loading the sample, the channel was first filled with buffer, after which residual solutions in both reservoirs were withdrawn; upon pipetting $\sim 5\text{ }\mu\text{L}$ of sample solution, flow was readily initiated without external pump because of the height difference ($\sim 5\text{ mm}$) in the fluid level between the two

reservoirs, which made operation simple and robust. The length of the illumination laser line was around 20 μm for 633 nm laser (Figure 1a). The 633 nm laser line was made to be ten times longer than the width of the channel so that the laser illumination was homogeneous across the width of the channel; this was important for single-molecule counting to minimize any variability in detection sensitivity as the molecules passed through the channel at different lateral positions. The 405 nm laser was made shorter ($\sim 5 \mu\text{m}$) because we only needed to detect the presence of the Pdot as they transited the channel and did not need to perform single-molecule counting at this laser excitation wavelength. Thus, the homogeneity of the laser illumination was not critical as long as we could detect each and every transiting Pdot. The two illumination laser lines were co-linear and were positioned around 6 μm into the constricted channel.

Figure 1b shows the detection sensitivity for single-dye molecules (Alexa647 attached to a biotin); top trace is buffer only and bottom trace shows the detection of three biotin-Alexa647 molecules. To set the threshold for detection, the average intensity and the standard deviation of the background were analyzed. The threshold was set as the background plus five times standard deviation (See Data analysis and statistics).

To demonstrate the single-molecule flow quantification platform, we took a two-stage approach. In the first stage, we validated the results provided by the flow method using a well characterized model system: the number of bound biotin to a single streptavidin. For this first-stage experiment, because of the small size of a streptavidin molecule ($\sim 5 \text{ nm}$ diameter) we had to quantify the amount of dye-dye quenching present within a single streptavidin (Figure 2a). Based on the dye-dye quenching efficiency, we determined the half maximal distance (R_0) to be $\sim 2.4 \text{ nm}$. In the second stage, we applied the flow platform to quantify the number of streptavidin per Pdot. Here, we did not need to consider the quenching that existed within each streptavidin because we used single streptavidin-biotin-dye complex on a nanoparticle as our calibration and thus any quenching within the streptavidin was accounted for in our singly labeled Pdot measurements.

In the bulk measurement, we first carried out a fluorescence titration assay between streptavidin and biotin-Alexa647 (Figure 2a). In this titration curve, we see the fluorescence of biotin-Alexa647 did not increase linearly as increasing number of the molecule was bound to streptavidin. This deviation was caused by the intramolecular self-quenching between biotin-Alexa647 molecules, due to their close proximity to each other when bound to streptavidin. There are four binding sites for biotin on a single streptavidin and self-quenching became apparent when two or more biotin-Alexa647 molecules were bound according to the molar ratio.^[7,15]

For example, when streptavidin had an average of 2.1 biotin-Alexa647 molecules bound (data point 1), the fluorescence intensity of a biotin-Alexa647 molecule was quenched to 57.6% of its original unquenched value (data point 2). From this bulk titration measurement, we could calculate the intensity ratio between a streptavidin and biotin-Alexa647 complex (self-quenching present) with free biotin-Alexa647 molecules in solution (self-quenching absent). For example, with 2.1 biotin-Alexa647 molecules bound to a streptavidin, the ratio was 1.21 ($57.6\% \times 2.1$).

Using these same samples, we then used the single-molecule flow platform to quantify the average number of bound biotin-Alexa647, and compared with that obtained using bulk measurements. Figure 2b shows the fluorescence intensity distribution of the sample determined by bulk measurements to have an average of 2.1 biotin-Alexa647 per streptavidin (top panel, showing a median photon count of 160) as well as the fluorescence intensity distribution of single biotin-Alexa647 in solution (bottom panel; median count of 131), from which we calculated an average fluorescence intensity ratio of 1.23 (160/131). The ratio is in good agreement with that obtained from bulk measurements (1.21) for an average of 2.1 biotin-Alexa647 per streptavidin. In summary, quantifying the average number of biotin-Alexa647 per streptavidin using bulk and single-molecule flow experiments served as our initial validation of the single-molecule flow platform. Next, we applied the single-molecule flow method to quantify the number of bound streptavidin (as reported by biotin-Alexa647) on the surface of individual Pdot-streptavidin conjugates.

As shown in Figure 3a, Pdot-streptavidin conjugates were first incubated with saturating concentrations of biotin-Alexa647. Afterwards, Pdot-streptavidin-biotin-Alexa647 complexes were purified using a size-exclusion column to remove free biotin-Alexa647. This purified sample containing Pdot-streptavidin-biotin-Alexa647 was introduced into the microfluidic channel and the fluorescence signals were recorded.

Although free biotin-Alexa647 should have been removed by the size-exclusion column, it is inevitable that some of the free biotin-Alexa647 still remained in solution. Therefore, we detected the fluorescence separately from both Pdots (405 nm excitation laser; 455/50 nm emission band-pass filter) and Alexa647 (633 nm excitation laser; 675/50 nm band-pass) (Figure 3). The 405 nm and 633 nm excitation lines were colinear and thus fluorescence from both Pdot and biotin-Alexa647 arrived at their respective detectors at the same time. As a result, detection of two correlated spikes (Pdot emission and biotin-Alexa647 emission) in both the blue and red color channel would signal the passing of a Pdotstreptavidin-biotin-Alexa647 complex, while a spike in only the red color channel would correspond to free biotin-Alexa647 and a spike only in the blue color channel would be caused by a Pdot without streptavidin conjugation. We chose Alexa647 to use with this particular Pdot with 405 nm excitation because these two fluorescent probes should have minimal crosstalk, if any, given they have non-overlapping spectral regions for both excitation and emission (Figure S1). Because Pdots are much brighter than single organic dyes, we were still concerned there may be some leakage of Pdot fluorescence into the Alexa647 color channel. To test this possibility, we compared the single-molecule trace with the 633nm laser on and then off (Figure 3b), and confirmed there was indeed no fluorescence leakage. We next confirmed there was no energy transfer from Pdot to Alexa647. As expected, because there was minimal spectral overlap between Pdot emission and Alexa647 absorption (Figure S1), we did not observe FRET when 633 nm laser was off (Figure 3b). Finally, we tested whether there was non-specific binding of biotin-Alexa647 to Pdots, which would generate false-positive signals. According to both our bulk and single-molecule co-localization assays, we did not observe any nonspecific binding events (See Figure S4).

With the two-color-co-localization scheme and the use of gold nanoparticles as an internal standard (Figure S2), we quantified first the average number of streptavidin molecules on the

Pdot-streptavidin conjugates. To accomplish this analysis, we had to measure the average fluorescence intensity of the Pdot-streptavidin-biotin-Alexa647 complex, then divide this value by the average fluorescence intensity of Pdots labeled with only a single streptavidin-biotin-Alexa647. We could not use the average fluorescence intensity we measured previously for free streptavidin-biotin-Alexa647 in solution and unattached to Pdots because we found streptavidin attached to a nanoparticle surface had fewer accessible biotin binding sites than free streptavidin in solution.

We first prepared a singly labeled Pdot-streptavidin sample by reducing the ratio between streptavidin and Pdot (Figure 4a) during conjugation to a sufficiently low level such that most Pdots were not labeled with any streptavidin while a small percentage were labeled with only one streptavidin. We then used size exclusion chromatography both to remove any un-reacted streptavidin and to enrich Pdot-streptavidin from unlabeled Pdots. It was difficult, however, to separate well Pdots from Pdot-streptavidin complexes, and thus, many unlabeled Pdots remained in solution. To address this issue, we used the two-color-co-localization scheme to distinguish unlabeled Pdots (only blue spike) from Pdot-streptavidin (both blue and red spikes). For example, figure 3b shows the spike trace for the singly labeled Pdot-streptavidin sample, in which 77% of the Pdots present were unlabeled and only 23% of the Pdots had conjugated streptavidin, and thus a correlated red spike accompanying the blue spike. In contrast, the fully streptavidin-labeled sample showed a spike trace where nearly every Pdot (blue spike) had at least one streptavidin (a correlated red spike) (Figure S5).

We obtained the fluorescence intensities in the red color channel (Alexa647) of both the singly labeled and fully labeled sample (Figure 4a). The average streptavidin number on the Pdot-streptavidin conjugate was calculated by dividing the median fluorescence intensity of the fully labeled sample (1391 photon counts; Figure 4c) by that of the singly labeled sample (107 photon counts; Figure 4b). The average number of streptavidin molecules per Pdot of 26nm diameter (as measured by dynamic light scattering (DLS), Figure S6) was thus 13 (1391/107). We next prepared larger Pdots (34 nm diameter by DLS) and followed the same procedure to conjugate streptavidin to the Pdot surface. For this sample, we found the average number of streptavidin per Pdot was 19 (Figure 4d,e). This result is consistent with the increase in surface area of the larger Pdots; the ratio of surface area for 34 nm and 26 nm diameter Pdots is 1.7, similar to the ratio of the average streptavidin number per Pdot (19/13 ~ 1.5).

Beyond the average number of streptavidin per Pdot, the single-molecule flow approach can provide information about the variability in streptavidin number between Pdots, information that is inaccessible to bulk measurements. To achieve this, we deconvolved the intensity distribution of the fully labeled sample using the singly labeled sample. We note that to obtain the average number of streptavidin per Pdot, we only needed to use the median fluorescence intensity from the distribution; but to determine the number of streptavidin on each Pdot, we had to use all the information contained in the distribution, not just the median value. The deconvolution method was described in detail in our previous publications^[9,10,11], which we developed for counting single-molecules on synaptic vesicles with fluorescence imaging. Here, we adapted the same approach for analyzing flow data. Briefly, we used the singly labeled sample as the calibration distribution, which showed

what the intensity distribution was like when there was only one streptavidin per Pdot ($n=1$). To generate distribution for $n>1$, the singly labeled distribution was multiplied with integers (Figure S7)^[9,11], from which we created the basis set for subsequent fitting and deconvolution of the fully labeled sample.

Figure 5 shows the results of the deconvolution (Figure 5a, c), and plots the probability of Pdots with a given number of streptavidin (Figure 5b, d), for both 26 nm and 34 nm diameter Pdots. The variance in the number of streptavidin per Pdot was caused by both variabilities in Pdot diameter (Figure S6) and in conjugation efficiency. Finally, we note the importance of obtaining a large number of single-particle intensities to minimize noise in the resultant distributions. While the number of single-particle intensities required to obtain a reliable median value is fairly low (e.g. hundreds), the number of single-particle intensities needed to build a high-quality distribution for subsequent deconvolution is usually large (thousands) depending on the width of the distribution. This requirement to measure a large number of single-particles, however, was fairly straightforward to fulfill using the flow platform, which offered a throughput of hundreds to thousands of detected particles per second.

CONCLUSION

Our results demonstrated a new approach to quantify the copy number of biomolecules attached to nanoscale objects, such as nanoparticles or subcellular organelles. In summary, the approach provides several important advantages: 1) With single-dye molecule sensitivity, this platform offers information about both the average value per particle and the variability between particles; 2) With high-throughput (hundreds to thousands of particles detected per second), a large number of single-particle fluorescence intensity can be obtained within a short time, an important requirement to obtain high quality intensity distributions for deconvolution; 3) With gravity flow and a built-in internal standard, this method is robust and easy to use; 4) This platform requires minimal sample, usually just 5 μL at a particle concentration at 10 pM; and 5) This platform is capable of measuring precisely the concentration of a sample by carrying out high-throughput counting of individual particles within a known volume of sample, or using a known volumetric flow rate, or relative to a known concentration of the internal standard (e.g. by comparing spike frequencies). This single-molecule flow approach, however, does require sensitive fluorescence detection for counting the biomolecules on each particle. This requirement can be met readily for most biomolecules (e.g. proteins or nucleic acids), because most should have specific fluorescent binders. For example, fluorescently tagged secondary antibody can be used to detect attached primary antibody or fluorescently tagged DNA can be used to hybridize to the attached nucleic acid molecules on the nanoparticles. Additionally, this method requires the binder/ dye to stay on the nanoparticle during the labeling and flow-measurement process, because if any binder falls off, then this method would underestimate the number of bound molecules. Fortunately, for most binders that are used to conjugate to nanoparticles, the binding affinity is usually high (e.g. antibodies or DNA), because these same binders are usually used to attach the nanoparticle to a biological cell or to bind an analyte of interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Giljohann DA, Seferos DS, Daniel WL, Massich MD, Patel PC, Mirkin CA. *Angew. Chem. Int. Ed. Engl.* 2010; 49(19):3280–3294. [PubMed: 20401880]
2. Lu AH, Salabas EL, Schüth F. *Angew. Chem. Int. Ed. Engl.* 2007; 46(8):1222–1244. [PubMed: 17278160]
3. Wu C, Chiu DT. *Angew. Chem. Int. Ed. Engl.* 2013; 52:3086–3109. [PubMed: 23307291]
4. Pan L, He Q, Liu J, Chen Y, Ma M, Zhang L, Shi J. *J. Am. Chem. Soc.* 2012; 134(13):5722–5725. [PubMed: 22420312]
5. Stender AS, Marchuk K, Liu C, Sander S, Meyer MW, Smith EA, Neupane B, Wang G, Li J, Cheng JX, Huang B, Fang N. *Chem. Rev.* 2013; 113(4):2469–2527. [PubMed: 23410134]
6. Sapsford KE, Algar WR, Berti L, Gemmill KB, Casey BJ, Oh E, Stewart MH, Medintz IL. *Chem. Rev.* 2013; 113(3):1904–2074. [PubMed: 23432378]
7. Mittal R, Bruchez MP. *Bioconjug. Chem.* 2011; 22(3):362–368. [PubMed: 21314110]
8. Stoner SA, Duggan E, Condello D, Guerrero A, Turk JR, Narayanan PK, Nolan JP. *Cytometry A.* 2016; 89(2):196–206. [PubMed: 26484737]
9. Mutch SA, Kensel-Hammes P, Gadd JC, Fujimoto BS, Allen RW, Schiro PG, Lorenz RM, Kuyper CL, Kuo JS, Bajjalieh SM, Chiu DT. *J. Neurosci.* 2011; 31(4):1461–1470. [PubMed: 21273430]
10. Mutch SA, Gadd JC, Fujimoto BS, Kensel-Hammes P, Schiro PG, Bajjalieh SM, Chiu DT. *Nat. Protoc.* 2011; 6(12):1953–1968. [PubMed: 22094731]
11. Mutch SA, Fujimoto BS, Kuyper CL, Kuo JS, Bajjalieh SM, Chiu DT. *Biophys. J.* 2007; 92(8):2926–2943. [PubMed: 17259276]
12. Yu J, Yu J, Rong Y, Kuo CT, Zhou XH, Chiu DT. *Anal. Chem.* 2017; 89(1):42–56. [PubMed: 28105818]
13. Wu C, Schneider T, Zeigler M, Yu J, Schiro PG, Burnham DR, McNeill JD, Chiu DT. *J. Am. Chem. Soc.* 2010; 132(42):15410–15417. [PubMed: 20929226]
14. Wu C, Jin Y, Schneider T, Burnham DR, Smith PB, Chiu DT. *Angew. Chem. Int. Ed. Engl.* 2010; 49(49):9436–9440. [PubMed: 20979060]
15. Schiro PG, Kuyper CL, Chiu DT. *Electrophoresis.* 2007; 28(14):2430–2438. [PubMed: 17577880]
16. Lorenz RM, Fiorini GS, Jeffries GDM, Lim DSW, He M, Chiu DT. *Anal. Chim. Acta.* 2008; 639(2):124–130.
17. Miller MM, Lazrides AA. *J. Opt. a-Pure. Appl. Op.* 2006; 8(4):S239.
18. Jung S, Nam J, Hwang S, Park J, Hur J, Im K, Park N, Kim S. *Anal. chem.* 2013; 85(16):7674–7681. [PubMed: 23883363]
19. Zhu S, Yang L, Long Y, Gao M, Huang T, Hang W, Yan X. *J. Am. Chem. Soc.* 2010; 132(35):12176–12178. [PubMed: 20707319]
20. Kuyper CL, Budzinski KL, Lorenz RM, Chiu DT. *J. Am. Chem. Soc.* 2006; 128(10):730–731. [PubMed: 16417357]
21. Kuyper CL, Fujimoto BS, Zhao Y, Schiro P, Chiu DT. *J. Phys. Chem. B.* 2006; 110(48):24433–24441. [PubMed: 17134198]
22. Schiro PG, Gadd JC, Yen GS, Chiu DT. *J. Phys. Chem. B.* 2012; 116(35):10490–10495. [PubMed: 22574902]

Figure 1.

Single-molecule flow platform. a) Schematic of the optical setup of the single-particle flow platform. APD1 and APD2 are for fluorescence detection (e.g. of Pdots and Alexa647 dye); APD3 is for detecting the backscatter of gold nanoparticles. Top inset is a microscope image of the flow channel and the back reflected light of the laser line, showing the placement of the laser line with respect to the flow channel. Bottom inset is a schematic of the flow channel, showing the dimensions of the constricted region of the channel where single-molecule line-confocal detection occurs. b) Fluorescence intensity traces for buffer without (top) and with (bottom) biotin-Alexa647 molecules; here a single biotin was covalently

conjugated with a single Alexa 647 dye (“Biotin-Alexa647”). Blue line indicates threshold we used for analysis, which was the sum of the background average and 5 times the standard deviation of the mean (28.1 in this trace). A small amount of biotin-Alexa647 (10 pM) flowed through the channel and three individual biotin-Alexa647 were detected during the 500 ms time window (bin time was 0.33 ms). We found the background noise mostly came from the PDMS substrate.

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Figure 2.

Measurements obtained at the bulk and singlemolecule level. a) Fluorescence titration assays of varying numbers of biotin-Alexa647 bound to streptavidin. The control curve (red, without streptavidin) was measured in the absence of streptavidin. It shows a linear increase in the fluorescence intensity with increasing concentrations of free biotin-Alexa647. The data were fitted with a linear function. The sample curve (light blue; drawn to guide the eye) was measured in the presence of streptavidin (0.033 μM). b) Fluorescence intensity distribution of a single streptavidin-biotin-Alexa647 complex at a ratio of 1:2.1 (top) and of single free biotin-Alexa647 molecules without streptavidin (bottom). The total number of

events detected for streptavidin-biotin-Alexa647 complex and free biotin-Alexa647 were 1314 and 1433, respectively. According to the Wilcoxon test, the two histograms are significantly different ($P < 0.0003$).

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Figure 3.

Quantification of the number of streptavidin on the surface of Pdot-streptavidin (SA) conjugates. a) Schematic showing the labelling and measurement procedures. Signal 1 from APD1 is from PFO Pdots with blue fluorescence emission; signal 2 from APD2 is from biotin-Alexa647 with red fluorescence emission; signal 3 from APD3 is back-scatter light from gold nanoparticles. b) Fluorescence intensity traces of single-particle flow measurements of Pdot-SA-biotin-Alexa647 complex; blue (top) and red (bottom) traces were from PFO Pdots and biotin-Alexa647, respectively. The dotted red line shows when the

633nm laser was turned off. The five labeled peaks indicate Pdot-SA-biotin-Alexa647 complex since both blue (Pdot) and red (biotin) fluorescence were detected at the same time.

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Figure 4.

Fluorescence intensity distributions of singly and fully labeled Pdot-streptavidin (SA)-biotin-Alexa647 complexes. a) Determination of the concentration ratios of streptavidin to Pdot to produce singly (green arrows) and fully (blue arrows) streptavidin-labeled Pdots. The top and bottom graphs were from 26 nm diameter and 34 nm diameter Pdots, respectively. Inserts are intensity median with streptavidin and Pdot ratios of 0.5 and 1, respectively. b,c) Distribution of fluorescence intensity of 26 nm-diameter Pdot complexes labeled with a single streptavidin (b; total number of events = 951) and multiple copies of streptavidin (c; total number of events = 3013). d,e) Distribution of fluorescence intensity of

34 nm-diameter Pdot complexes labeled with a single streptavidin (d; total number of events = 1468) and multiple copies of streptavidin (e; total number of events = 1581). Median fluorescence intensities of each distribution are shown in the individual panels.

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Figure 5. Quantification of the number of streptavidin on individual Pdot-streptavidin (SA)-biotin-Alexa647 complexes. a,c) Experimental data (black line) and best-fit results (red line); the dotted blue line is a plot of the residuals of the fit compared to the experimental data. b,d) Probability histograms displaying the number of streptavidins per Pdot for the 26 nm-diameter (b) and 34 nm-diameter (d) Pdots. Error bars are standard error of mean among 5 (for 26 nm Pdots) or 4 (for 34 nm Pdots) data sets.