Determining fluorescence Limit of Detection with Nanoparticle Tracking Analysis (NTA)

FLUORESCENCE DETECTION

PARTICLE SIZE

PARTICLE CONCENTRATION

Introduction

The ability to detect nanoparticle fluorescence is important when attempting to distinguish a particular subset of particles from within a complex background. Particles which are fluorescent can be measured in terms of particle size and concentration to understand the nature of those particles. A typical application for this is in understanding the fate of drug delivery vectors as well as understanding the toxicology of nanoparticles in biological environments. Alternatively, fluorescently labeled antibodies may be used to bind specifically to target particles within a sample, thus providing a mechanism to identify specific particle populations within an unknown sample. This has implications in diagnostic applications such as research into exosomes and microvesicles, where specific markers can be identified and monitored in the detection of disease.

Nanoparticle Tracking Analysis (NTA), available on the NanoSight range of instruments, can operate in light scatter mode where all particles within a sample are visualized and measured (and thus does not require the sample to be fluorescently labeled to take a measurement). Alternatively, the instruments can also operate in fluorescence mode where only fluorescently labeled particles are detected and measured.

The NanoSight system uses either a 405 nm (violet), 488 nm (blue) or 532 nm (green) laser source to excite particles labelled with appropriate fluorophores, whose fluorescence can then be determined using suitably matched long or short-pass filters.

This application note works through an experiment to approximately establish a baseline in terms of the fluorescence limit of detection for a given fluorophore.

The biotin-streptavidin interaction is known to be a very strong non-covalent binding interaction, K_d ~10^{-14} mol/L. Each streptavidin molecule has 4 available

sites for biotin binding, one for each of the monomer units that comprise the quaternary structure. This ratio was used to calculate a maximum possible number of fluorescent streptavidin molecules that each gold nanoparticle could have bound to the surface (Figure 1).

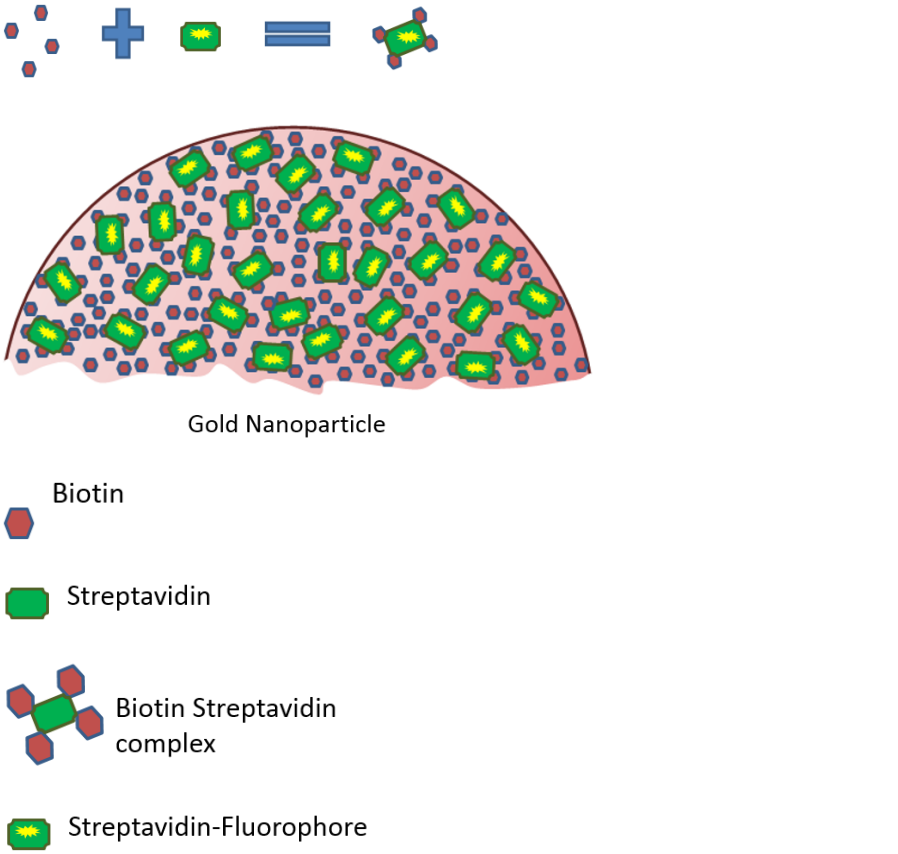

Figure 1: Graphic showing the formation of the strepatividn-biotin complex and it's binding to a gold nanoparticle

Materials

Nominally 80 nm spherical gold nanoparticles with biotin attached to the surface (GNP) were purchased from NanoPartz (product code C11-80-TB-50), with a specified biotin/particle density. The size of the particles was determined by DLS and TEM and was supplied at a concentration of approximately 9.4×10^{11} particles/mL.

Streptavidin (F*) fluorescently labelled with NorthernLights™ 557 dye was purchased from R&D Systems (product code NL999) at a stock concentration of 1 mg/mL (~10^{16} particles/mL). This dye has an absorption peak at 557 nm and an emission peak of 574 nm.

Methods

A NanoSight LM10 system equipped with a 532 nm LM14 laser module, 565 nm long pass emission filter, high sensitivity (HS) sCOMS camera and NTA syringe pump were used for all data collection. Data were analyzed using NTA3.1 software.

All dilutions were carried out in 0.02 µm filtered PBS. Firstly, an appropriate concentration of GNP for NTA was prepared by serial dilution. The GNP working stock was prepared by diluting the original sample by a factor of 100 in PBS. Aliquots of this stock along with F* were taken and diluted in PBS to give an overall sample volume between 1200-2000 µL and NP concentration of ~4 x 10^8 particles/mL. Samples were prepared such that F* was added at the ratio 0-1000000 F* per 1 GNP and mixed by pipetting. From preliminary experiments, and the knowledge that a stable biotin-streptavidin complex forms rapidly, each sample was allowed to incubate for 30 minutes in the dark prior to measurement at room temperature.

The total concentration of particles under scatter and fluorescence was then measured using NTA. Camera settings were kept constant for measurement type – one for all fluorescent measurements and another for all scatter measurements. The NTA syringe pump was used to ensure analysis under slow and constant flow was carried out in both scatter and fluorescence modes, allowing data from both modes to be compared directly. (N.B. Analysis in flow is necessary in fluorescence mode to prevent the fluorophore from photobleaching, and additionally improves the statistical robustness of the data by ensuring a greater proportion of the sample is measured). 3 videos of 60 seconds were captured for each sample under both the scatter and fluorescence mode.

A number of controls were also measured as shown in Table 1.

Table 1: Controls measured in both scatter mode and fluorescence mode, and their significance.

Sample	Scatter Mode	Fluorescence Mode
Biotin-gold nanoparticles	Confirmation of concentration	No signal expected
Fluorescent streptavidin	Checking for aggregates	Checking for aggregates
Biotin-Gold-non-fluorescent streptavidin	Checking for aggregates	No signal expected

Assumptions and Caveats

The nanoparticles were supplied with biotin specified as being 2 molecules per nm^2 of the particles' surface. For an 80 nm spherical particle the amount of biotin was calculated as 40,000 biotin molecules per particle.

A streptavidin molecule has 4 biotin binding sites, thus if all biotin molecules on each gold particles are bound ~10,000 streptavidin molecules would be required to fully cover the particle (100% coverage).

When the nanoparticles are incubated with F* it is assumed that the F* is distributed homogeneously over all the nanoparticles present such that all particles have bound the same number of F* molecules.

Results

The gold nanoparticles were measured initially under both scatter and fluorescence modes under a constant flow of 50 a.u. The NTA size distribution profile of the particles is shown in Figure 2.

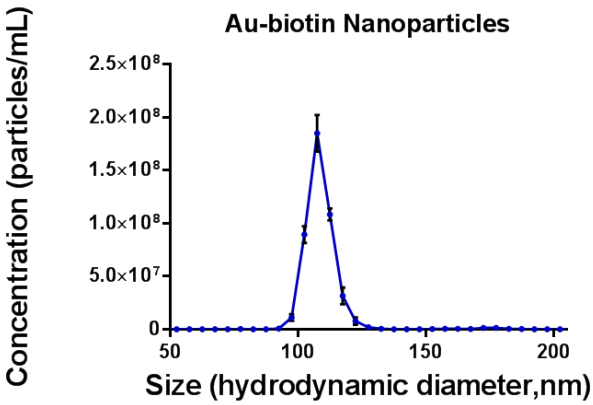

Figure 2: Size distribution profile of gold-biotin nanoparticles measured under a constant flow of 50. Only data from light scatter mode are shown as the unlabelled particles did not exhibit a fluorescent signal.

To find an appropriate incubation time a preliminary time course study was carried out. Nanoparticles were incubated with the equivalent of approximately 1000 F* molecules per nanoparticle and allowed to incubate at room temperature in the dark for different times. As shown in the Table 2, the labelling was nearly instant and a 30 minutes incubation was selected to ensure the incubation for binding biotin-streptavidin was complete.

Table 2: Incubation time labelling efficiency data from biotin-gold NP labelled with F* in ratio of approximately 1000F* molecules to one gold NP.

Time from labelling [minutes]	3	20	35	60	150
Fluorescence efficiency [%]	62	67	64	67	69

When different ratios of F* per gold nanoparticle were measured the percentage of the total population measured in fluorescence mode increased with increasing ratios of F* until “100% coverage” was achieved. Selected ratio data are shown in Figure 3.

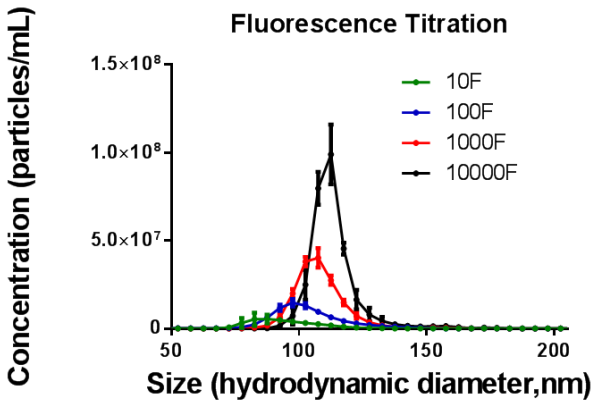

Figure 3: Size distribution data for 10F* per 1GNP (green), 100F* per 1GNP (blue), 1000F* per 1GNP (red) and 10000F* per 1GNP (black) measured in fluorescence mode

When increasing the ratio of F* beyond “100% coverage” the number of particles measured in fluorescence mode decreased with increasing amounts of fluorescence. The image background became brighter making the particles more difficult to visualize and track with the NTA software. The overall proportion of fluorescently labelled particles measured in fluorescence mode NTA increased up to "100% coverage", whilst adding further fluorescence to the sample caused a decline in the number of particles measured Figure 4.

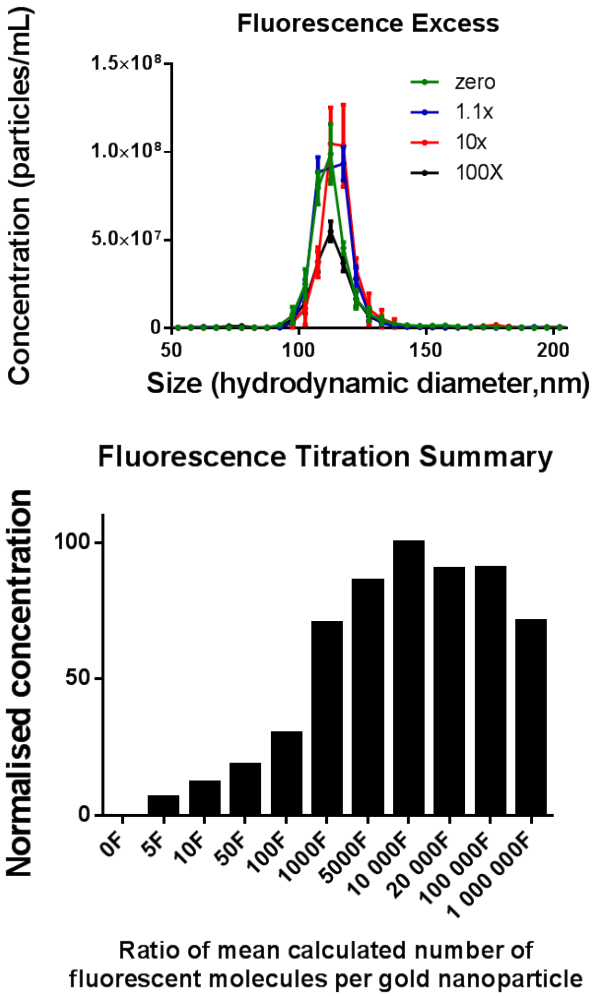

Figure 4: Top) Size distribution data for 10000F* per 1GNP (zero excess fluorophore, green), 11000F per 1GNP (1.1x excess fluorophore, blue), 100000F* per 1GNP (10x excess fluorophore, red) and 1000000F* per 1GNP (100x excess fluorophore, black) measured in fluorescence mode. Bottom) Overall summary of fluorescently labelled GNP measured in fluorescence mode of NTA. The x axis shows the ratio of fluorescence molecules per GNP (Data combined from three separate assays).

Conclusion

This short study aims to look at the sensitivity of NTA in terms of measuring fluorescently labelled particles using standard materials with known affinity strengths. It makes a number of assumptions (gold particle surface area, biotin coverage, complete streptavidin binding, homogeneous sample incubations), but demonstrates that with an equivalence of 5 fluorescent streptavidin molecules per gold nanoparticle some gold particles are detected as being fluorescently labelled. There are a number of reasons why 100% recovery may not have been measured at this level:

1. These particles will be noticeably dimmer than those with greater loadings, therefore they may only be observed in a smaller scattering volume.

2. Uneven loading of the particles, i.e. at the 5F/particle loading level, some particles may have 10F attached whereas others may have 0F attached. It may be that only the brighter particles are detectable.
3. Photo bleaching would have a greater effect on particles with a lower number of fluorophores than particles with a high number of fluorophores.

In this example the particles with 5F* per gold nanoparticle were very dim and were challenging to visualize and focus in fluorescence mode in comparison to gold nanoparticles with 600F or more which were very bright in comparison. In addition, increasing the ratio of fluorescent molecules at a level greater than 10% above that of a theoretical "100% coverage" gives rise to a raised unbound fluorescence background. This raised background makes particle visualization and tracking much more difficult and thus the number of particles measured decreases as the fluorescence excess increases.

The titration of fluorophore to target nanoparticle is crucial to establish a labelling protocol that will lead to the high fluorescence signal detection. Having too few or too many fluorophore particles in the sample will affect the efficiency of the fluorescence measurement with NTA.

Depending on the fluorophore used and its binding efficiency, the particle size and the laser wavelength, a recommended minimum number of 10 – 20 organic fluorophore molecules per nanoparticle will be required to successfully visualize, track and measure fluorescently labelled particles in fluorescence mode using NTA.

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