Enumerating virus-like particles in an optically concentrated suspension by fluorescence correlation spectroscopy

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Abstract: Fluorescence correlation spectroscopy (FCS) is one of the most sensitive methods for enumerating low concentration nanoparticles in a suspension. However, biological nanoparticles such as viruses often exist at a concentration much lower than the FCS detection limit. While optically generated trapping potentials are shown to effectively enhance the concentration of nanoparticles, feasibility of FCS for enumerating field-enriched nanoparticles requires understanding of the nanoparticle behavior in the external field. This paper reports an experimental study that combines optical trapping and FCS to examine existing theoretical predictions of particle concentration. Colloidal suspensions of polystyrene (PS) nanospheres and HIV-1 virus-like particles are used as model systems. Optical trapping energies and statistical analysis are used to discuss the applicability of FCS for enumerating nanoparticles in a potential well produced by a force field.

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OCIS codes: (350.4855) Optical tweezers or optical manipulation; (140.7010) Laser trapping; (290.1990) Diffusion; (180.2520) Fluorescence microscopy; (170.1790) Confocal microscopy.

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14. Assuming the trapping potential has an isotropic Gaussian distribution $U(r) = U(0)\exp(-2r^2/R^2)$, where $R$ is the beam waist of the 1064 nm trapping laser, estimated to be 0.97 $\mu$m. The experimentally determined trapping potential $U_{trap}$ is the integration of $U(r)$ in the illumination volume with beam waist 0.23 $\mu$m. Therefore, $U_{trap} = 0.97U(0)$.
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1. Introduction

Sensitive detection of pathogenic nanoparticles, such as viruses, is key to prevention of infectious diseases. Compared to sensing their molecular fingerprints, direct detection of whole particle viruses offers the advantage of easy sample preparation and fast assay. Fluorescence correlation spectroscopy (FCS) is one of the most sensitive methods for detecting suspended nanoparticles [1, 2]. However, the detection limit of FCS, on the order of sub nano-molar [3], does not meet the needs for clinical analysis of viral samples. The use of force fields, such as dielectrophoresis [4, 5] and optical trapping, to concentrate nanoparticles [6, 7], are an appealing approach to lower the FCS detection limit. Lacking an analytical expression for the FCS autocorrelation function, we need to examine experimentally the applicability of existing models for using FCS to measure the concentration of nanoparticles by enriching them in a potential well produced by a force field.

Multiple attempts have been made to interpret FCS data obtained from colloidal nanoparticles in a gradient force field. The experiment of Osborne et al. [8] showed that optical trapping can affect diffusion of molecules in the vicinity of the optical trap. Hosokawa et al. [9] used optically biased diffusion to study the cluster formation of nanoparticles in a highly focused laser beam. They demonstrated that it might be possible to quantify the relationships between the cluster size, trapping energy and the decay time of the FCS autocorrelation function (ACF). More recently, Wang et al. [10] studied the diffusion dynamics of gold nanoparticles in optical confinement and reported a laser-induced rise in temperature changed the diffusion behavior and the average number of gold nanoparticles in the focal area of the laser beam. Ito et al. [11] used a Brownian dynamics simulation to investigate the FCS ACF of colloidal nanoparticles in an optical gradient force field. They found the standard FCS ACF for free diffusing particles is applicable for particles in an optical trap with potential energies lower than $1k_BT$. Meng et al. [12] compared the ACF obtained by numerical calculation and Monte Carlo simulation for non-interacting particles in an isotropic Gaussian potential with trapping energies up to 1.8$k_BT$ and showed the ACF amplitude is equal to the inverse of the mean number of particles in the
optical trap.

In this paper we report an experimental study that combines optical trapping and FCS to examine the influence of trapping potential energy on the ACF amplitude. We compare the experimental ACF amplitude with the experimentally determined $1/\langle N \rangle$ using colloidal suspensions of polystyrene nanoparticles and HIV-1 virus-like particles (VLPs) to verify the predictions by Meng et al. [12]. Our experimental results are also compared with the Brownian dynamics simulation results by Ito et al. [11]. We further explain that the equality of the ACF amplitude to $1/\langle N \rangle$ under optical trapping requires that the particle number density fluctuation follows the Poisson statistics.

2. Experimental

2.1. FCS data analysis

A typical FCS experiment measures ACF of the fluorescence intensity from a detection volume defined by the focus of a laser beam. The FCS ACF $G(\tau)$ is defined as:

$$G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2},$$  \hspace{1cm} (1)

where $\tau$ is the correlation delay time, $F(t)$ is the instantaneous fluorescence intensity at time $t$, and $\delta F(t) = F(t) - \langle F(t) \rangle$ is the fluctuation of the fluorescence intensity around its mean value. For a dilute solution of non-interacting, point-like particles, $G(\tau)$ can be expressed as [2]:

$$G(\tau) = \frac{\langle \Delta N^2 \rangle}{\langle N \rangle^2} \left( 1 + \frac{4D\tau}{w_0^2} \right)^{-1} \left( 1 + \frac{4D\tau}{z_0^2} \right)^{-1/2}$$  \hspace{1cm} (2)

where $\langle \Delta N^2 \rangle$ and $\langle N \rangle$ are the variance and the mean number of particles in the volume, respectively; $D$ is the diffusion coefficient of the particles; $w_0$ and $z_0$ are the radial and axial dimensions of the detection volume, respectively. $G(\tau)$, the amplitude of the ACF at zero delay time, is the normalized variance of the particle number fluctuations $\langle \Delta N^2 \rangle/\langle N \rangle^2$. As moving particles pass the observation volume, the fluorescence intensity fluctuates and thus affects the amplitude of ACF. When the probability of finding a particular particle in the laser focus is much less than 1, the number fluctuation of particles in this region follows a Poisson distribution in which the variance of the distribution $\langle \Delta N^2 \rangle$ equals the mean number density $\langle N \rangle$, and

$$G(0) = \langle N \rangle^{-1}$$  \hspace{1cm} (3)

2.2. Optical instrument

A schematic of the optical apparatus is illustrated in Fig. 1. An IR laser (1064 nm, TREM-C1, Spectra-Physics) is used for trapping fluorescent nanoparticles. A blue laser (488 nm) of a confocal laser scanning microscopy (FV-1000, Olympus, Japan) is used for fluorescence excitation. A half-wave plate and a polarization beam splitter are used for adjusting the power of the trapping laser. The power of the 488 nm excitation laser at the focal point is maintained at a low power of 5 $\mu$W to minimize photo bleaching of the fluorescent particles in the observation volume. The trapping and excitation beams are combined by a dichromatic mirror and confocally aligned at the detection point. A microscope objective lens (UPlanFluor 100X, NA 1.3, Olympus, Japan) is used to focus the laser beams and to collect fluorescence emission. The fluorescence emission passing through a band-pass filter and a beam splitter is detected by two photon-counting avalanche photo diodes (APD: SPCM-AQRH-13-FC, Perkin Elmer). Since the fluorescence emission from the two channels are correlated, while the dark counts produced by
thermal noise in the two APD are not, we can minimize the adverse effects due to the APD dark counts by taking the cross-correlation of the two APD outputs to obtain $G(\tau)$. The cross-correlation calculation is made by the use of a digital correlator (Flex02-01D, correlator.com, USA). The geometrical parameters of the FCS detection volume $w_0$, $z_0$ are determined by using a 10nM solution of Alexa 488 dye. Using the known diffusivity of Alexa 488 molecules in water (430 µm$^2$/s) in fitting Eq. (2) to ACF, $w_0$ and $z_0$ are determined to be 0.22 µm and 2 µm, respectively.

2.3. Sample preparation

2.3.1. Colloidal polystyrene (PS) nanoparticles
An aqueous suspension of 100 nm diameter PS particles with a volume fraction of 1% is purchased from Thermo Scientific (Fremont, USA). The PS particles are fluorescently labelled with Firefli red (excitation maximum 543 nm, emission maximum 612 nm). The ionic (salt) concentration of the stock suspension is estimated to be 2 mM. The stock suspension is diluted 100 times in deionized water for all the FCS measurements. The ionic strength of the diluted sample solution was estimated to be 20 µM. A 20 L sample is held between a microscope cover-glass and a slide and sealed with wax. The chamber thickness of the gap between the two glass substrates is 60 ± 10 µm. FCS measurements are positioned at ~ 10 µm above the cover glass.

2.3.2. Preparation of virus-like particles (VLP)
HEK 293T cells (ATCC No. CRL-11268) are cultured in DMEM supplemented with 10% fetal calf serum at 37°C and 5% CO$_2$. The cells at 60% confluency are transfected with the GAG-EGFP plasmid (AIDS reagent program Cat. No. 11468) and incubated for 48 hours. The supernatant containing virus-like particles is then collected, filtered using 0.2 µm filter and centrifuged for 3 hours at 20,000 rpm to concentrate the virus-like particle at the bottom of the test tube by sucrose density centrifugation. The pellet containing the virus-like particles is then resuspended in the cell culture medium. These particles have a similar capsid structure to the wild-type HIV virion, but lack some of the viral genome, which minimizes their pathogenicity. Moreover, these particles are genetically engineered to carry green fluorescent protein (excitation maximum at 488 nm and emission maximum at 509 nm) tagged to Gag, the primary structural protein of HIV.

SEM and confocal microscope images are acquired to examine the size and fluorescence of the VLPs [13]. To prepare SEM samples, glutaraldehyde is added to the VLPs suspension to a final concentration of 2.5% followed by incubation at room temperature for 1 hour. A 20 µL
suspension is then pipetted onto an autoclaved cover glass, dehydrated in ethanol, dried at room temperature and sputter-coated with gold palladium for 60 seconds before imaging.

3. Results and discussion

3.1. Fluorescence correlation spectroscopy in an optical trap

The ACF $G(\tau)$ from 110 nm PS nanoparticle suspensions in the presence of optical trapping with laser powers between 0 and 18 mW are shown in Fig. 2. Each ACF curve represents an average of 10 independent measurements. Fitting Eq. (2) to the trap-free (0 mW) curve yields a particle number density $\langle N_0 \rangle = 0.165$ in the observation volume and diffusivity $D = 4.5 \mu m^2/s$. The measured diffusivity agrees with that obtained by the Stokes-Einstein equation $D = k_B T / (6\pi\eta a) = 4.35 \mu m^2/s$, where $\eta$ is the viscosity of water at the ambient temperature and $a$ is the particle radius.

![Fig. 2. FCS autocorrelation functions of 0.01% (v/v) 110 nm PS particle suspensions at selected trapping laser powers. Each curve represents an average of 10 measurements.](image)

![Fig. 3. The experimental ACFs (black) are fitted using Eq. (2) (red) for trapping laser powers of (a) 0 mW; (b) 6 mW; (c) 12 mW; (d) 18 mW.](image)

To examine the range of trapping powers at which Eq. (2) can be used to describe the exper-
imental ACFs, we plot in Fig. 3 the same data shown in Fig. 2 and fit curves using Eq. (2) for various trapping energies, 0 mW, 6 mW, 12 mW and 18 mW, respectively. The excellent match between Eq. (2) and the experimental ACF data suggests that the conventional ACF can be used to describe the motion of the nanoparticles in a potential well with a depth up to $1.8k_B T$. This range of trapping powers exceeds that examined by Ito et al. through Brownian dynamic simulation [11], where Eq. (2) is shown to be applicable for trapping energies only up to $1k_B T$.

The trapping energy will be discussed later.

In the presence of the optical trap, the average number of particles in the trap can be determined independently by total photon counts:

$$\langle N_{\text{trap}} \rangle = \langle F_{\text{trap}} \rangle / \varepsilon.$$ 

Here, $\varepsilon$ is the average fluorescence luminosity per particle at fixed excitation light intensity, which can be determined by the ratio $\langle F_0 \rangle / G(0)$, where $\langle F_0 \rangle$ is the average fluorescence photon counts at zero trapping laser power, and $G(0)$ at zero trapping laser power is equal to the mean number of particle in the FCS observation volume.

Figure 4(a) shows a plot of $G(0)^{-1}_{\text{trap}}$ vs. $\langle N_{\text{trap}} \rangle$. The linearity in Fig. 4(a) with a slope of 1 suggests $G(0)^{-1}_{\text{trap}}$ equals the average number of particles in the presence of optical trapping for all trapping powers tested between 0 mW to 18 mW.

A semi-log plot of $G(0)^{-1}_{\text{trap}} / G(0)^{-1}_0$ vs. the power of trapping laser is shown in Fig. 4(b). Since $G(0)^{-1}_{\text{trap}} / G(0)^{-1}_0 = \langle N_{\text{trap}} \rangle / \langle N_0 \rangle$, Fig. 4(b) implies the number of particles in the optical trap follows a Boltzmann distribution with the trapping potential energy:

$$\langle N_{\text{trap}} \rangle = \langle N_0 \rangle \exp(U_{\text{trap}} / k_B T)$$

where $U_{\text{trap}}$ [14], the average single-particle trapping potential energy is the slope of the semi-log plot in Fig. 4(b). The Boltzmann distribution is expected when particle interactions are negligible. The trapping energies at which the particle distribution are no longer Boltzmann depends on the bulk concentration and the ionic strength of the buffer [6, 7]. The trapping energy of the 110 nm PS spheres is found to be $0.1 \pm 0.04 k_B T$ per mW of laser. This result is in good agreement with trapping energies reported previously by other methods [7, 15].

#191164 - $15.00 USD  Received 24 May 2013; revised 14 Jul 2013; accepted 23 Jul 2013; published 14 Aug 2013  (C) 2013 OSA  1 September 2013 | Vol. 4,  No. 9 | DOI:10.1364/BOE.4.001646 | BIOMEDICAL OPTICS EXPRESS  1651
Figure 4(c) shows a constant ratio of $G(0)^{-1}_{trap}/\langle N_{trap} \rangle = 1$ for trapping potential energies up to $1.8k_BT$, suggesting our experimental result agrees with the theoretical prediction by Meng et al. [12].

The decay time $\tau = \omega_0^2/4D$ of our experimental ACF decreases with increasing trapping laser power as shown in Fig. 4(d). This trend agrees with the result reported by Hosokawa et al. [9]. However, a theoretical model will be needed to describe the influence of the particle motion in an optical trap to the FCS ACF.

3.2. Optical trapping of VLPs

Since the size of the fluorescent objects could affect the accuracy of the FCS measurements [13], we determine the sizes of the VLPs by SEM imaging and compare them with measurements by FCS. Figure 5(a) shows a SEM image of the VLPs on a glass slide. The average diameter of the particles is $110 \pm 11 \text{nm}$ (standard deviation from 10 VLPs), which agrees with previous studies [16, 17]. A fluorescence image (Fig. 5(b)) taken by a 100X oil immersion objective lens (UPlanFluor, Olympus, Japan) shows intrinsic green fluorescence.

A FCS autocorrelation curve of the VLPs suspended in culture medium is shown in Fig. 5(c). By fitting the curve by Eq. (2), the ACF yields a diffusion coefficient $3.79 \mu m^2/s$. Using the Stokes-Einstein equation $D = k_BT/(6\pi \eta a)$ and medium viscosity $\eta = 0.98 cP$, we determine the diameter of the VLPs to be $115 \pm 14 \text{nm}$ (standard deviation from 10 independent measurements), which is consistent with results obtained by SEM.

We use the procedures described in Section 3.1 to determine the trapping energy of VLP. Selected ACF curves for different laser powers are shown in Fig. 5(a). A semi-natural log plot of $G(0)^{-1}$ vs. trapping laser power is shown in Fig. 5(b). For PBS buffer solutions containing $135 mM$ NaCl, the Debye screening length is on the order of $1 \text{nm}$. Since the estimated average distance between two VLPs in our experiments is more than $1 \mu m$, particle interactions can be neglected, and the number of particles in the optical trap follows Eq. (4). A semi-natural log plot of the average number of particles vs. the trapping power is shown in Fig. 5(b). The trapping energy for the VLPs is calculated to be $0.02 \pm 0.003 k_BT/mW$.

Because VLPs are hollow particles surrounded by capsid proteins and a thin shell of phospholipid bilayer their trapping energy is significantly smaller than that of comparably sized solid PS particles. We use a discrete dipole approximation method [18] to numerically calculate the optical trapping energies of these particles. The trapping beam used in the calculation is a fifth-order-corrected monochromatic Gaussian beam with beam waist $0.5 \mu m$. For the $110 \text{nm}$ diameter PS spheres (refractive index 1.59) in water (refractive index 1.33), the calcu-
lated trapping energy is $0.13 k_BT/mW$. Modeling VLPs as 100nm inner diameter vesicles with a 10nm lipid bilayer wall (refractive index 1.46) [19], we calculate their trapping energy to be $0.017 k_BT/mW$, in reasonable agreement with the experimentally determined value for VLP.

Since the relationship $G(0) = \langle N \rangle^{-1}$ is derived from the Poisson statistics, the fact that the relation holds for particles in an optical trap implies that the conditions required for Poisson distribution are satisfied. These conditions are i) the probability a particular particle in the trap is much less than one, and ii) the particle movements near the trap are uncorrelated. Thus, for extremely low particle concentrations, one can estimate the trapping energies under which $G(0) = \langle N \rangle^{-1}$ is valid. As an example, for a sample of concentration $C = 10^9$ particle/cm$^3$ in a volume of $V = 10^6 \mu m^3$, the probability for a particular particle to be in the laser illuminated volume of $v = 1 \mu m^3$ is $p = 10^{-6}$, and the average number of particles in the illuminated volume is $\langle N \rangle = v \times C = 10^{-3}$. Limited by the intrinsic dark-count of a typically APD detector, $\langle N \rangle \geq 0.1$ is needed to ensure a statistical meaningful FCS measurement, which requires an enhancement of $p$ by a factor of 100. As a result, $p$ is enhanced from $10^{-6}$ to $10^{-4}$, at which the required condition (i) for Poisson distribution is still satisfied. Since the particle interactions are negligible at such low concentration the concentration enhancement by trapping follows a Boltzmann distribution of the trapping potential energy. The trapping energy needed for the concentration enhancement of 100 can be estimated by $\ln(100) = 4.6k_BT$.

4. Conclusions

This paper describes an experimental investigation of the use of FCS for analyzing the behavior of colloidal nanoparticles in an optical trap. Our experiments show the standard FCS autocorrelation function for free diffusing particles describes the behavior of particles in an optical trap with trapping energies up to 1.8$k_BT$. We also found $G(0) = \langle N \rangle^{-1}$ to be valid under such trapping energies, which is in good agreement with the results obtained by Meng et al. by Monte Carlo simulation [12].

Our experiments demonstrate that the average number of nanoparticles in the trap increases exponentially with the power of the trapping laser for non-interacting particles in suspension. In this case, the concentration of the particles in the trap follows a Boltzmann distribution. We determine the trapping energy of HIV-1 virus-like particles (VLPs) to be 0.02$k_BT$ per mW of laser power, a value in good agreement with a discrete dipole approximation by modeling VLP as a hollow sphere with a thin shell of phosphor-lipid bilayer. Using probability theory and the requirements for Poisson statistics, we give an example to illustrate how to estimate the range of force-induced concentration enhancement within which the relationship $G(0) = \langle N \rangle^{-1}$ can be used to determine particle concentration.

Acknowledgments

The authors wish to thank Ming-Tzo Wei, Drs. Denis Pristinski, Ya Liu and Professor Dimitrios Vavylonis for their helpful suggestions during the course of this study, and Professor Olga Latinovic for her training on virus production. This project is supported in part by funds from NIAID-IR21AI081638, PA Department of Health CURE Formula Funds, NSF DMR 0923299, Lehigh University Center for Optical Technologies and Emulsion Polymers Institute.