

Quantification of Receptor Targeting Aptamer Binding Characteristics Using Single-Molecule Spectroscopy

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ABSTRACT: This experimental design presents a single molecule approach based on fluorescence correlation spectroscopy (FCS) for the quantification of outer membrane proteins which are receptors to an aptamer specifically designed to target the surface receptors of live *Salmonella typhimurium*. By using correlation analysis, we also show that it is possible to determine the associated binding kinetics of these aptamers on live single cells. Aptamers are specific oligonucleotides designed to recognize conserved sequences that bind to receptors with high affinity, and therefore can be integrated into selective biosensor platforms. In our experiments, aptamers were constructed to bind to outer membrane proteins of *S. typhimurium* and were assessed for specificity against *Escherichia coli*. By fluorescently labeling aptamer probes and applying FCS, we were able to study the diffusion dynamics of bound and unbound aptamers and compare them to determine the dissociation constants and receptor densities of the bacteria for each aptamer at single molecule sensitivity. The dissociation constants for these aptamer probes calculated from autocorrelation data were 0.1285 and 0.3772 nM and the respective receptor densities were 42.27 receptors per μm^2 and 49.82 receptors per μm^2 . This study provides ample evidence that the number of surface receptors is sufficient for binding and that both aptamers have a high-binding affinity and can therefore be used in detection processes. The methods developed here are unique and can be generalized to examine surface binding kinetics and receptor quantification in live bacteria at single molecule sensitivity levels. The impact of this study is broad because our approach can provide a methodology for biosensor construction and calculation of live single cell receptor-ligand kinetics in a variety of environmental and biological applications.

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In the United States, there are approximately 1.4 million cases of food-borne illness annually resulting from *Salmonella* poisoning causing 16 000 hospitalizations and 550 deaths per year (Mead et al., 1999). While significant strides have been made in emergent biosensors, the challenge still exists in the development of technologies that can detect pathogens at single cell resolution. While antibodies have predominantly been used in biosensors, aptamers have significant appeal because of their specificity, high-binding efficiency, stability, and ability to be conjugated to a variety of probes (Chen et al., 2009a; Dwarakanath et al., 2004; Joshi et al., 2009; Poglitsch and Thompson, 1990). Aptamers are single-stranded nucleotides with three-dimensional structures capable of binding to a variety of biological surfaces with high specificity and are constructed via a chemical procedure called systematic evolution of ligands by exponential enrichment (SELEX) (Chen et al., 2009b; Dwarakanath et al., 2004; Joshi et al., 2009). Using these specific probes, we demonstrate for the first time a quantitative method based on a single molecule approach utilizing fluorescence correlation spectroscopy (FCS) to quantify receptor density and binding kinetics which can be used for sensor development and studies relating to detection of foodborne pathogens.

Fluorescence correlation spectroscopy was developed in the 1970s to study the concentration and diffusion times of fluorescently labeled species by monitoring the fluorescence fluctuations. FCS utilizes nanomolar concentrations of fluorescent probes to ensure that primarily one molecule's trajectory is monitored at a time as it flows through the sub femtoliter confocal volume providing single molecule resolution (Elston and Webb, 1975; Koppel et al., 1976; Magde et al., 1972). With the development of faster data acquisition systems, more stable lasers, and detectors, including avalanche photodiodes (APDs) and photomultiplier tubes (PMTs), the role of FCS in the study of chemical kinetics is becoming more prominent (Webb, 2006). FCS has also been applied in studying singlet and triplet

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dynamics and in the study of cell membranes including localized regions within the membrane (Pramanik and Rigler, 2001; Vukojevic' et al., 2005; Widengren et al., 1995; Wohland et al., 2001). The advantage of FCS in cell membrane dynamics experiments is that it is not necessary to remove the unbound species, as this state can be accounted for by fitting for two components in the autocorrelation analysis (Rigler et al., 1999). Previous studies on quantifying surface receptor densities have primarily been conducted in cultured mammalian cells using methods such as fluorescence subtraction counting, liquid scintillation, and total internal reflection fluorescence (TIRF) (Chen et al., 2009b; Haustein et al., 2003; Lieto et al., 2003; Thompson et al., 1981; Thompson et al., 2002; Wolfrum et al., 2007). Fluorescence subtraction is a quantification method based on the removal of the unbound fluorescent target molecule into the supernatant. The difference between the fluorescence of the supernatant and the initial fluorescence, based on a linear curve fitting is used to determine the amount of bound targets. The disadvantages of this method include the prolonged (longer than the half-life of the receptors) time periods between each wash and high background or low receptor numbers can lead to erroneous results in certain cases (Chen et al., 2009b). Liquid scintillation counting uses Beta-emitting radioactive analytes, which are capable of converting kinetic energy to light energy. The membranes are washed and the bound radioactivity is measured (Chen et al., 2009b; Wolfrum et al., 2007). TIRF can also be utilized to for cell surface studies (Haustein et al., 2003; Lieto et al., 2003; Thompson et al.,

1981; Thompson et al., 2002). In this work, we show for the first time the use of FCS for receptor quantification and binding kinetics assessment in a live bacterium.

Our research is important from two perspectives, (i) the ability to quantify the number of surface binding sites has a direct relevance to developing highly sensitive single cell biosensor platforms, and (ii) the ability to study single molecule kinetics at single cell resolution will enhance our basic understanding of the number of active surface binding sites and their activities from a theoretical perspective, with a possible relation to pathogenesis.

Figure 1a–d are set to the same relative intensities for direct comparison. Figure 1e is a time correlated single photon counting (TCSPC) curve that can be fitted with an exponential decay function which can then be used to determine the fluorescence lifetime of the bacteria. Fluorescence lifetime measures the decay kinetics of fluorescence emission after excitation (Bastiaens and Squire, 1999). The significance of this image is to show that there is a change in lifetime between measurements from samples that consisted of *Salmonella* alone and the bacteria targeted with aptamer even at the lowest aptamer concentration of 0.25 nM. The lifetime of aptamer-bound bacteria was approximately 4.015 ± 0.1170 ns while the lifetime of bacteria alone was approximately 3.158 ± 0.1248 ns estimated using five images with approximately 50 cells. Our results compare well with the existing results which report the lifetime of Alexa 488 as 4.1 ns (Rusinova et al., 2002) and the lifetime of bacteria due to auto-fluorescence (lifetime is between 2.5–3.5 ns).

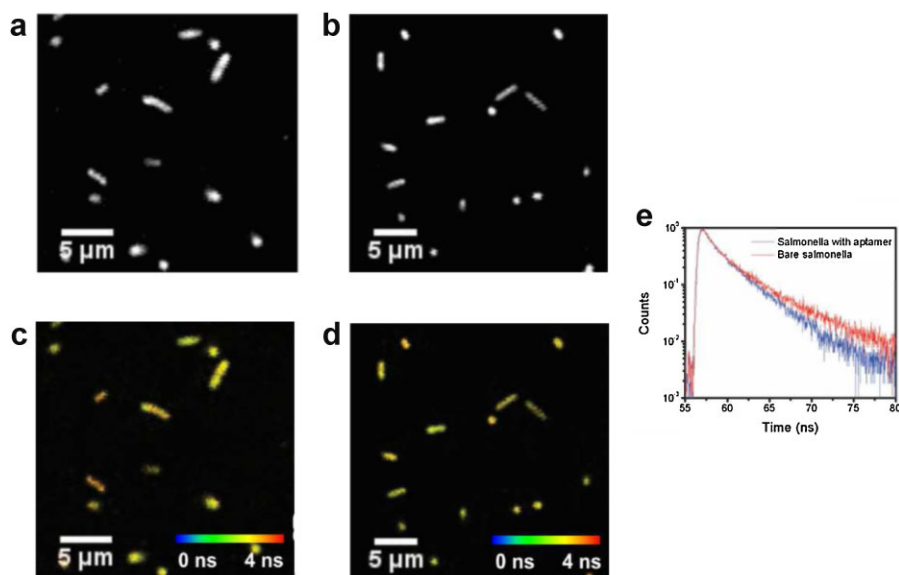


Figure 1. Photon illumination intensity confocal images of *S. typhimurium* bound Aptamer 33 and Aptamer 45 are shown in (a) and (b), respectively. Images were taken using a 465 nm pulsing laser at a power of approximately $8 \mu\text{W}$ with the same intensity scales for comparison. The corresponding lifetime images for Aptamer 33 and Aptamer 45 bound *S. typhimurium* are provided in (c) and (d), respectively. The fluorescent lifetime of the aptamer attached to the bacterial surface was estimated to be approximately 4 ns, indicative of Alexa 488 and thereby ensuring that the probe is present and measurements are conducted in these localized regions [21]. The TCSPC curve of the bacteria alone compared to bacteria with bound aptamer are represented in (e). From the change in decay profile, a significant decrease in lifetime can be detected.

Figure 2a and b show the autocorrelation curves fitted with Equation (5). These curves measure the molecular fluctuations of the aptamer itself. A significant increase in the autocorrelation function, $G(0)$ for the aptamer bound to bacteria can be observed from Figure 1a and b. However, the increase is greater for aptamer 33, noted by comparing Figure 2a and b. The change in signal is due to the change in diffusion time which demonstrates a change in molecular weight of the diffusing species depicting the binding of the aptamer to the bacteria. While, low laser power was used, approximately $8\ \mu\text{W}$, Figure 2c and d provide evidence that no photobleaching occurred, because the trace amplitude

remains constant for the duration of the recording. The dissociation constants (K_d) in Figure 2e and f were obtained by fitting the binding fraction percentage, which was determined by the original fitting of the data with Equation (5) with respect to the concentrations studied. The fitting was accomplished using the following one-site binding equation (Equation 1) where “ x ” denotes the concentration and “ b ” the percentage of bound molecules. Then, the values for the bound and unbound aptamer-bacteria complex were compared (Equation 2) to calculate the final dissociation constant for each aptamer ligand. The dissociation constants of the two aptamers designed for

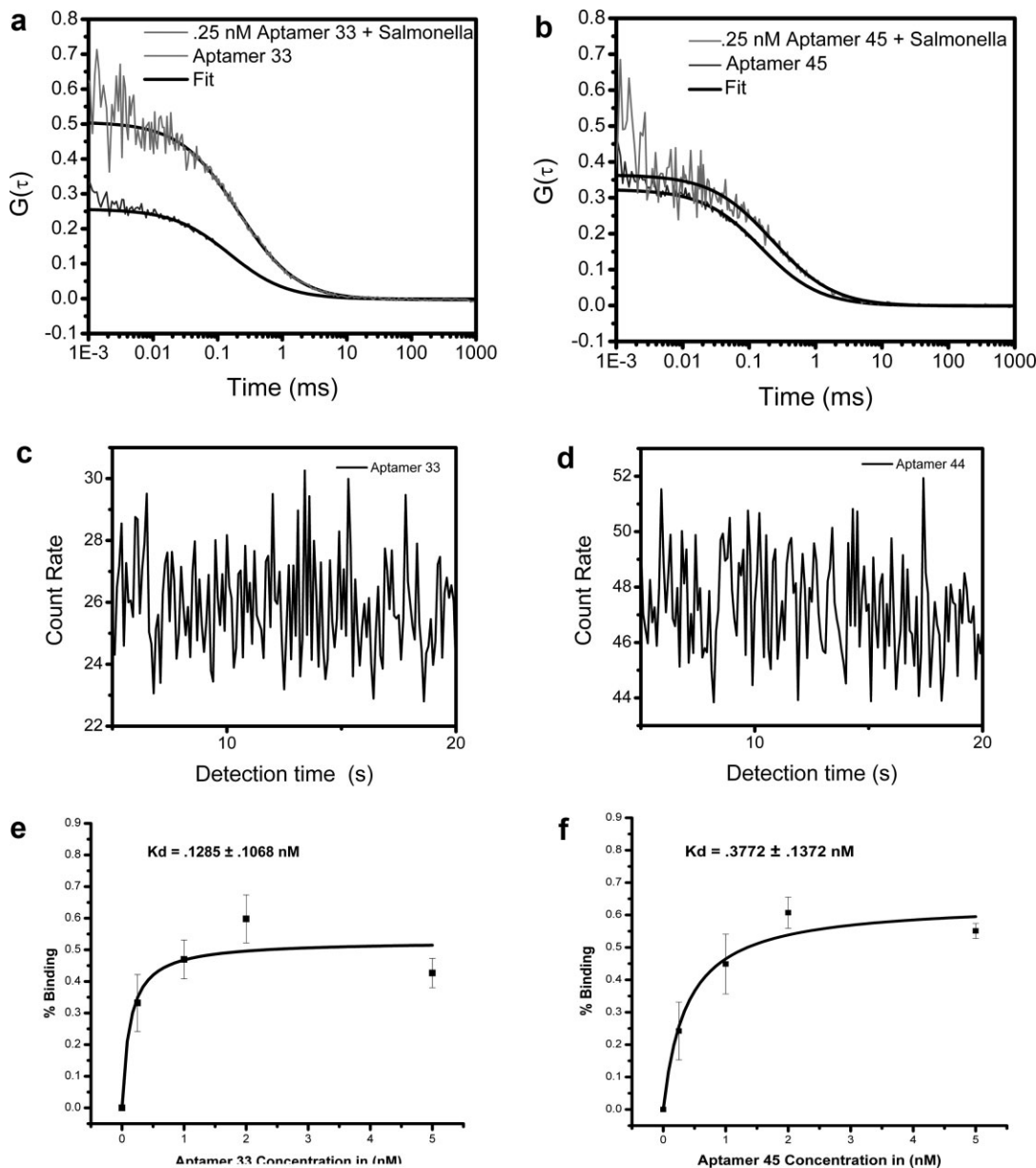


Figure 2. FCS autocorrelation curves comparing aptamers bound to bacteria using the lowest aptamer concentration of 0.25 nM and free control aptamers (aptamer 33 (a) and aptamer 45 (b)). (c) and (d) depict the count rates over time which provides evidence that there is no decrease in fluorescence due to photobleaching of the probes. The calculated K_d values with varying concentrations of aptamers, 33 and 45 are provided in (e) and (f), respectively.

targeting *Salmonella typhimurium* were calculated to be 0.1285 nM (aptamer 33) and 0.3772 nM (aptamer 45), demonstrating that aptamer 33 has a slightly higher affinity for its receptor. Therefore, both of the aptamers studied have the potential to be selective ligands for targeting *S. typhimurium* and can be used to target and detect the pathogen. These values are comparable to typical aptamer dissociation constants which usually spans pico to nanomolar range and are comparable and at times improved from typical antibody dissociation constants (Jayasena, 1999). The Poly-lysine coating of the coverslips improved bacteria affinity for the coverslip by facilitating electrostatic binding for consistent measurements. However, as time elapsed minor movement of the bacterium was noted, as reflected in the standard deviations of the dissociation constants.

$$y = \frac{B_{\max}x}{(K + x)} \quad (1)$$

$$K_d = \frac{[\text{Free Aptamer}][\text{Free Bacteria}]}{[\text{Bound Aptamer}]} \quad (2)$$

Figure 3.

Even for high-affinity detection agents such as aptamers, it is crucial to have a significant number of receptor targets in order to create an efficient detection mechanism. Therefore, the receptor density was calculated using the following equation:

$$\text{Density} = \frac{(N)(y)}{\omega^2\pi} \quad (3)$$

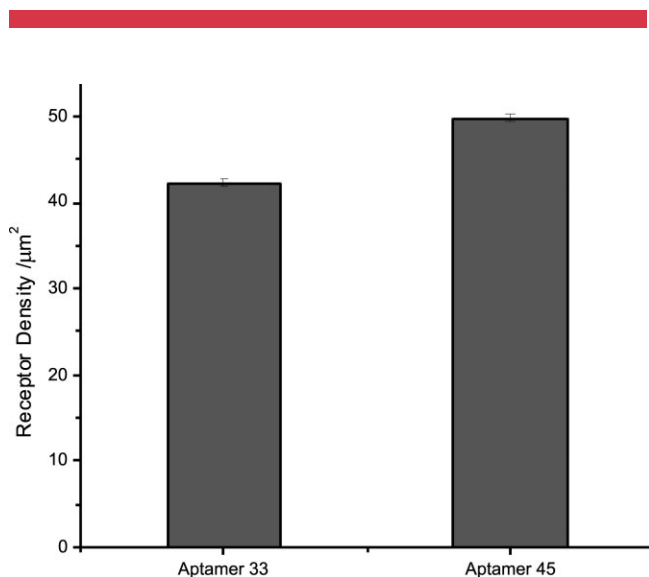


Figure 3. *S. typhimurium* outer membrane protein receptor density.

Here, $N \times y$ correlates to the number of bound aptamers and can be acquired from the correlation fitting function (Equation 5). The surface area of the bacterium can be estimated from,

$$SA = \omega^2\pi \quad (4)$$

where ω is the beam width which is determined from the confocal volume calibrations using the well characterized Rhodamine 123, and was determined to be 200 nm (Varghese et al., 2008; Chen et al., 2009a). The receptor densities were calculated using an aptamer concentration of 5 nM to ensure that all of the surface receptors were bound, since at this concentration the aptamer has reached the saturation limit with respect to the receptor (Figure 2). The receptor densities were also verified using the 2 nM concentration (data not shown) which yielded consistent results. The receptor density for aptamer 33 was 42.27 ± 0.49 receptors per μm^2 and 49.82 ± 0.45 receptors per μm^2 for aptamer 45.

Using FCS, the dissociation constants of the two aptamer ligands designed for *S. typhimurium* were calculated to be 0.1285 nM (aptamer 33) and 0.3772 nM (aptamer 45) demonstrating high-binding affinity for both the aptamers. The receptor targeted by aptamer 33 was ~ 42 receptors per μm^2 and 49 receptors per μm^2 for aptamer 45. Due to the nanomolar dissociation constants and similar receptor densities, both aptamers are optimal detection ligands for use in a pathogen biosensor. The experimental results depict the capabilities of FCS for the quantification of bound and unbound target ligands in live bacteria so as to allow for the calculation of receptor densities and binding kinetics at a single molecule level in live bacterium. This platform has broad implication as it can provide a methodology for biosensor construction and assessment of live single cell receptor–ligand kinetics in a variety of environmental and biological applications.

Materials and Methods

Aptamers

Two 28-mer aptamer sequences (Aptamer 33 and 45) were acquired from Dr Streevetsan's Lab at the University of Minnesota (Joshi et al., 2009). These aptamers were constructed to interact with outer membrane protein sequences on the surface of *S. typhimurium* and contained the following sequences,

Aptamer 33: TATGGCGGCGTCACCCGACGGGGACTT
GACATTATGACAG

Aptamer 45: GAGGAAAAGTCTATAGCAGAGGAGATGT
GTGAACCGAGTAA

The aptamers were tested against *Escherichia coli* outer membrane proteins and lipopolysaccharides to guarantee

bacteria specificity. The aptamers were tested against *Escherichia Coli* outer membrane proteins and lipopolysaccharides to guarantee bacteria specificity in previous work by Dr. Sreevatsan's lab (Joshi et al., 2009).

Bacteria

Slides were prepared by cleaning with Piranha solution for 30 min at room temperature followed by coating with the positively-charged polymer, Poly-L-lysine for 1 h at room temperature. Then, *S. typhimurium* were cultured, placed on the prepared slides, and allowed to attach to the slide for 30 min at room temperature. The slides were coated with Poly-L-lysine to cause electrostatic binding of the bacteria to the surface. This reduces the movement of the bacteria allowing imaging and spectroscopy measurements to take place. The cells were then incubated for 1 h with varying concentrations of aptamers ranging from 0 to 5 nM suspended in phosphate buffer solution (PBS). As the bacteria are alive, the interaction of the diffusing aptamers with the receptors at the surface of the bacteria can be measured by FCS in this study. Nanomolar concentrations of aptamers were selected to limit the number of molecules flowing through the confocal volume close to one, ensuring single molecule measurements.

FCS Measurements and Instrumentation

Fluorescence fluctuation measurements were acquired using a scanning confocal time-resolved microscope, Microtime 200 (Picoquant GmbH, Germany) as detailed in (Varghese et al., 2008). The excitation source for these experiments was a 40 MHz pulsing picosecond laser which emitted at 465 nm.

The laser beam was then focused with a dichroic mirror onto a 60 $\times$ water immersion objective lens with a numerical aperture of 1.2. The resulting fluorescence from the sample was passed back through the same objective lens and the out of focus light was removed using a 50 μ m pinhole. Dichroic mirrors were utilized to focus the emission onto the single photon photo avalanche diode (SPAD) and filtered with a 500–540 nm emission filter (Varghese et al., 2008). Figure 4a and b give the schematics for the FCS probing volume and the instrument set-up. The bacteria were first imaged by bidirectional scanning and fluorescence intensity images and fluorescence lifetime images (FLIM) were acquired. Fluorescent lifetime images were used to detect the fluorescent probe, Alexa 488 conjugated to the aptamers. By FLIM, we first distinguished the fluorescent molecules from background autofluorescence using the specific lifetimes associated with the fluorophore and established a measurement window for subsequent single molecule analysis by FCS. Two images were taken per sample to detect a stable bacteria specimen, which was then selected from the image and a fluorescence correlation scan was taken at a point on the bacteria. FCS measurements were recorded for 60 s and five correlation curves were obtained from each sample.

The correlation curve was constructed from the point scan using Equation (5) and Origin Labs analysis software (OriginPro 7.5). In Equation (5), N represents the number of fluorescently tagged molecules emitting photons, τ represents the diffusion time, D the diffusion coefficient, γ the fraction of bound molecules, ω_0 the distance from the optical axis, and z_0 is the distance along the optical axis where the laser intensity is diminished by $\frac{1}{e^2}$ which denotes κ (Rigler, 1995; Rigler et al., 1999; Rusinova et al., 2002). Using Equation (5), the concentration of the

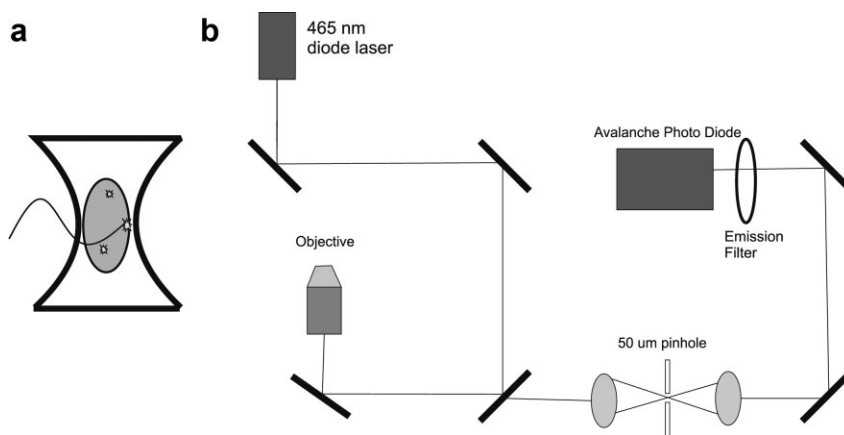


Figure 4. The experimental set-up depicting a fluorescently labeled molecule diffusing through the confocal volume (a). Schematic of the FCS depicting the laser path into the system, its trajectory through the objective lens to interrogate fluorescent molecules diffusing through the confocal volume, signal collection optics, and detection using sensitive avalanche photo diode (b) is provided.

unbound and bound species can be separated and calculated.

$$G(\tau) = \frac{1}{\langle N \rangle} \left((1-y) \left(1 + \frac{\tau}{\tau_d^{\text{free}}} \right)^{-1} \left(1 + \frac{\tau}{\tau_d^{\text{free}} K^2} \right)^{-1/2} + y \left(1 + \frac{\tau}{\tau_d^{\text{bound}}} \right)^{-1} \right) \quad (5)$$

$$K = \frac{z_o}{\omega_o} \quad (6)$$

$$\tau_D = \frac{\omega_o^2}{4D} \quad (7)$$

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