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Fluctuation correlation spectroscopy and photon histogram analysis of light scattered by gold nanospheres

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Abstract

Fluorescence correlation spectroscopy (FCS) is a valuable tool in biological research. In recent years there has been growing interest in using light scattered from metallic colloids in place of organic fluorophores. Metallic colloids display optical cross sections for scattering that are orders of magnitude brighter than fluorophores. We used the FCS method to study the scattering properties of varying sizes of gold colloids 38-100 nm in diameter. The optical cross sections of the gold colloids increase rapidly with size, as can be seen by both the $G(0)$ value of the autocorrelation function and the scattering intensity distributions. In mixtures of different size gold colloids the autocorrelation function is dominated by the larger (brighter) colloids, even when present at a small fractional population. We show that it is possible to detect one 100 nm gold colloid in the presence of 10^3 - 10^4 smaller 39 nm diameter colloids. Because the scattering cross sections of colloids will increase with aggregation, we believe that FCS can be used to detect a small number of associated bio-labeled colloids in the presence of a much larger population of non-associated colloids.

1. Introduction

Fluorescence correlation spectroscopy (FCS) is widely used in the biosciences. FCS has many applications because a wide variety of processes can result in intensity fluctuations [1,2]. These processes include translational diffusion, photophysical effects (e.g. fluorophore blinking) and association or aggregation kinetics, to name a few. FCS has its origins in the 1970s [3-6], but technical limitations resulted in low signal-to-noise ratios and prevented its widespread adoption. The availability of confocal optics, high efficiency detectors and improved lasers has resulted in renewed interest in FCS and its applications [7-10]. Because of the need to observe only a few molecules, and the unavoidable presence of Raman scattering, FCS measurements are limited to a relatively narrow range of optical conditions and observation volumes. Typically, the analyte concentrations range from picomolar to nanomolar; concentrations which are much less do not produce enough fluorescence to be detected, and concentrations that are higher do not yield enough intensity fluctuations for correlation analysis. The excitation intensity is of the order of a few microwatts at the back aperture of the high numerical aperture objective, which produces an effective illumination volume of about a femtoliter or less. Increasing the excitation intensity allows one to probe lower concentrations, but care must be taken not to over-saturate the fluorophores and induce intersystem crossing into the triplet state, or even worse, photobleach the molecules before they traverse the excitation volume.

We now describe a new approach to FCS using light scattered by metal colloids. Herein we use the term 'FCS' to refer to fluctuation correlation spectroscopy of light scatterers. The use

of metallic colloids and FCS provide several new opportunities not available with conventional fluorophores and labeled biomolecules. The optical cross sections for light scattering are orders of magnitude larger than the cross sections for fluorophores [11,12]. As a result, lower concentrations of colloids can be observed while maintaining an adequate signal-to-noise ratio. Additionally, the light scattering cross sections for the colloids themselves can vary by at least 100-fold based on colloid size or extent of aggregation. This range of observable cross sections is not available using fluorophores and provides a unique opportunity for detection of a small population of larger colloids or colloid clusters in a much larger population of smaller colloids. Furthermore, the light scattered from these colloids are stable and are not prone to blinking or photobleaching.

In this paper we describe FCS measurements of gold colloids in aqueous suspension. We examined colloids ranging in size: 39, 62, 83 and 100 nm in diameter. The correlation functions are shown to reflect their associated diffusion coefficients. Examination of binary mixtures of different size colloids shows that the larger colloids contribute to the correlation functions well in excess of their relative populations in the mixture. The photon counts histogram of binary mixtures is shown to be a combination of the histograms from each colloid size. These results show that it is possible to detect a 1% or less fractional population of a more highly scattering species, which can be of value in bioassays to detect a small number of analyte molecules in mixtures.

2. Theory

2.1. Fluorescence correlation spectroscopy

FCS is usually performed in a diffraction-limited volume (defined by the objective and a confocal pinhole) which is ellipsoidal with a minor axis w_0 and major axis z_0 . The ratio of z_0/w_0 is typically near 3. Assume the solution contains a scattering species with a diffusion coefficient D . The autocorrelation function for the scattering intensity fluctuation is given by

$$\begin{aligned} G(\tau) &= G(0) \left(1 + \frac{4D\tau}{w_0^2}\right)^{-1} \left(1 + \frac{4D\tau}{z_0^2}\right)^{-1/2} \\ &= G(0) G_D(\tau), \end{aligned} \quad (1)$$

where τ is the lag time and $G(0)$ is the amplitude at $\tau = 0$. For simple spheres, the diffusion coefficient is evaluated by the Stokes-Einstein relation

$$D = \frac{kT}{6\pi\eta r}, \quad (2)$$

where k is the Boltzmann constant, T is absolute temperature, η is the solution viscosity and r is the radius of the sphere.

The autocorrelation function provides a measure of the average number of molecules N in the observed volume. The number of molecules is given by the inverse of the intercept at $\tau = 0$ when $G(0) = 1/N$. Hence the amplitude of the correlation function is larger for a smaller number of molecules in the confocal volume. The fluorophore brightness affects the signal-to-noise ratio of the measurements and the ability to see the fluorophore over the background counts. However, the time constants of the autocorrelation function for a single non-interacting species is independent of the brightness and $G(0)$ depends only on the average number of observed fluorophores.

Now assume the solutions contain two different size colloids with diffusion coefficients D_1 and D_2 and brightness B_1 and B_2 . The autocorrelation function for these mixtures is then given by [13]

$$G(\tau) = \frac{N_1 B_1^2 G_{D_1}(\tau) + N_2 B_2^2 G_{D_2}(\tau)}{(N_1 B_1 + N_2 B_2)^2}, \quad (3)$$

where $G_D(\tau)$ is defined in equation (1).

The brightness of each species is the number of photons per time interval observed under the chosen experimental conditions. These values are proportional to the optical cross sections of the fluorophores. The presence of two species with different brightness results in a more complex interpretation of the $G(0)$ intercept. The intercept does not yield the number of diffusing molecules, but rather an apparent number:

$$N_{\text{app}} = \frac{(\sum N_i B_i)^2}{\sum N_i B_i^2}. \quad (4)$$

Examination of the above equation reveals an important property of the correlation function for mixtures: each species contributes to the autocorrelation function in proportion to the square of its brightness. This means that the autocorrelation function is strongly weighted by the brightest species in the sample. For instance, suppose the sample containing one molecule with $B_1 = 1$ and one molecule with $B_2 = 10$. The fraction of the signal due to B_2 will be about 99%.

2.2. Mie scattering theory

Noble metal nanoparticles show strong optical activity in the visible range of the electromagnetic spectrum. Light incident on such particles will cause their electrons to oscillate and radiate light of the same frequency as the incident electromagnetic wave, and this process is called scattering. The incident light can also be absorbed and converted into heat, thereby removing photons from the incident beam. The extinction cross section σ_{ext} is defined as the sum of the scattering cross section σ_{sca} and the absorption cross section σ_{abs} of a particle, and represents the energy removed from the incident light. The electromagnetic fields absorbed and scattered by such particles are well described by classical electrodynamics. For the special case of spherical particles, Mie theory [14] yields the following expressions for the scattering and extinction coefficients:

$$\sigma_{\text{sca}} = \frac{2\pi}{k^2} \sum_{n=1}^{\infty} (2n+1) (|a_n|^2 + |b_n|^2), \quad (5)$$

$$\sigma_{\text{ext}} = \frac{2\pi}{k^2} \sum_{n=1}^{\infty} (2n+1) \text{Re}(a_n + b_n). \quad (6)$$

In the above equations $k = 2\pi n_{\text{med}}/\lambda$, where λ is the wavelength of the incident light (in vacuum) and n_{med} is the refractive index of the medium (water in our experiments). The scattering coefficients a_n and b_n are defined by the Riccati-Bessel functions and are dependent on the size and the refractive index of spherical particles. An algorithm for numerical computation of the scattering coefficients is provided in [14]. The dielectric functions for gold, silver and

copper have been previously determined experimentally [15] and can be used to calculate the wavelength-dependent scattering and absorption cross sections for various sizes of nanoparticles. It is also informative to normalize the scattering and absorption cross sections by the physical cross section of the nanosphere, thus defining 'efficiency factors': $Q_{\text{sca}} = \sigma_{\text{sca}}/\pi r^2$ and $Q_{\text{abs}} = \sigma_{\text{abs}}/\pi r^2$, where r is the radius of the nanoparticle.

Recently, Etchegoin *et al* have developed an analytical model for the dielectric function of gold as a function of the incident illumination wavelength, λ , using the Drude model and the two interband gold transitions that occur in the UV/visible part of the spectrum [16]:

$$\varepsilon(\lambda) = \varepsilon_{\infty} - \frac{1}{\lambda_p^2(1/\lambda^2 + i/\gamma_p\lambda)} + \sum_{i=1,2} \frac{A_i}{\lambda_i} \times \left[\frac{e^{-i\pi/4}}{1/\lambda_i - 1/\lambda - i/\gamma_i} + \frac{e^{i\pi/4}}{1/\lambda_i + 1/\lambda + i/\gamma_i} \right]. \quad (7)$$

In the above equation, λ_{∞} represents the high-frequency limit dielectric constant. From the Drude contribution, $\lambda_p = 2\pi c/\omega_p$ is the resonance plasmon wavelength in terms of the plasmon frequency ω_p and the speed of light c , and γ_p is the associated plasmon damping constant. The two gold interband transitions occur at $\lambda_1 = 468$ nm and $\lambda_2 = 331$ nm, and have associated broadenings γ_1, γ_2 and critical point amplitudes A_1, A_2 . The values of these parameters are presented in [16] and are in excellent agreement compared to published experimental data [15]. Using the above expression, the real and imaginary components of the dielectric function were calculated to be -3.362 20 and 2.609 23 for $\lambda = 515$ nm. The dielectric constant of gold was used to calculate Q_{sca} and Q_{abs} given by Mie theory, using the MieCalc 1.5 software package (Simuloptics, GmbH).

3. Results and discussion

The extinction spectra of different size gold nanospheres were obtained using a standard UV-visible spectrophotometer. The normalized extinction spectra are presented in figure 1. For the 39, 62, 83 and 100 nm diameter colloids, the peaks in extinction represent the particles' dipole resonances and occur at 526, 537, 551 and 566 nm, shifting towards the red as the size of the colloid increases. Mie theory was used to estimate the absorption and scattering components of the extinction spectra (figure 2). The theoretical and experimental extinction spectra are in excellent agreement with each other to within 6 nm (comparing the peaks of the extinction spectra from figures 1 and 2). Note the large differences in the scattering and absorption components as the colloid size increases. For the smallest 39 nm sphere, photon absorption dominates over scattering. As the colloid size increases, the scattering component increases and photon absorption decreases. The peak scattering wavelength is also size-dependent and shifts towards the red part of the spectrum as the colloid diameter increases.

In our microscope set-up, a colloidal suspension is illuminated by focusing the 515 nm line of an Ar⁺ laser with a 40×1.2 NA water immersion objective. The backscattered light is collected by the objective and passed through a 515 ± 3 nm bandpass filter. A $50 \mu\text{m}$ pinhole is used to reject out-of-focus light and the confocal volume was imaged by a single-photon avalanche photodiode. The expected scattering intensities for this set-up can be estimated using Mie theory. Because we use monochromatic illumination, only the scattering intensity at 515 nm is considered. Figure 3 shows a plot of intensity versus scattering angle for the four different size colloids. The scattering intensity increases with colloid size and is greatest in the forward (0°) and backward (180°) directions relative to the incident beam. We estimate that, with a 1.2 NA objective, the collection angle is about 65° , and a significant amount of light can be collected even from the smallest scatters (see figure 3).

The diffusion coefficients for the different size colloids at 5 pM concentrations were measured using FCS. The confocal volume dimensions were measured using several known concentrations of rhodamine 6G, and assuming the fluorophore's diffusion coefficient of $280 \mu\text{m}^2 \text{s}^{-1}$, which gave half-axis dimensions $w_0 = 0.4 \mu\text{m}$ and $z_0 = 1.2 \mu\text{m}$. For the colloidal suspensions, the illumination intensity was approximately 5 nW as measured before the entrance to the back aperture of the objective. Photons were detected at a sampling rate of 40 kHz and the collection time was 120 s. A relatively long FCS acquisition time was required because of the diluteness of the sample. It is impressive to note that, with 5 nW illumination and 25 μs time bins, the average photon count rate for a 5 pM concentration of 100 nm colloids (~ 10 kHz) is comparable to the fluorescence emitted from a 5 nM concentration of dye with 5 μW excitation power in 1 ms time bins. Thus, a 100 nm colloid is of the order of 10^7 times brighter than a single dye molecule. The normalized autocorrelation function was determined for ten separate runs and averaged together as shown in figure 4 (left four panels). The autocorrelation functions were fit to double-exponential decays. The major time decays ($\sim 70\%$) agree very well with the expected diffusion coefficient of spheres as given by the Stokes-Einstein expression (figure 4, right panel). Although ideal monodispersed spheres should only exhibit a single characteristic lag time, the presence of an additional longer correlation time may suggest optical trapping of the colloids in the highly focused illumination volume, or it may indicate inhomogeneity in the sample, for instance from the presence of larger particles.

The photon counting histogram (PCH) is another statistical analysis of the intensity trajectories. With this method the detected photoelectrons are represented as a histogram of the number of photoelectrons detected in a defined time bin. For the different size colloids, the PCH shows an increase in counts as the colloid diameter increases (figure 5). In a typical PCH analysis, the histogram is modeled by a convolution of two Poisson distributions describing the distribution of the number of particles in the volume and the photon detection statistics [17]. However, for the case of dilute large colloids, the PCH are not well approximated by a Poisson distribution for the following reasons. First, the low concentration of the sample (5 pM) yields a zero probability of observing more than one colloid in the volume per time bin. Note that the peaks of the PCHs occur at zero counts, indicating the diluteness of the sample. Second, the scattering of diffusing colloids is more complicated than for the case of a fluorophore. For a fluorophore, the emission is considered independent of its position in the volume, i.e. fluorescence is approximately uniform throughout the volume. For a (large) colloid, the scattering intensity is not uniform as it traverses the illumination volume because scattering is highly dependent on the angle of the incident beam. Furthermore, using epi-collection restricts the collection angle to about 65° relative to the incident beam, and so the collection efficiency is also a function of the colloid position in the volume. Still, a quantitative treatment of the photon intensities can be performed by analyzing the total scattered photons detected. The total photon counts were found to increase linearly with the scattering cross sections, σ_{sca} calculated from Mie theory (figure 5, inset). The total counts were 2.21×10^3 and 9.42×10^5 for the 39 and 100 nm colloids, respectively, yielding more than a 400-fold difference in their scattering intensities.

For the smallest 39 nm colloids, $G(\tau)$ and the PCH were determined as a function of concentrations C ranging from 5 pM to 2.5 nM (figure 6, left panel). The $G(0)$ value was observed to decrease as $(\nu C)^{-0.88 \pm 0.06}$, where ν is a normalization constant and $G(0)$ is approximated by $G(25 \mu\text{s})$. The exponent is in good agreement with a homogeneous solution of particles where $G(0)$ is inversely proportional to the number of particles in the observation volume. For PCH analysis, the raw data were re-binned to a 2.5 ms sampling time so that the peak counts per bin occur at non-zero values and is a measure of the most probable brightness of the samples (figure 6). The brightness were plotted as a function of concentration, which was linear from 5 to 250 pM. At 2.5 nM, the brightness was found to be lower than the expected

linear relation, which we interpret as a quenching phenomenon similar to the inner filter effect commonly seen with fluorophores, and also observed with gold colloids [12].

Binary mixtures of 39 and 100 nm colloids were analyzed in order to understand their contributions to the autocorrelation function and the photon counting histogram. Molar ratios of 1:100 and 1:1000 (39 nm:100 nm) were analyzed with the 100 nm colloid concentration kept at 2.5 pM. Because it was found that the FCS curves were multi-exponential for the homogeneous solutions, the diffusion coefficients of the colloidal mixtures could not be readily recovered. However, a large difference in the $G(0)$ values could easily be observed. From figure 7(a), the $G(0)$ values are 49.8, 17.2 and 1.2 for the 2.5 pM solution of the 100 nm colloids, the 1:100 mixture, and the 250 pM solution of 39 nm colloids, respectively. Even when the 39 nm colloid concentration was increased to 2.5 nM, the presence of one 100 nm colloid in a background of 1000 smaller colloids yields a $G(0)$ value of 2.2, about 10-fold greater than $G(0)$ for a homogeneous solution of smaller colloids (figure 7(b)). A simple determination of the colloid ratios can be accomplished by comparing the PCHs. Figure 7(c) shows the PCHs for the 39 and 100 nm homogeneous solutions and their mixture. The differences in the collected photons between the two colloid sizes can readily be seen. For the small colloid at high concentration (2.5 nM), the histogram shows a peak at 28 counts per 2.5 ms. The large dilute (2.5 pM) colloids show a very different count distribution with a major fraction of the counts at relatively low values (representing the diluteness of the sample) which slowly decays to very high count rates with low probabilities of occurrences. Although the functional forms of the individual species are quite complicated, the PCH of the binary mixture is approximated very well by simple summation of the PCH for the individual colloids at their respective concentrations (figure 7(c), circles). Note, however, that because the binary mixture is not dilute due to the high concentration of smaller colloids, the measured photoelectron count occurrence will be lower in the range of 0-15 counts/bin compared to that obtained by summation of the individual PCHs.

4. Conclusions and outlook

We have used a combination of fluctuation correlation spectroscopy and photon counting histograms to investigate light scattered from gold colloids using a confocal, epiilluminated microscope set-up. As individual colloids traverse the illumination volume, light is backscattered by the colloids, producing intensity fluctuations that correlate with their dwell time in the volume (i.e. diffusion coefficient). The experimental scattering intensities are found to be proportional to the scattering cross sections predicted by Mie theory. Because of the large differences (>400-fold) in the scattering intensities of the smallest (39 nm) and largest (100 nm) colloids examined, one 100 nm colloid can be detected in the presence of 1000 smaller 39 nm colloids from analysis of the autocorrelation functions and photon histograms. For future work, we propose to use the large differences in scattering intensities of large and small colloids to monitor molecular association events. We speculate that dimerization of two small colloids can effectively produce large scattering cross sections in excess of the sum of the two small colloids. Specific dimerization of the colloids can be accomplished by coincidence hybridization of two colloids containing oligonucleotides complementary to a target DNA, or the colloids can be labeled with antibodies specific to a protein of interest. Even greater scattering intensities can theoretically be achieved with higher multimer aggregates, for instance with colloids that are highly labeled with oligonucleotides or polyclonal antibodies that can bind to multiple epitopes per molecule. We are currently working on developing such assays for the rapid identification of low abundance biomolecules in a high-throughput format.

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Figure 1.

Extinction spectra of Au colloids. The peak extinction wavelengths are 526, 537, 551 and 566 nm for the 39, 62, 83 and 100 nm diameter colloids, respectively. The gray line at 515 nm is a reference to the illumination wavelength used in the experiments.

Figure 2. Scattering (Sca), absorption (Abs) and extinction (Ext) efficiency factors for different size Au colloids (diameter) determined by Mie theory. The peak extinction values occur at the wavelengths indicated next to the arrows.

Figure 3.

Intensity as a function of scattering angle for different size Au colloids (diameter) illuminated by 515 nm monochromatic light. The dashed and solid lines represent s- and p-polarizations. The scattering intensities are normalized according to Bohren and Huffman [14]. The shaded gray region represents the portion of light that is collected by a 1.2 NA microscope objective.

Figure 4.

Autocorrelation analysis of different sized Au colloids. All colloids are 5 pM concentration in water. The illumination power was ~ 5 nW measured before the objective back aperture. Sampling rate was 40 kHz and the total acquisition time was 120 s. The left four plots show the average of 10 FCS runs, normalized (circles) and double-exponential fits to the data (line). The fits yield diffusion coefficients ($\mu\text{m}^2 \text{s}^{-1}$) and amplitudes (%): $D_1 = 11.05 \pm 0.51$ (73%) and $D_2 = 1.11 \pm 0.11$ (27%) for 39 nm; $D_1 = 7.06 \pm 0.53$ (71%) and $D_2 = 1.30 \pm 0.20$ (29%) for 62 nm; $D_1 = 5.37 \pm 0.27$ (65%) and $D_2 = 0.77 \pm 0.06$ (35%) for 83 nm; $D_1 = 4.49 \pm 0.11$ (66%) and $D_2 = 0.49 \pm 0.02$ (36%) for 100 nm. The right panel is a plot of the colloid size versus the major diffusion coefficient (D_1). Error bars represent standard deviations, which for the colloid diameters were given by the manufacturer. The dashed line is a theoretical curve based on the Stokes-Einstein relation.

Figure 5.

Photon count histogram of Au colloids: 39 nm (squares), 62 nm (diamonds), 83 nm (circles) and 100 nm (triangles). The colloid concentration is 5 pM for all samples. The illumination intensity was ~ 5 nW as measured at the back aperture of the objective. Bin time was $25 \mu\text{s}$ and the total collection time was 120 s. The histograms represent an average of 10 separate runs. The gray area is the PCH for water. Inset: the total counts versus the scattering cross-section area of the colloids (σ_{sca} determined from Mie theory). Total counts are determined by integrating the PCHs and are shown as open symbols, with the error bars representing the standard deviation from 10 runs. The dashed curve is a line fit with a slope of $7.61 \times 10^7 \pm 2.08 \times 10^6$ photons μm^{-2} .

Figure 6.

FCS of various concentrations of 39 nm Au colloids (left panel). The inset shows the FCS curves at concentrations (upper curves to lower curves): 5 pM, 50 pM, 250 pM, 500 pM and 2.5 nM. The main figure is a plot of the $G(0)$ values versus concentration, C . The dashed line is a power law fit to the data: $G(0) = (\nu C)^{-0.88 \pm 0.06}$, where ν is a normalization constant. The right panel displays the PCH of the same data sets, showing increasing counts with larger colloids. Bin time was 2.5 ms. The inset is a plot of the peak counts per bin versus colloid concentration. The dashed curve is a linear fit to the data with slope = 0.0233 ± 0.0001 counts pM^{-1} . Note the log scale on the x axis.

Figure 7.

FCS and PCH of binary mixtures of Au colloids. (a) Autocorrelation function for 100 nm colloids at 2.5 pM concentration (upper curve); 39 nm colloids at 250 pM (lower curve), and a mixture of 2.5 pM of 100 nm colloids and 250 pM of 39 nm colloids (middle curve). (b) Autocorrelation function for 39 nm colloids at 2.5 nM concentration (lower curve) and a mixture of 2.5 pM of 100 nm colloids and 2.5 nM of 39 nm colloids (upper curve). (c) PCH for 2.5 nM of 39 nm colloids (gray line); 2.5 pM of 100 nm colloids (black line), and a mixture of 2.5 nM of 39 nm colloids and 2.5 pM of 100 nm colloids (dashed line). The open circles represent a histogram generated by adding the PCHs of the 39 and 100 nm colloids.