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Using Image Attributes to Assure Accurate Particle Size and Count Using Nanoparticle Tracking Analysis

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ABSTRACT

Nanoparticle tracking analysis (NTA) obtains particle size by analysis of particle diffusion through a time series of micrographs and particle count by a count of imaged particles. The number of observed particles imaged is controlled by the scattering cross-section of the particles and by camera settings such as sensitivity and shutter speed. Appropriate camera settings are defined as those that image, track, and analyze a sufficient number of particles for statistical repeatability. Here, we test if image attributes, features captured within the image itself, can provide measurable guidelines to assess the accuracy for particle size and count measurements using NTA. The results show that particle sizing is a robust process independent of image attributes for model systems. However, particle count is sensitive to camera settings. Using open-source software analysis, it was found that a median pixel area, 4 pixels^2, results in a particle concentration within 20% of the expected value. The distribution of these illuminated pixel areas can also provide clues about the polydispersity of particle solutions prior to using a particle tracking analysis. Using the median pixel area serves as an operator-independent means to assess the quality of the NTA measurement for count.

Introduction

The growth of protein-based therapeutics drives the need to develop facile and robust analytical tools to characterize protein formulations.[1] Analytical methods such as light obscuration, flow cytometry, and electrical sensing zone have been applied to measure particle sizes in the range of 1-10 μm.[2-6] Recent emphasis has been placed on extending particle characterization to smaller particle sizes in the submicrometer range (less than 1 μm) because of the potential negative impact on drug efficacy and safety.[7] Dynamic light scattering (DLS) is the most common approach for particle sizing in this size regime due to its high throughput and ease of use. However, it is only accurate for samples that are monodisperse and within a concentration range for a given particle size.[8,9] An alternative method, nanoparticle tracking analysis (NTA) has been developed to measure and quantify particle-size distributions in the submicrometer range that overcomes the limitations of DLS.[10,11]

An attractive feature of nanoparticle tracking is the visualization and imaging of scattered light from individual particles in solution by laser illumination. NTA is not an ensemble measurement, unlike DLS, which only reports the average particle size of the measured sample.[10,12] Comparative studies of NTA and DLS have shown that NTA can resolve particle sizes for polydisperse mixtures of polystyrene (PS) particles with a size diameter ratio of 2:1[10,13] versus diameter ratio of 5:1 for DLS.[10,13,14] DLS does not have capability to detect and distinguish individual particles, which can affect particle size and count.

Laser illumination of particles is universal to all NTA instrumentation that allows for the imaging of the scattered light from a particle. Tracking the Brownian motion of these distinguishable and detected particles over a time series of micrographs directly gives the mean squared displacement of the particles. This allows for the determination of individual diffusion coefficients. Assuming spherical particles, the Stokes-Einstein equation is used to calculate individual particle sizes (sphere-equivalent hydrodynamic radius) from the diffusion coefficient.[15]

The concentration range where reliable NTA measurements may be obtained is determined on the lower end by the need to obtain sufficient counts for good statistical repeatability.[10,16,17] The upper limit of the concentration range is limited by the need to have sufficient particle separation which allows for long tracks from clearly distinguishable particles. However, adjusting the camera

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sensitivity to increase or decrease the number of tracked particles visible can have the unintended consequence of altering the ratio of visible particles per unit volume to the actual number of particles per milliliter volume. The number of detected particles is dependent on the details of instrument set up, particle properties, and the location of the particle in the focused laser beam path. These factors influence particle illumination, which affects the acquired images needed for particle size. NTA has the capability to measure particle concentration by counting the number of particles observed in the sample volume. This feature is understudied because interest in NTA has initially been focused on analyzing the feasibility of NTA for particle size and distribution.

A common conclusion in studies using NTA is the need for a skilled operator to assess whether the imaged particles are suitable for tracking to measure particle size.[9,16,18] Qualitative guidelines have been reported to help users, but operator training will be necessary as instrumentation is changed or software is upgraded.[10,19,20] Optimization of the instrument to obtain reliable particle sizing may not give reliable particle concentration. One may assume that certain image attributes such as image intensity, individual particle illumination, or the imaged area of the scattered light are suitable for sizing particles because they yield the correct particle size across a large range of attribute values. However, caution should be used when reporting particle concentration because it can be affected by these image attributes. Without a better understanding of the connection between image attributes and particle count, it is difficult to assess if particle solutions are sized and measured for concentration properly.[21] In this study, we hypothesize submicrometer particle size, and count is impacted by image attributes captured by NTA. We investigate how image acquisition camera settings such as sensitivity and shutter speed impact particle size and concentration measurements for model monodisperse particle systems. In particular, we find that the median pixel area of a measured particle correlates well with the measured concentration of a particle suspension, for both PS and silica particles. This finding suggests that the accuracy of NTA reported particle concentrations can be improved by monitoring the median pixel area and implementing suitable adjustments to that value or corrections based on that value.

Experimental Section

Use of commercial products and instrumentation within this publication is for information only; it does not imply recommendation or endorsement by NIST.

Particle Systems

PS latex particles with NIST-traceable diameter were purchased from Thermo Fisher Scientific with a reported diameter, $d = 102.0$ nm ± 3.0 nm (coefficient of variation <3.0%). The estimated concentration for a 1 wt. % of particles in solution is 1.8×10^{13} 1/mL. Silica particles were purchased from nanoComposix with a reported $d = 97.0$ nm ± 4.8 nm measured by transmission electron microscopy. The supplier also reported the hydrodynamic radius, $d_h = 121.2$ nm and a particle concentration of 9.8×10^{12} 1/mL. The particle concentration or number density will be referenced to represent particles per milliliter, which has SI units of 1/mL.

The wt. % solids of the stock solutions were measured by gravimetric analysis and were 1.03 ± 0.11% and 0.098 ± 0.001% for the stock solution of PS, and this was solution diluted by 100 times respectively. For silica particles, the manufacturer listed the wt. % solid content by volume as 10.3 mg/mL. The wt. % solids content was measured by gravimetric analysis to be 10.8 ± 0.19 mg/mL and 0.12 ± 0.01 mg/mL for the stock solution, and this solution diluted by 100 times respectively.

For NTA measurements, the stock solutions were diluted to the recommended concentration range used for NTA, 1×10^7 1/mL to 1×10^9 1/mL. The concentrations used to map out the image attributes were estimated to be 5.6×10^7 1/mL and 5.9×10^7 1/mL for PS and silica respectively, based on dilution volumes using the estimated or reported concentrations provided by the particle supplier. These expected concentrations, $C_{particle\ type}$, were used to normalize the measured concentration, $C_{method\ type,\ particle\ type}$, and the values are reported as the ratio $N = C_{method\ type,\ particle\ type}/C_{particle\ type}$. $N = 1$ corresponds to an NTA concentration measurement in agreement with the calculated dilution concentration. The particle type is either PS or silica, and the method type is the measured concentration analyzed by NTA using either the manufacturer (Z) or open-source software (ImageJ).

Instrumentation to Measure Particle Size

DLS reported that the particle sizes were $d_{h,\ PS} = 108.5$ nm ± 1.1 nm, $d_{h,\ Si} = 121.0 \pm 12.4$ nm with a polydispersity index value of 0.01 and 0.02, for PS and silica respectively. The uncertainty reported is standard deviation unless noted. DLS measurements were performed with a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) equipped with a 633-nm He-Ne laser and operating at an angle of 173°. Particle size was also verified using quasi elastic light scattering measured by Wyatt Technologies.

The particle size and count were measured by a commercial NTA instrument, Zetaview® (Particle Metrix, Meerbusch, Germany). This instrumentation allowed for collection and export of unedited images suitable for image analysis with multiple software packages. The sample was loaded by injecting ≈2 mL of sample into the sample cell. Particles were illuminated with a 405-nm laser sheet (45 mW) and imaged with a video camera mounted on a 10× microscope objective oriented 90° to the laser sheet. The camera imaged 11 independent positions in the sample cell, probing ≈0.4 nL of volume at each position. The aspect ratio of the camera is 640 pixels × 480 pixels, with an effective pixel size of 0.7 μm/pixel.

Before NTA measurements of the PS and silica, a reference stock solution provided by the manufacturer was used to optimize the camera settings for alignment and focus. This is not same solution as described previously and was only used to determine if the instrumentation was functioning properly per the guidance by the manufacturer. The provided reference was diluted recommended by the manufacturer to particle count of 6.0×10^7 1/mL. If needed, the live video was used to manually focus the camera to eliminate the appearance of diffraction or halo patterns after the standard instrument focusing step.

Methodology

A map of the image attributes acquired at different camera settings was generated by adjusting the camera gain (sensitivity) and exposure time (shutter). The sensitivity amplifies the scattering intensity by an electronic gain, whereas the shutter is the inverse of exposure time observed by the camera. The settings available by the manufacturer's software are nominal values for sensitivity, 0 to 100, and shutter, 32 to 500. The videos were recorded at 30 fps (frames per second), and the temperature controller was set at 25°C. The camera recorded video at each of the eleven positions within the sample cell for 2 s for a total recording time of 22 s. All measurements for each camera setting were done in triplicate. The uncertainty reported is standard deviation unless noted.